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Multiple Charged and Aromatic Residues in CCR5 Amino-terminal Domain Are Involved in High Affinity Binding of Both Chemokines and HIV-1 Env Protein*

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CCR5 is a functional receptor for MIP-1α, MIP-1β, RANTES (regulated on activation normal T cell expressed), MCP-2, and MCP-4 and constitutes the main coreceptor for macrophage tropic human and simian immunodeficiency viruses. By using CCR5-CCR2b chimeras, we have shown previously that the second extracellular loop of CCR5 is the major determinant for chemokine binding specificity, whereas the amino-terminal domain plays a major role for human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus coreceptor function. In the present work, by using a panel of truncation and alanine-scanning mutants, we investigated the role of specific residues in the CCR5 amino-terminal domain for chemokine binding, functional response to chemokines, HIV-1 gp120 binding, and coreceptor function. Truncation of the amino-terminal domain resulted in a progressive decrease of the binding affinity for chemokines, which correlated with a similar drop in functional responsiveness. Mutants lacking residues 2–13 exhibited fairly weak responses to high concentrations (500 nM) of RANTES or MIP-1β. Truncated mutants also exhibited a reduction in the binding affinity for R5 Env proteins and coreceptor activity. Deletion of 4 or 12 residues resulted in a 50 or 80% decrease in coreceptor function, respectively. Alanine-scanning mutagenesis identified several charged and aromatic residues (Asp-2, Tyr-3, Tyr-10, Asp-11, and Glu-18) that played an important role in both chemokine and Env high affinity binding. The overlapping binding site of chemokines and gp120 on the CCR5 amino terminus, as well as the involvement of these residues in the epitopes of monoclonal antibodies, suggests that these regions are particularly exposed at the receptor surface.

Chemokines constitute a large family of polypeptides that regulate the trafficking of immune cell populations (1). They mediate these functions through the activation of G protein-coupled receptors (GPCRs)[1] (1–3). Chemokines and their receptors have been implicated in a variety of human diseases, including acute and chronic inflammatory diseases, atherosclerosis, and cancer (4–8).

CCR5 is a functional receptor for the CC-chemokines MIP-1α, MIP-1β, RANTES, MCP-2, and MCP-4 (9–12) and is expressed in memory T-cells, B-cells, macrophages, dendritic cells, and microglial cells (13–15). CCR5 plays a major role in AIDS pathogenesis (16). Indeed, human immunodeficiency virus (HIV) entry is initiated by the interaction between the viral envelope glycoprotein (gp120), the host cell factor CD4, and one of several coreceptors (CCR5, CXCR4, and others) that belong to the chemokine receptor family (16, 17). This trimolecular interaction promotes conformational changes in the gp120-gp41 complex, leading to the ill-defined fusion process between the viral and host cell membranes (18). The fact that some lentivirus strains are able to enter CD4-negative cells through a direct interaction with chemokine receptors has led to the suggestion that chemokine receptors constitute the primordial receptor for primate lentiviruses (19–21). Transmission of HIV infection is typically mediated by viral strains that use CCR5 as a coreceptor (R5 or macrophage tropic strains), whereas development of AIDS is often associated with the emergence of strains that use CXCR4 exclusively (X4 or T-tropic strains) or in addition to CCR5 (R5X4 or dual tropic strains) (22, 23).

The key role of CCR5 in HIV pathogenesis was demonstrated by the observation that individuals homozygous for a 32-base pair deletion in the CCR5 coding region (CCR5Δ32), resulting in the production of a truncated and non-functional receptor, are highly resistant to HIV infection (24, 25), although this resistance is not complete (26–28). The mutant allele, at the

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[1] The abbreviations used are: GPCRs, G protein-coupled receptors; mAb, monoclonal antibody; FACS, fluorescence-activated cell sorter; HIV, human immunodeficiency virus; SIV, simian immunodeficiency virus; gp, glycoprotein; RANTES, regulated on activation normal T cell expressed; Fmoc, fluoremethyloxycarbonyl; CHO, Chinese hamster ovary; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; wt, wild type.

heterozygous state, is also associated with delayed AIDS progression in HIV-1-seropositive individuals (29–32).

Natural CCR5 ligands (MIP-1α, MIP-1β, RANTES, and MCP-2), chemokine analogs (truncated RANTES and aminooxypentane-RANTES), as well as monoclonal antibodies directed to CCR5 extracellular domains can inhibit HIV-1 infection *in vitro* as well as *ex vivo* (10, 33–37). Their effectiveness *in vivo* has not, however, been demonstrated so far. The understanding at the molecular level of how CCR5 interacts with chemokines and HIV could help the design of new drugs and vaccines endowed with more potent HIV suppressive activities. For example, broadly cross-reactive neutralizing antibodies have been generated by immunization with an HIV Env protein conformationally modified by its interaction with CD4 and coreceptors (38). A small molecule inhibitor of CCR5 has also been described recently (39). By using CCR5-CCR2b chimeras, we have shown previously that the amino terminus of CCR5 plays a dominant role in coreceptor activity but that all other extracellular domains of CCR5 contribute to this mechanism as well (40). Other groups have since extended this observation to other coreceptors and have identified more precisely amino acids involved in viral docking (41–49). We have also identified the second extracellular loop of CCR5 as the major determinant of ligand specificity (50). The study of other chemokine receptors such as CXCR1 and CCR2, however, suggested the existence of two sites contributing to the high affinity binding of chemokines, one located in the amino terminus, the other within the extracellular loops and possibly the transmembrane segments of the receptors (51–53). The high affinity binding site of CCR2 was reported within the amino-terminal domain of the receptor (50, 54, 55). Also, studies dealing with chimeras between structurally related receptors do not adequately investigate the regions that are conserved between the two molecules. Therefore, the contribution of shared determinants can easily be overlooked.

In the present study, we have investigated the role of the distal region of CCR5 amino terminus in the interaction of the receptor with chemokines and gp120, as well as the functional consequences of these interactions, using a panel of amino-terminal truncations and alanine substitution mutants. We showed that multiple charged and aromatic residues spread along CCR5 amino terminus contribute significantly to the high affinity binding of both chemokines and HIV gp120.

EXPERIMENTAL PROCEDURES

Plasmids—CCR5 amino-terminal truncations (Δ2–5, Δ2–9, Δ2–13, and Δ2–17) and alanine substitution mutants were previously described (40, 56). Additional truncations (Δ2, Δ2–3, and Δ2–4) were generated as described (40). The constructs were cloned in a bicistronic expression vector as described previously (50) for generation of stable cell lines and in pcDNA3 (Invitrogen) for envelope binding assays. All constructs were verified by sequencing before transfection.

Chemokines—RANTES was chemically synthesized as described (57). MIP-1β was synthesized on an automated peptide synthesizer (Pioneer, Perspective-Perkin Elmer Biosystems) using fluoremethyloxycarbonyl (Fmoc) chemistry, *O*-(7-azabenzotriazol-1-yl)-1,1,3,3-tetramethyluroniumhexafluorophosphate/diisopropylethylamine activation, and an Fmoc Asn(tri)-polyethylene glycol-phosphatidylserine resin (0.1-mmol scale). All amino acids were coupled twice. The polypeptide was released from the resin by trifluoroacetic acid/phenol/H_2O/ethanedithiol/triisopropylethylsilane (85/5/5/2.5/2.5), precipitated in cold diethyl ether, dissolved in aqueous 0.08% trifluoroacetic acid, and kept for 1 h at room temperature in order to remove the indole protective group from trytophan. After lyophilization, the crude polypeptide was dissolved in 6 M guanidine hydrochloride, 0.1 M Tris acetate, pH 8.5, and 16% 2-mercaptoethanol, stirred at 37 °C for 2 h, and then acidified to pH 4. The reduced chemokine was purified on a preparative medium pressure liquid chromatography C18 column (313 × 26 mm, 100-Å, 20-μm Nucleoprep, Macherey-Nagel, Düren, Germany) using a 20–80% linear gradient of acetonitrile in 0.08% aqueous trifluoroacetic acid. Fractions of the major 214-nm UV-absorbing peak were analyzed by high pressure liquid chomatography, and pure fractions were pooled and lyophilized. The reduced chemokine was solubilized in 6 M guanidine hydrochloride, 0.1 M Tris acetate, pH 8.5, then rapidly diluted into 0.1 M Tris acetate buffer, pH 8.5. The final chemokine concentration was 0.4 mg/ml in 1 M guanidine hydrochloride, 0.1 M Tris acetate, pH 8.5. The solution was stirred overnight to allow chemokine folding and gentle air oxidation of the four cysteines. The folded chemokine was submitted to medium pressure liquid chromatography purification as described above. Final purity of MIP-1β was superior to 95% as judged by high pressure liquid chromatography. The molecular weight determined by ion spray mass spectrometry was 7814.23 ± 0.27 (calculated, 7814.79). Protein concentration was determined by amino acids analysis (6300 Beckman amino acid analyzer) following 6 N HCl, 0.2% phenol hydrolysis with norleucine as internal standard. The total yield was 3.5 mg. All chemicals used for the synthesis were purchased from Perspective-Perkin-Elmer. The binding properties of synthetic RANTES and MIP-1β as well as their biological activity were identical to those of commercially available preparations. Other chemokines were purchased from R & D Systems.

Expression of Mutant Receptors in CHO-K1 Cells—CHO-K1 cells were cultured using Ham's F-12 medium supplemented with 10% fetal calf serum (Life Technologies, Inc.), 100 units/ml penicillin, and 100 μg/ml streptomycin (Life Technologies, Inc.). A plasmid encoding apoaequorin under control of the SRα promoter (58) was transfected into CHO-K1 cells, using Fugene 6 (Roche Molecular Biochemicals) and zeocin (250 μg/ml, Invitrogen), and selection was initiated 2 days later. Individual clones were isolated 3 weeks after transfection, and the most responding clone was selected on the basis of its functional response (luminescence signal) to ionomycin A (100 nM) and ATP (10 μM). Constructs encoding wild-type CCR5 or mutant receptors in the pEFIN bicistronic vector were further transfected using Fugene 6 in this apoaequorin-expressing cell line. Selection of transfected cells was made for 14 days with 400 μg/ml G418 (Life Technologies, Inc.), and the population of mixed cell clones expressing wild-type or mutant receptors was used for binding and functional studies. The level of receptor expression was measured by quantitative flow cytometry using standardized microbeads (Sigma) and three antibodies directed against CCR5. The phycoerythrin-conjugated 2D7 antibody was obtained from PharMingen, and the phycoerythrin-conjugated 45531 and fluorescein-conjugated 45549 monoclonals were obtained from R & D Systems.

^{125}I-MIP-1β Binding Assays—CHO-K1 cells expressing wild-type or mutant CCR5 were collected from plates with Ca^{2+}- and Mg^{2+}-free PBS supplemented with 5 mM EDTA, gently pelleted for 2 min at 1000 × *g*, and resuspended in binding buffer (50 mM Hepes, pH 7.4, 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA). Competition binding assays were performed in Minisorb tubes (Nunc), using 0.08 or 0.24 nM ^{125}I-MIP-1β (2200 Ci/mmol, NEN Life Science Products) as tracer, variable concentrations of competitors, and 40,000 cells in a final volume of 0.1 ml. Total binding was measured in the absence of competitor, and nonspecific binding was measured with a 100-fold excess of unlabeled ligand. Samples were incubated for 90 min at 27 °C, and then bound tracer was separated by filtration through GF/B filters presoaked in 1% BSA. Filters were counted in a β-scintillation counter. Binding parameters were determined with the PRISM software (Graphpad Software) using nonlinear regression applied to a one-site competition model.

Functional Assays—Functional response to chemokines was analyzed by measuring the luminescence of aequorin as described (59). Cells were collected from plates with Ca^{2+}- and Mg^{2+}-free DMEM supplemented with 5 mM EDTA, pelleted for 2 min at 1000 × *g*, resuspended in DMEM at a density of 5×10^6 cells/ml, and incubated for 2 h in the dark in the presence of 5 μM coelenterazine H (Molecular Probes, Eugene, OR). Cells were diluted 7.5-fold before use. Agonists in a volume of 50 μl of DMEM were added to 50 μl of cell suspension (33,000 cells), and luminescence was measured for 30 s in a Berthold luminometer.

Inhibition of HIV Fusion—Inhibition of HIV fusion was performed as described previously (60). PA317-T4 cells cotransfected with CCR5 mutants and a T7 polymerase-dependent luciferase gene were mixed with HeLa cells expressing an R5 strain Env and T7 polymerase in the presence or absence of RANTES (1 μg/ml). After 8 h, cells were lysed and assayed for luciferase activity.

Infection Assay—Plasmids encoding the HIV-1 ADA and JRFL Envs were provided by John Moore (Aaron Diamond AIDS Research Center). The NL4–3 luciferase virus backbone (pNL-Luc-E$^-$ R$^-$) was provided by Ned Landau (Aaron Diamond AIDS Research Center, New York). Luciferase reporter viruses were prepared as described previously by cotransfecting 293T cells with the indicated Env and the NL4–3 luciferase virus backbone (61). Target cells were prepared by cotransfecting

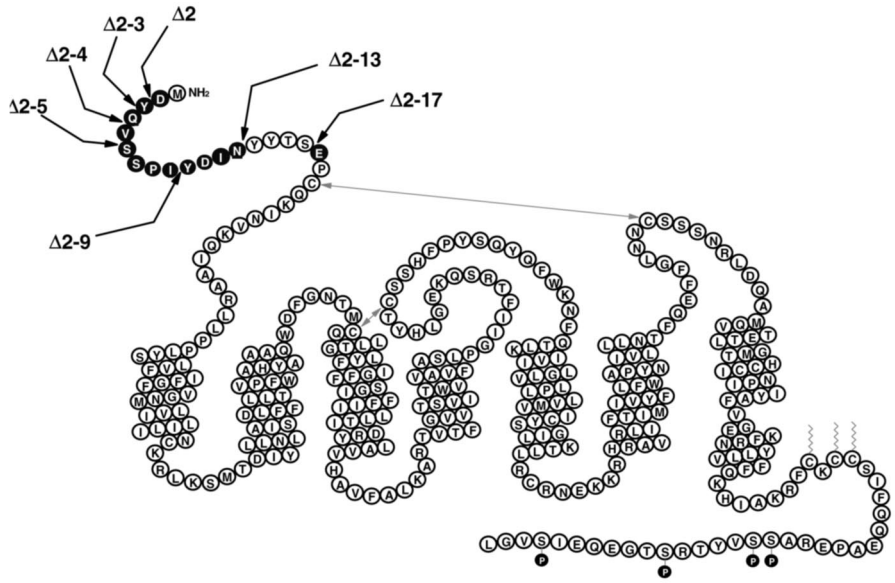

FIG. 1. **Schematic representation of CCR5.** The putative transmembrane organization of CCR5 is represented, as well as the location of deletions and alanine-substituted amino acids (in *black*) within the amino-terminal extracellular domain of the receptor.

293T cells with CD4 and a constant amount of coreceptor-encoding plasmid. Incubation was done at 37 °C. Three days post-infection, cells were lysed with 0.5% Triton X-100 in PBS and analyzed for luciferase activity.

^{125}I-gp120 Binding Assays—Soluble JRFL gp120 was iodinated as described elsewhere (56). Env binding assays were performed by resuspending 2×10^5 transfected 293T cells in a total volume of 100 μl of 50 mM Hepes, pH 7.4, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 5% BSA. Iodinated JRFL gp120 and 100 nM sCD4 were added to cells, and indubation was carried out at room temperature for 1 h. Cells were filtered through Whatman GF/C filters presoaked in 0.2% polyethyleneimine (Sigma), and washed twice with 4 ml of 50 mM Hepes, pH 7.4, 500 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$. Filters were counted in a Wallac 1470 Wizard gamma counter.

RESULTS

Truncation of the CCR5 Amino-terminal Domain Impairs Chemokine Binding and Signaling—By using chimeras between CCR5 and the closely related receptor CCR2b, we have previously identified the second extracellular loop (ECL2) of CCR5 as the main determinant of ligand specificity. Indeed, CCR5 ECL2, when introduced into a CCR2b background (2252), was able to confer high affinity binding for MIP-1α, as well as functional responses to MIP-1α, MIP-1β, and RANTES (50). Moreover, the replacement of the amino-terminal domain of CCR5 by the corresponding region of other chemokine receptors, such as CCR1, CCR2b, CXCR2 or CXCR4, did not affect MIP-1β, MIP-1α, or RANTES binding (62). These experiments suggested that the amino-terminal domain of CCR5 was not involved in ligand selectivity as is the amino terminus from other chemokine receptors such as CCR2 and CXCR2 but did not rule out that structural determinants shared by other receptors could play an important role in the high affinity binding of chemokines.

To investigate the potential involvement of the CCR5 amino terminus in ligand binding and receptor activation, stably transfected cell lines expressing amino-terminal deletions (CCR5Δ2–5, Δ2–9, Δ2–13, and Δ2–17) were generated (Fig. 1). Cell surface expression of these constructs was tested by quantitative FACS analysis using 2D7, an anti-CCR5 mAb mapping to ECL2 (36). All mutant receptors were found to be expressed at high and similar levels (Table I), allowing us to perform binding and functional studies. Quantitative FACS analysis using two other monoclonals, one (45531) directed against another region of ECL2 and the other recognizing a conformational epitope (56), did label the mutants similarly, demonstrating that the conformation of these mutant receptors is not significantly altered (data not shown). In contrast to wtCCR5, none of these truncation mutants bound detectable amounts of ^{125}I-MIP-1β when a standard tracer concentration (0.08 nM) was used. By increasing the tracer concentration to 0.24 nM, a low level of ^{125}I-MIP-1β specific binding (15% of total binding capacity) could be detected for CCR5Δ2–5, but not for CCR5Δ2–9, Δ2–13, or Δ2–17 (data not shown).

Because high affinity ligand binding is not a prerequisite for receptor activation, we also tested the ability of the truncated CCR5 constructs to respond functionally to chemokines

TABLE I
Surface expression, binding, and functional parameters of CCR5 amino-terminal mutants

Cell surface expression of CCR5 mutants was measured by quantitative FACS analysis using 2D7, a mAb mapping to the second extracellular loop (Fig. 4*A*). Values represent the number of specific antibody-binding sites (ABS) per cell and are representative of three independent experiments. The pIC_{50} ($-$log M) values were obtained from competition binding assays using 0.1 nM ^{125}I-MIP-1β as tracer (Fig. 4, *C* and *D*). Values represent the mean ± S.E. of two independent experiments. The pEC_{50} ($-$log M) values were obtained from functional dose-response curves using the aequorin assay (Figs. 3*C* and 4, *D–F*). Values represent the mean ± S.E. of three independent experiments. ND, not determined, due to the low level of ^{125}I-MIP-1β binding or weak functional response.

Construct	Surface expression 2D7 mAb 10^3 ABS	Binding affinity pIC_{50} ± S.E. $-$log (M)	Functional response pEC_{50} ± S.E. $-$log (M)
CCR5	519	9.29 ± 0.09	8.44 ± 0.33
Δ2	413	ND	7.61 ± 0.22
Δ2–3	545	ND	7.65 ± 0.25
Δ2–4	388	ND	7.22 ± 0.03
Δ2–5	440	ND	7.21 ± 0.11
Δ2–9	362	ND	ND
Δ2–13	235	ND	ND
Δ2–17	328	ND	ND
D2A	462	ND	7.62 ± 0.18
Y3A	357	ND	7.62 ± 0.11
Q4A	606	9.18 ± 0.04	8.15 ± 0.22
V5A	460	9.49 ± 0.02	8.09 ± 0.38
S6A	322	9.66 ± 0.05	8.26 ± 0.42
S7A	437	9.44 ± 0.22	8.42 ± 0.07
P8A	395	9.27 ± 0.02	8.65 ± 0.46
I9A	495	9.56 ± 0.01	8.21 ± 0.02
Y10A	385	ND	7.34 ± 0.33
D11A	272	ND	6.92 ± 0.22
I12A	488	9.35 ± 0.02	8.38 ± 0.63
N13A	409	9.39 ± 0.11	8.35 ± 0.92
E18A	329	ND	7.08 ± 0.01

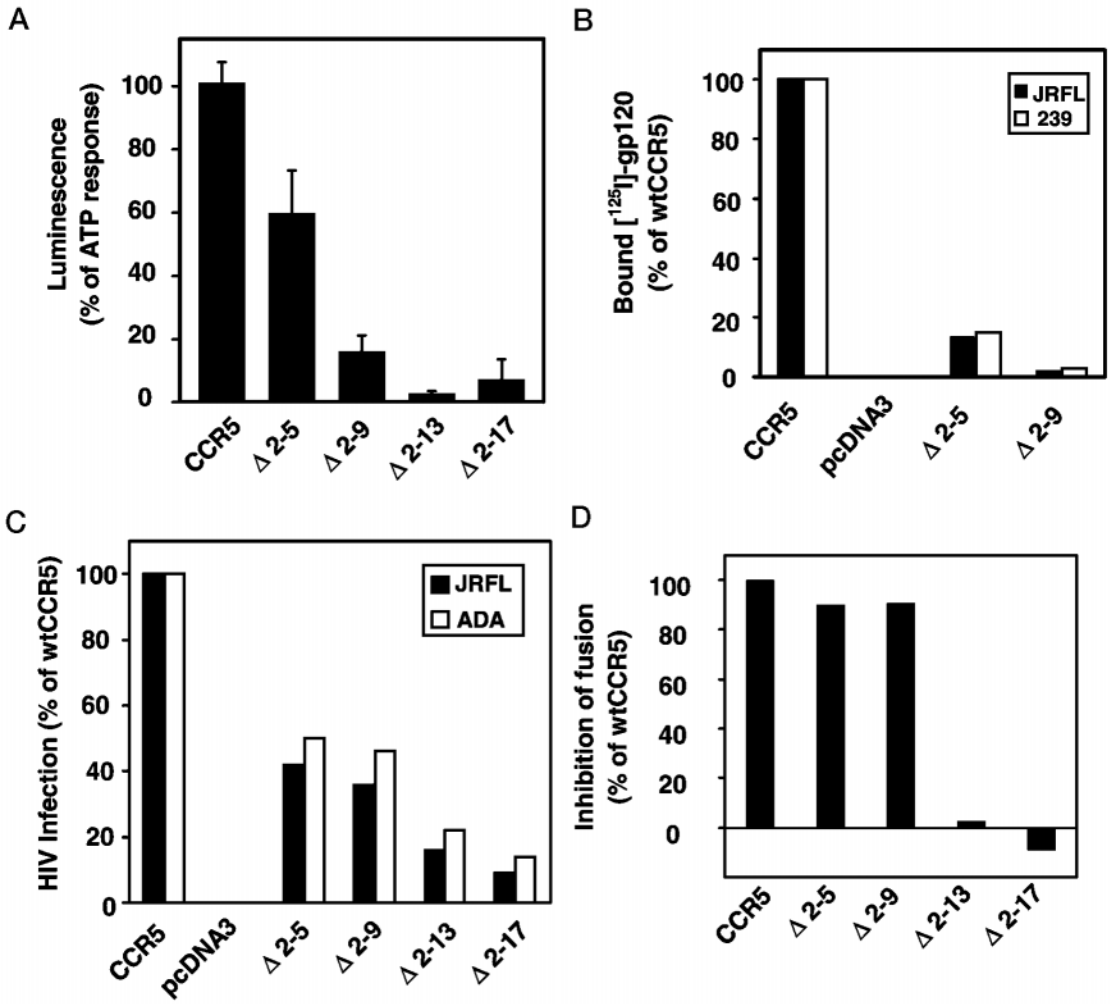

FIG. 2. **Functional analysis of CCR5 amino-terminal deletion mutants.** *A*, the functional response of amino-terminal truncation mutants (CCR5Δ2–5, Δ2–9, Δ2–13, and Δ2–17) to 100 nM MIP-1β was assayed using cell lines coexpressing the receptor and apo-aequorin. Light emission resulting from the activation of the apo-aequorin-coelenterazine complex following intracellular calcium release was recorded by a luminometer. The data were normalized to basal luminescence (0%) and maximal luminescence as determined by the activation of endogenous P2 receptors by saturating concentration (10 μM) of ATP (100%). All points were run in triplicate (*error bars,* S.E.). The data shown represent a typical experiment out of 3 performed independently. *B,* binding of ^{125}I-gp120 from R5 strains of HIV-1 (JRFL) and SIV (239) was assayed on 293T cells expressing wtCCR5 and deletion mutants in the presence of sCD4. Results were normalized as the percentage of gp120 specific binding to wtCCR5 and represent the mean of two independent experiments. *C*, the ability of luciferase reporter viruses pseudo-typed with R5 HIV-1 Envs (JRFL or ADA) to infect U87 cells expressing CD4 and CCR5 deletion mutants was assayed. The data were normalized to the luminescence obtained with wtCCR5 (100%) and represent the mean of two independent experiments. *D,* the ability of RANTES to inhibit the coreceptor function of CCR5 amino-terminal truncations was also investigated. PA317 cells expressing CD4, CCR5 mutants, and luciferase under control of the T7 promoter were mixed with HeLa cells expressing T7 polymerase and the JRFL Env glycoprotein in the presence or absence of 1 μg/ml RANTES. Cell fusion was quantified by measuring the luciferase activity 8 h after mixing. The results were normalized as the percentage of RANTES-mediated inhibition using wtCCR5 as coreceptor.

(MIP-1β or RANTES) by using two assays based on different principles as follows: a microphysiometer assay that measures the rise in metabolic activity following activation of intracellular cascades (9), and a reporter assay in which a luminescent signal is produced following the activation of an apo-aequorin-coelenterazine complex by the intracellular release of calcium (59). Both functional assays gave similar results that correlated well with the chemokine binding. The metabolic response to 100 nM MIP-1β (Fig. 2*A*) or RANTES (not shown) was reduced by 40% for CCR5Δ2–5, 80% for CCR5Δ2–9, and more than 90% for CCR5Δ2–13 and Δ2–17. The EC_{50} was evaluated to 62 nM for CCR5Δ2–5 and well over 500 nM for CCR5Δ2–9, Δ2–13, and Δ2–17, as compared with 4.4 nM for wtCCR5. Thus, truncation of the CCR5 amino-terminal domain largely abrogated high affinity chemokine binding as well as functional response to these ligands.

Amino-terminal Truncations Impair Coreceptor Activity and the Chemokine-mediated Inhibition of This Activity—The same constructs were tested for their ability to bind ^{125}I-gp120 in the presence of saturating concentrations of soluble CD4. Env-specific binding was decreased by more than 80% for CCR5Δ2–5, and no binding was detected for further truncations (Fig. 2*B* and data not shown). We next investigated whether the reduction of Env binding was correlated with coreceptor function. Luciferase reporter viruses pseudotyped with two R5 strain Envs (ADA and JRFL) were used to infect human U87 cells expressing CD4 and CCR5 truncations. As shown in Fig. 2*C*, truncation of the first 4 amino acids resulted in a 50% decrease of infection efficiency. A further drop in coreceptor activity was observed for CCR5Δ2–13 (20% of wtCCR5), suggesting that residues 2–5 and 9–13 played an important role in coreceptor function.

We further investigated whether the lower affinity of CCR5 mutants for chemokines correlated with an impaired ability of the ligands to inhibit HIV fusion. In accordance with gp120 binding and infection assays, sequential truncation of the

CCR5 amino terminus gradually impaired the fusion activity supported by these constructs (data not shown). Inhibition of HIV fusion by RANTES (1 μg/ml) was kept at a level similar to that of wtCCR5 for CCR5Δ2–5 and Δ2–9 (Fig. 2*D*), in keeping with the ability of these mutants to bind RANTES. In contrast, CCR5Δ2–13 and Δ2–17 were insensitive to the fusion inhibitory properties of RANTES, in keeping with the inability of these mutants to bind RANTES. Thus, as expected, inhibition of HIV fusion appeared to be correlated with the ability of chemokines to bind and activate CCR5 mutants.

From this first set of data, multiple residues within the first 13 amino acids of CCR5 appeared to be important for chemokine binding as well as in the interaction with the HIV Env glycoprotein. However, the contribution of the amino-terminal domain to chemokine binding may involve structural determinants shared by other receptors, since substitution of the CCR5 amino-terminal domain with the corresponding region from divergent receptors has little effect on chemokine binding and receptor activation (50, 62).

Structural Determinants Composed of Charged and Hydrophobic Residues in CCR5 Amino Terminus Are Necessary for Both Chemokine and gp120 High Affinity Binding—Since residues 2 to 5 appeared necessary for high affinity binding of chemokines, we investigated the effects of intermediate truncations (CCR5Δ2, Δ2–3 and Δ2–4) on chemokine binding and functional responses. All constructs were expressed at high levels in stable cell lines as revealed by FACS analysis (Table I). Specific binding of ^{125}I-MIP-1β (0.24 nM) was reduced by 60% for CCR5Δ2 and Δ2–3 and by more than 80% for CCR5Δ2–4 and Δ2–5 as compared with wtCCR5 (Fig. 3*A*). Competition binding experiments confirmed the reduced affinity of CCR5Δ2–3 for MIP-1β as compared with wtCCR5 (Fig. 3*B*). It was not possible to perform competition binding curves for CCR5Δ2, CCR5Δ2–4, and CCRΔ2–5, and binding parameters could not be determined accurately for any of these constructs. In the aequorin-based functional assay (Fig. 3*C* and Table I), the EC_{50} for CCR5Δ2 and Δ2–3 was shifted 5-fold, whereas that of CCR5Δ2–4 and Δ2–5 by over an order of magnitude, in good agreement with the binding data.

We also assessed the relative contribution of each amino acid from Asp-2 to Asn-13, as well as Glu-18, to chemokine and Env binding using alanine-scanning mutagenesis. As for truncation mutants, stable cell lines coexpressing apo-aequorin and the mutants were established and tested for surface expression by FACS analysis, using the 2D7 mAb. In each pool, a majority of transfected cells expressed high receptor levels (Table I), although in some cases a small proportion of cells expressed undetectable levels of receptor mutants (Fig. 4*A*). FACS analysis using conformation-dependent antibodies was allowed to exclude obvious alteration of the receptor tridimensional structure (data not shown).

As shown in Fig. 4*B*, specific binding of 0.08 nM ^{125}I-MIP-1β was similar to wtCCR5 for the V5A, S6A, P8A, I9A, and I12A mutants, increased for Q4A, slightly decreased (40 to 50% reduction) for Y3A, S7A, and N13A, and more severely affected (over 70% reduction) D2A, Y10A, D11A, and E18A. Competition binding experiments showed that the CCR5wt, Q4A, V5A, S6A, S7A, P8A, I9A, I12A, and N13A cell lines were characterized by a similar IC_{50} (Fig. 4, *C* and *D*, Table I), demonstrating that differences in receptor expression levels could explain the variations in bound ^{125}I-MIP-1β, in agreement with FACS results. We could not obtain reliable binding parameters for the D2A, Y3A, Y10A, D11A, and E18A constructs from competition binding experiments as a consequence of the low level of specific ^{125}I-MIP-1β binding, even when higher tracer concentrations were used.

In order to test whether receptor activation could be dissociated from ligand binding in some mutants, the functional response to chemokines was tested using the aequorin-based assay. Alanine substitution mutants that bound MIP-1β with affinities similar to that of wtCCR5 responded functionally with comparable concentration-response curves (Fig. 4, *E—G,* and Table I). The EC_{50} of the D2A- and Y3A-expressing cells was shifted to higher concentrations (25 nM) as compared with wtCCR5 (3.6 nM) (Fig. 4*E*). The EC_{50} of the Y10A-, D11A-, and E18A-expressing cells was more severely affected (EC_{50} of 45.7, 120, and 83.2 nM, respectively) (Fig. 4*G* and Table I), once again consistent with the binding data. These data suggest that multiple hydrophobic and negatively charged residues within the first 20 amino acids of CCR5 contribute to the binding site for chemokines.

We next investigated the importance of these amino-terminal residues in the binding of macrophage tropic HIV-1 (JRFL) and SIV (239) gp120. 293T cells transfected with wtCCR5 or alanine substitution mutants were tested for their ability to bind ^{125}I-JRFL gp120 (0.5 nM) in the presence of an excess of sCD4 (100 nM) (Fig. 5). These constructs were shown to be expressed at levels comparable to wtCCR5 in 293T cells by FACS analysis (56). Interestingly, the residues involved in chemokine binding were found to be involved as well in gp120 binding. The D2A and Y3A mutants displayed over 50% reduction of specific binding for both M-tropic envelopes, whereas more than 90% reduction was observed for Y10A and D11A. The substitution of Glu-18 (E18A) affected more severely JRFL Env binding (90%) than SIVmac239 Env binding (50%). We note, however, that the global structure of these mutants is unlikely to be dramatically altered since they react at near wild-type levels with conformational antibodies directed to the loops of the receptor (Ref. 56 and data not shown).

DISCUSSION

CCR5, a receptor activated by MIP-1α, MIP-1β, RANTES, MCP-2, and MCP-4, also constitutes the main coreceptor for macrophage tropic strains of HIV-1. Because CCR5 ligands and analogs are potent inhibitors of HIV infection, understanding how CCR5 interacts with chemokines and gp120 could help in the design of more potent inhibitors of virus entry. By using CCR5-CCR2b chimeras, we have previously shown that the amino-terminal domain of CCR5 plays an important role in coreceptor function (40), whereas the second extracellular loop (ECL2) of the receptor is the major determinant of ligand specificity (50). Other studies have shown the involvement of specific residues, particularly within the amino-terminal region, involved in coreceptor function (44–49). In order to analyze further the role of specific amino acids in the chemokine binding properties of CCR5 and to determine whether common amino acids are involved in the interaction with chemokines and gp120, we have generated truncation and alanine substitution mutants of the CCR5 amino-terminal domain.

Our present and previous (50) results, as well as those from others, clearly implicated the second extracellular loop of CCR5 as being critically important for chemokine binding and selectivity. For example, the 2252 chimera that contains the second extracellular loop of CCR5 in a CCR2b background exhibits high affinity binding and normal functional response to CCR5 ligands (50). In addition, a number of point mutations within the ECL2 loop were found to dramatically affect CCR5 function.[2] Moreover, mAbs recognizing ECL2 compete much more efficiently for chemokine binding than mAbs directed to other parts of the receptor, including the amino-terminal domain (36,

[2] C. Blanpain, J. Vakili, and M. Parmentier, unpublished observations.

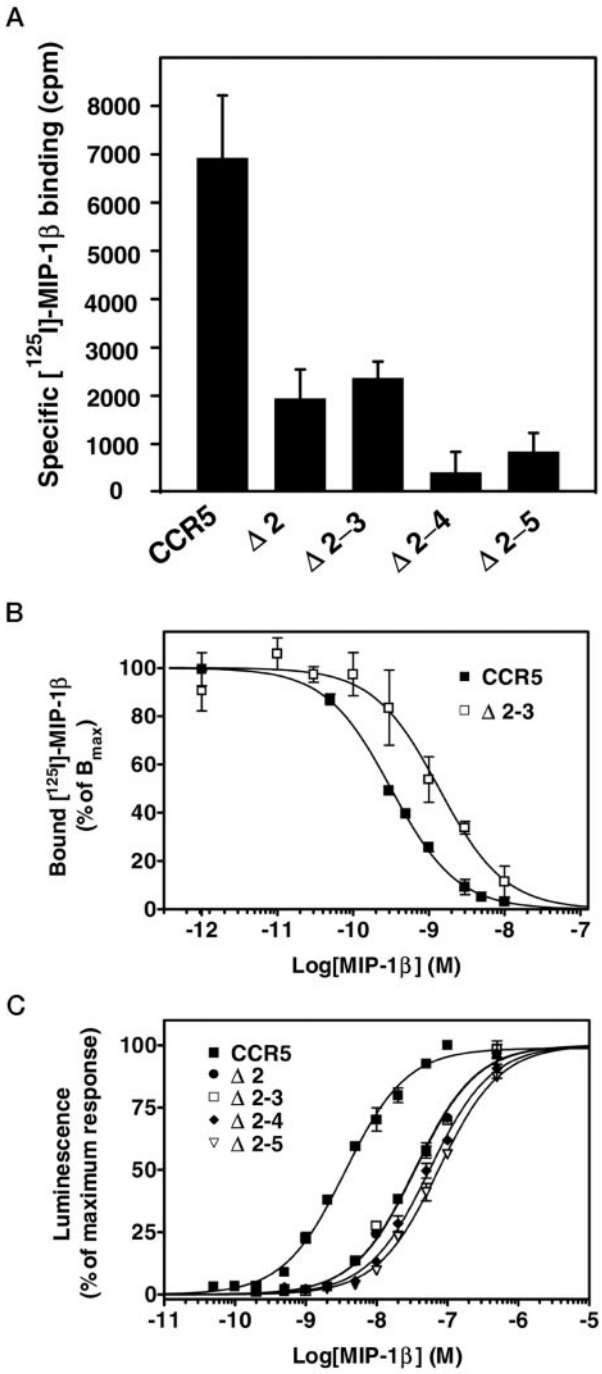

FIG. 3. **MIP-1β binding and functional properties of CCR5 amino-terminal truncations.** *A,* CHO-K1 cell lines stably transfected with apo-aequorin and CCR5 amino-terminal truncations (Δ2, Δ2–3, Δ2–4, and Δ2–5) were tested for their ability to bind ^{125}I-MIP-1β. Specific binding was determined using a tracer concentration of 0.24 and 24 nM unlabeled MIP-1β as competitor. All points were performed in triplicate (*error bars,* S.E.). The data are representative of two independent experiments. *B,* a competition curve was established for CCR5Δ2–3. The data were normalized to nonspecific (0%) and specific binding (100%) and analyzed by the Graphpad Prism software. All points were run in triplicate (*error bars,* S.E.). The displayed curves are representative of 2 independent experiments. *C,* the functional response of the cell lines coexpressing apo-aequorin and CCR5 mutants (Δ2, Δ2–3, Δ2–4, and Δ2–5) was tested following MIP-1β addition. The luminescent signal resulting from the activation of the apo-aequorin-coelenterazine complex was recorded for 30 s in a luminometer. Results were analyzed by non-linear regression using the Graphpad Prism software. The data were normalized for basal (0%) and maximal luminescence (100%). All points were run in triplicate (*error bars,* S.E.). The displayed curves represent a typical experiment out of 3 performed independently.

56). Thus, the progressive loss of binding and functional activity to chemokines resulting from amino-terminal truncations of CCR5 was surprising, whereas substitution of the CCR5 amino-terminal domain with divergent sequences is tolerated, elimination of the amino-terminal domain is not. These observations suggest that the amino-terminal domains of multiple chemokine receptors share conserved structural features that support chemokine binding. Alanine substitutions made it possible to determine more precisely which residues account for these complex interactions.

Three determinants formed by negatively charged and aromatic residues were found to be important for chemokine binding. The first determinant was composed of Asp-2 and Tyr-3. The role of Asp-2 in chemokine binding was demonstrated by the loss of function conferred by its deletion (Δ2) or substitution (D2A) both in binding and functional assays. The Tyr-3 substitution (Y3A) affected CCR5 function similarly to Asp-2 substitution, but no additive effect was observed following the simultaneous deletion of both Asp-2 and Tyr-3 (Δ2–3) as compared with Δ2 alone, suggesting that these two amino acids contribute to a single binding site. A second motif important for chemokine binding was composed of Tyr-10 and Asp-11. The failure of RANTES to abrogate the residual coreceptor activity of the CCR5Δ2–13 mutant and the very low functional response of this mutant to high concentrations (100 nM) of MIP-1β or RANTES suggested an important role of amino acids 10–13 in chemokine binding. Indeed, mutants Y10A and D11A exhibited a marked decrease in chemokine binding and signaling; almost undetectable levels of ^{125}I-MIP-1β binding were obtained for these mutants, and their EC_{50} for chemokines was shifted by about 1 log. In accordance with the important role of these residues, we have recently shown that CTC8, a mAb recognizing an amino-terminal epitope involving Tyr-10 to Asn-13, competed efficiently with ^{125}I-MIP-1β and ^{125}I-JRFL gp120 binding (56). The third motif involved Glu-18, since substitution of this residue (E18A) affected chemokine binding and the resulting functional response similarly. No aromatic residue is directly adjacent to Glu-18, but Tyr-15 was recently shown to contribute to MIP-1α binding (46).

Taken together, our results indicate that motifs of negatively charged and aromatic residues located within the CCR5 amino-terminal domain contribute to the high affinity binding of chemokines. It is interesting to note that some Tyr residues in the CCR5 amino-terminal domain can be sulfated (63), including Tyr-10, which would transform otherwise hydrophobic residues into negatively charged ones. However, it appears that the precise location of these motifs is not of primary importance, since the amino-terminal domain of CCR5 can be exchanged for that of other chemokine receptors, including CCR1, CCR2b, CXCR2, or CXCR4, without significant alteration of the chemokine binding ability (50, 62). The amino-terminal domain of each of these receptors contains similar motifs of negatively charged and aromatic residues, although these motifs cannot be aligned precisely with those present in the CCR5 sequence. Interestingly, the NMR structure of the complex formed by interleukin-8 and a peptide derived from the CXCR1 amino terminus revealed that the interacting domains involve hydrophobic and charged residues (64).

We have shown previously that chimeras consisting of the amino-terminal domain of CCR5 on a CCR2b background retained coreceptor activity for M-tropic HIV-1 strains, demonstrating the major role of the CCR5 amino terminus in this process (40). This observation was confirmed by other groups (42, 65), and the role of specific amino acids within the CCR5 amino terminus was further demonstrated (44, 46–48). By using our set of amino terminus truncations and alanine sub-

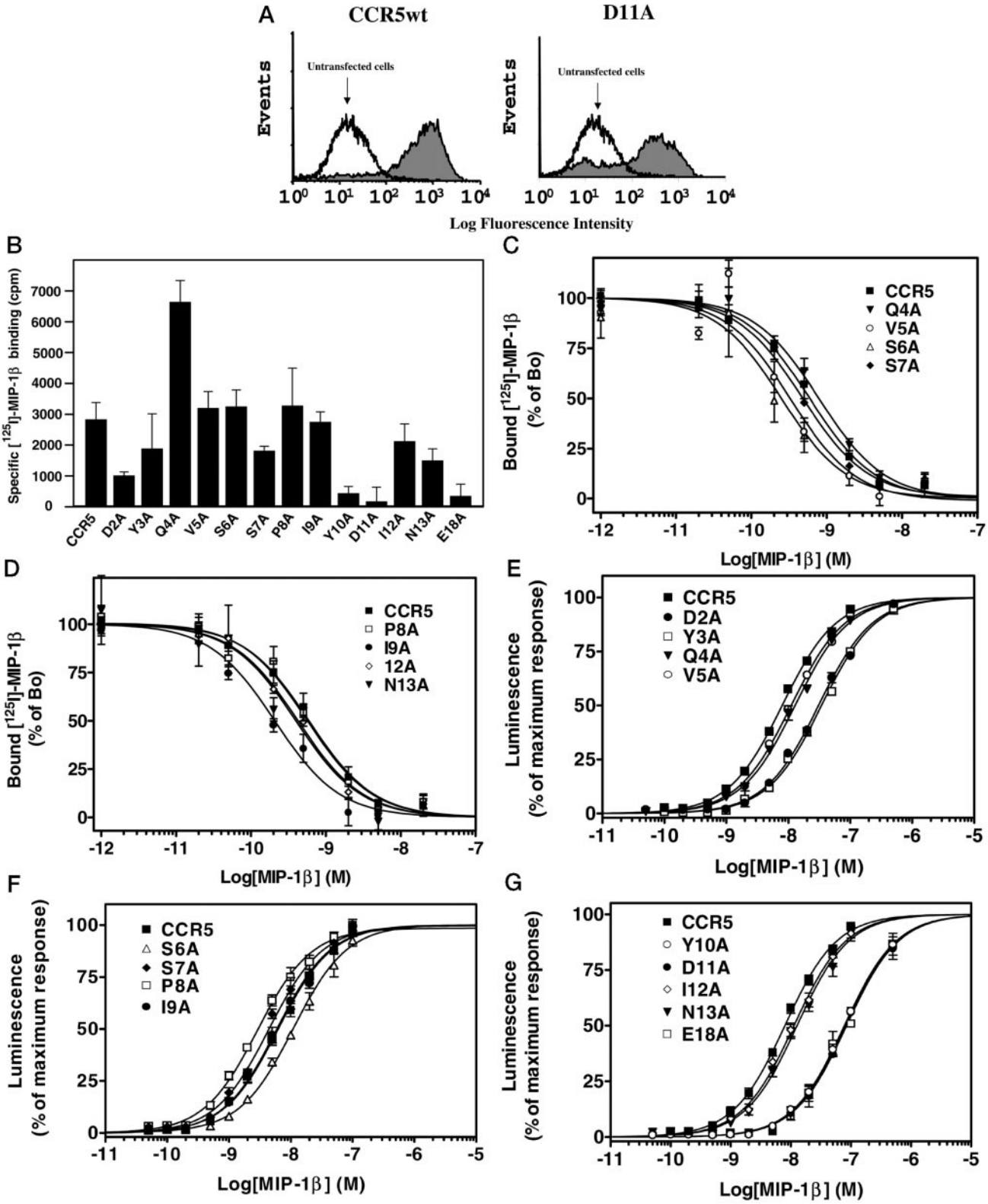

FIG. 4. **Surface expression and chemokine binding properties of alanine substitution mutants.** *A,* cell surface expression of wtCCR5 and the D11A mutant as analyzed by FACS using 2D7-PE, a mAb mapping to the second extracellular loop of the receptor. All mutant receptors were analyzed, and the displayed patterns are representative of the surface expression observed for the various mutants. Staining of untransfected cells with 2D7 was used as a negative control. These experiments were performed twice. *B*, CHO-K1 cell lines expressing CCR5 amino-terminal alanine substitution mutants were tested for ^{125}I-MIP-1β binding using 0.08 nM ^{125}I-MIP-1β as tracer. All points were performed in triplicates (*error bars,* S.E.). Data are representative of two independent experiments. *C* and *D,* competition binding curves were established for mutants whenever possible. Low binding levels made it impossible to derive reliable curves for D2A, Y3A, Y10A, D11A, and E18A. Results were analyzed by the Graphpad Prism software, using a single site model, and the data were normalized for nonspecific (0%) and specific binding in the absence of competitor (100%). All points were run in triplicate (*error bars,* S.E.). The presented curves are representative of 2 independent experiments. *E—G,* the functional response to MIP-1β of CHO-K1 cells expressing CCR5 mutants was tested in the aequorin assay as described for Fig. 3*C*. All points were run in triplicate (*error bars,* S.E.). The displayed curves represent typical experiments out of 3 performed independently for each mutant.

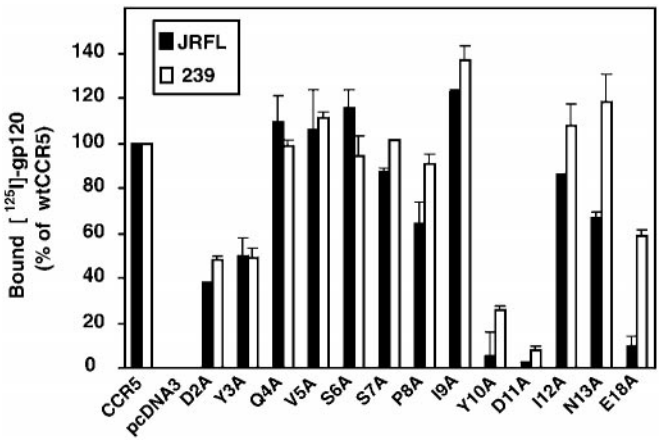

FIG. 5. **gp120 binding to CCR5 alanine substitution mutants.** 293T cells transfected with wtCCR5 or alanine substitution mutants were tested for their ability to bind ^{125}I-gp120 from the M-tropic HIV-1 strain JRFL and SIV strain 239, in the presence of soluble CD4 (100 nM). The data were normalized for the specific binding on wtCCR5 (100%), after deduction of nonspecific binding (bound ^{125}I-gp120 on cells transfected with the pcDNA3 vector alone). Results represent the mean and range of two experiments performed separately.

stitution mutants, and in accordance with other data in the recent literature (46), we have identified here a number of residues that play an important role in gp120 high affinity binding and coreceptor function. Interestingly, the substitution of amino acids involved in chemokine binding was found to affect gp120 binding and coreceptor activity as well (46–48). The D2A and Y3A mutants exhibited strong reductions (50–60%) in HIV-1 and SIV gp120 binding. In accordance with the role of this motif, the CTC5 mAb, mapping to Asp-2, blocked JRFL Env binding by more than 80% (56). Substitutions of Tyr-10 or Asp-11 by alanine resulted in a strong impairment (over 90%) of gp120 binding, in agreement with the reported alteration of coreceptor function of these mutants (46). Interestingly, rhesus macaque CCR5 is characterized by an Asp-13—Tyr-14 motif (instead of Asn-13—Tyr-14 in human) that has recently been shown to participate directly to the CD4-independent binding of SIV gp120 (19–21). Once again, Asp-Tyr motifs appear to be involved in the interaction between CCR5 and lentivirus gp120. The conserved structure of HIV gp120 glycoprotein that is involved in CCR5 binding (66) includes a number of basic and hydrophobic residues, and a conserved arginine in the gp120 V3 loop is essential for virus entry (67). It will be interesting to determine whether the hydrophobic and negatively charged residues of the amino terminus of HIV coreceptors and hydrophobic and positively charged residues of gp120 are involved in the direct interaction between these proteins.

The common residues involved in chemokine and gp120 binding also contribute to the epitopes recognized by mAbs directed at the CCR5 amino terminus. Asp-2 and Tyr-3 were shown to form the epitope of CTC9 and 502 mAbs, whereas Tyr-10 and Asp-11 are key residues of the antigenic determinant for mAbs CTC2, CTC8, and CTC12 (56). This suggests that these residues form highly exposed structural determinants.

In conclusion, we have shown in this work that a common motif of hydrophobic and negatively charged residues, highly exposed to the extracellular environment, contributes to the high affinity binding sites for both chemokines and HIV-1 envelope proteins. This amino-terminal binding site complements the role played by other regions of CCR5 in chemokine binding, particularly the second extracellular loop. It can, however, be substituted by the amino terminus of other chemokine receptors that share little primary sequence identity but contain similar motifs of hydrophobic and charged residues.

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