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Tyrosine Sulfation of the Amino Terminus of CCR5 Facilitates HIV-1 Entry

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Summary

Chemokine receptors and related seven-transmembrane-segment (7TMS) receptors serve as coreceptors for entry of human and simian immunodeficiency viruses (HIV-1, HIV-2, and SIV) into target cells. Each of these otherwise diverse coreceptors contains an N-terminal region that is acidic and tyrosine rich. Here, we show that the chemokine receptor CCR5, a principal HIV-1 coreceptor, is posttranslationally modified by O-linked glycosylation and by sulfation of its N-terminal tyrosines. Sulfated tyrosines contribute to the binding of CCR5 to MIP-1α, MIP-1β, and HIV-1 gp120/CD4 complexes and to the ability of HIV-1 to enter cells expressing CCR5 and CD4. CXCR4, another important HIV-1 coreceptor, is also sulfated. Tyrosine sulfation may contribute to the natural function of many 7TMS receptors and may be a modification common to primate immunodeficiency virus coreceptors.

Introduction

Chemokine receptors belong to a larger family of G protein–coupled seven-transmembrane-segment (7TMS) receptors, which have diverse and important roles in many biological processes (Premack and Schall, 1996). The chemokine receptor CCR5 serves as a coreceptor, together with CD4, for HIV and SIV and facilitates entry of these viruses into their target cells (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). Natural ligands for CCR5—MIP-1α, MIP-1β, RANTES, and MCP-2—inhibit replication of HIV-1 isolates that use CCR5 as a coreceptor (Cocchi et al., 1995; Gong et al., 1998). In humans, HIV-1 isolates that use CCR5 predominate during the asymptomatic stages of infection (Connor et al., 1997). As infection proceeds, viruses can emerge that use other 7TMS receptors, most commonly the chemokine receptor CXCR4 (Feng et al., 1996), as coreceptors.

Primate immunodeficiency viruses have been shown to use a number of other 7TMS receptors in vitro (Choe et al., 1998b; Littman, 1998). The chemokine receptors CCR2b, CCR3, CCR8, d6, C3XCR1, and the cytomegalovirus receptor US28, as well as several orphan receptors such as apj, gpr1, gpr15/BOB, dez/ChemR23, and strl33/Bonzo can be used by some HIV-1, HIV-2, or SIV isolates, with varying efficiencies (Choe et al., 1996, 1998a; Doranz et al., 1996; Deng et al., 1997; Farzan et al., 1997a; Liao et al., 1997; Pleskoff et al., 1997; Rucker et al., 1997; Horuk et al., 1998). Common to these coreceptors is the presence of several tyrosines in their N termini that are usually adjacent to a number of acidic amino acids, a motif similar to one described to predict tyrosine sulfation (Rosenquist and Nicholas, 1993; Bundgaard et al., 1997). Scanning mutagenesis experiments have shown that these tyrosines and acidic amino acids in the N terminus of CCR5 contribute to the ability of CCR5-using HIV-1 and SIV to fuse with and enter target cells (Dragic et al., 1998; Farzan et al., 1998; Rabut et al., 1998; Ross et al., 1998). The ability of soluble complexes of the HIV-1 gp120 exterior envelope glycoprotein and CD4 to bind CCR5 is likewise dependent on these N-terminal CCR5 residues. The same studies showed that MIP-1α, one of the natural ligands of CCR5, was also sensitive to alterations in this region (Farzan et al., 1998).

Sulfate moieties have been suggested to play several roles in HIV-1 entry. The HIV-1 envelope glycoprotein gp120 binds sulfated proteoglycans on the cell surface (Roderiquez et al., 1995; Mondor et al., 1998). This association has been demonstrated to occur at, or near, the third variable (V3) loop of gp120, which largely determines viral tropism and, equivalently, coreceptor use (Hwang et al., 1991; Roderiquez et al., 1995; Choe et al., 1996). In addition, several multiply sulfated compounds have been shown to inhibit HIV-1 infection in vitro by binding to gp120, again in regions proximal to its V3 loop (Yang et al., 1996; Este et al., 1997; Zhang et al., 1998). Finally, chemokines themselves bind heparan sulfate and related glycosaminoglycans, which are thought to anchor the chemokine for recognition by the receptor-bearing leukocyte (Tanaka et al., 1993; Webb et al., 1993; Luster et al., 1995). The presence of heparan sulfate on the surface of target cells has been shown to augment the ability of CCR5's natural chemokine ligands to inhibit HIV-1 replication (Oravecz et al., 1997).

We show here that CCR5 is modified by O-linked glycosylation and by tyrosine sulfation of its N terminus. An inhibitor of tyrosine sulfation does not affect CCR5 expression, but it substantially decreases the affinity of MIP-1α, MIP-1β, and gp120/CD4 complexes for the receptor. Moreover, CCR5 variants with conservative tyrosine to phenylalanine substitutions are not sulfated

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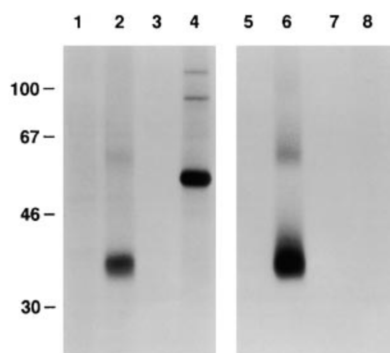

Figure 1. CCR5 Specifically Incorporates [^{35}S]Sulfate

Cf2Th cells (lanes 1, 3, 5, and 7) or Cf2Th-CD4/CCR5 cells (lanes 2, 4, 6, and 8) were labeled with [^{35}S]cysteine and methionine (lanes 1–4) or [^{35}S]sulfate (lanes 5–8). Cells were lysed, immunoprecipitated with the anti-CCR5 antibody 5C7 (lanes 1, 2, 5, and 6) or anti-CD4 antibody OKT4a (lanes 3, 4, 7, and 8), and analyzed by SDS-PAGE. Lanes 1–4 represent a 12 hr film exposure, whereas lanes 5–8 derive from the same gel exposed for 48 hr. Numbers on the left indicate the positions of molecular weight markers.

and are markedly less efficient in their ability to support the entry of several macrophage-tropic and dual-tropic viruses into target cells. We also show that full-length CXCR4, another major HIV-1 coreceptor (Feng et al., 1996), is sulfated. These data contribute to our understanding of the chemokine receptors' association with their natural ligands and with HIV-1 and SIV envelope glycoproteins.

Results

CCR5 Is Posttranslationally Modified by Sulfate

Both chemokines and HIV-1 envelope glycoproteins have been demonstrated to bind sulfate moieties (Tanaka et al., 1993; Webb et al., 1993; Roderiquez et al., 1995; Yang et al., 1996; Este et al., 1997; Mondor et al., 1998; Zhang et al., 1998). Additionally, a tyrosine-rich region in the amino terminus of CCR5, which has been shown to be important for HIV-1 and SIV entry (Dragic et al., 1998; Farzan et al., 1998; Rabut et al., 1998; Ross et al., 1998), shares similarity with tyrosine-sulfated regions of other proteins (Rosenquist and Nicholas, 1993; Bundgaard et al., 1997). We thus examined whether CCR5 was sulfated.

Cf2Th canine thymocytes that stably express CCR5 and CD4 (Cf2-CD4/CCR5 cells) were incubated with [^{35}S]cysteine and [^{35}S]methionine or sodium [^{35}S]sulfate for 24 hr. Cell lysates were immunoprecipitated with the anti-CCR5 antibody 5C7 or the anti-CD4 antibody OKT4a and analyzed by SDS-PAGE. As shown in Figure 1, although CCR5 and CD4 (lanes 2 and 4, respectively) were both clearly detectable in immunoprecipitates of cysteine- and methionine-labeled cells, only CCR5 was detected in immunoprecipitates of cells labeled with [^{35}S]sulfate (lane 6). These results show that [^{35}S]sulfate is specifically incorporated into native CCR5.

CCR5 Is Modified by O- but Not N-Linked Glycosylation

Sulfate can be incorporated into proteins at sites of N- or O-linked glycosylation or on tyrosines (Huttner, 1988). We first examined whether CCR5 was modified by N-linked glycosylation, as the third extracellular loop of the protein contains a potential N-linked glycosylation site. Consistent with other reports (Rucker et al., 1996), neither the N-glycosidase endo F nor tunicamycin treatment of Cf2Th-CD4/CCR5 cells resulted in a change in CCR5 mobility on SDS-PAGE (data not shown). By contrast, a shift in the mobility of CD4 was observed in the same tunicamycin-treated Cf2Th-CD4/CCR5 cells (data not shown).

Like most β-chemokine receptors, CCR5 also contains several extracellular regions rich in serines and threonines, which could be modified by O-linked glycosylation. In the case of CCR5, two such regions are present in its N terminus and one each is present in its second and third extracellular loops (Raport et al., 1996; Samson et al., 1996). The anti-CCR5 antibody 5C7 was used to precipitate lysates of Cf2-CCR5 cells radiolabeled with [^{35}S]cysteine and [^{35}S]methionine or with [^{35}S]sulfate. Immunoprecipitates were either treated with neuraminidase alone, a cocktail of O-glycosidases, or enzyme buffer only. As shown in Figure 2A, the mobility in SDS-PAGE of CCR5 labeled with [^{35}S]Cys/Met was increased by treatment with neuraminidase (lane 6) and more so when treated with a mixture of O-glycosidases (lane 7). The CCR5 signal from immunoprecipitates of cells labeled with [^{35}S]sulfate displayed an identical pattern with no loss of signal intensity (lanes 1–3). Thus, enzymatic treatments of CCR5 that remove O-linked glycosylation do not remove the sulfated moieties on CCR5. We conclude that CCR5 contains O-linked carbohydrates that include sialic acid, but these sugar moieties do not appear to account for the majority of CCR5 sulfation.

CCR5 Tyrosines Are Modified by Sulfate

To demonstrate that CCR5 was sulfated at its tyrosines, we extracted the [^{35}S]sulfate-labeled CCR5 band shown in Figure 1 (lane 6), hydrolyzed the peptide bonds with $Ba(OH)_2$, and performed amino acid analysis (Huttner, 1984). Hydrolyzed [^{35}S]sulfate-labeled CCR5 was mixed with unlabeled tyrosine sulfate to facilitate ninhydrin staining, and electrophoresis was performed on this mixture spotted on a thin layer chromatography (TLC) plate (Figure 2B, lanes 2 and 4). Unlabeled tyrosine and serine sulfate were run as standards (lanes 1 and 3). As shown in the autoradiograph of the TLC plate (lanes 3 and 4), most of the [^{35}S]sulfate signal comigrated with a tyrosine sulfate standard (lanes 2 and 4) faster than phenol red and slower than serine sulfate (lane 1). Taken together, these data indicate that the incorporation of [^{35}S]sulfate by CCR5 is largely or solely a result of the tyrosine sulfation.

Sulfation Inhibitor Chlorate Lowers Chemokine and gp120/CD4 Binding Affinity for CCR5 but Not CCR5 Expression

To assess the role of tyrosine sulfation in CCR5's association with its known ligands, we treated Cf2-CCR5-CD4 cells with 10 mM sodium chlorate or 10 mM magnesium sulfate for 16 hr in sulfate-free media. Chlorate is a relatively nontoxic inhibitor of sulfation (Mintz et al., 1994;

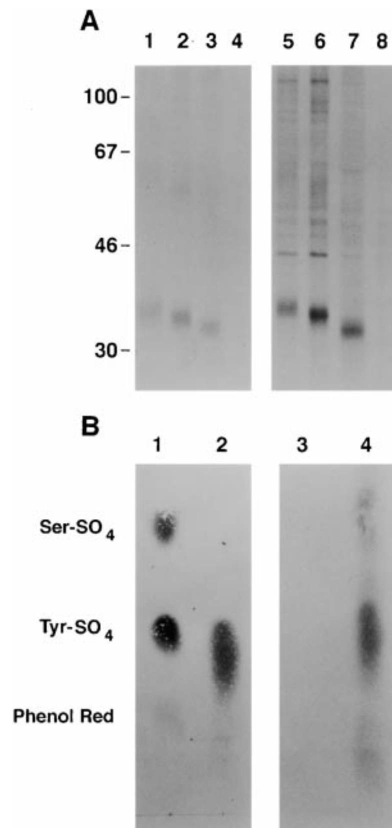

Figure 2. CCR5 Is Modified by O-Glycosylation and Tyrosine Sulfation

(A) O-glycosidases alter CCR5's mobility on SDS-PAGE. Cf2 cells (lanes 4 and 8) or Cf2Th-CD4/CCR5 cells (lanes 1–3 and 5–7) were labeled with [35S]sulfate (lanes 1–4) or [35S]cysteine and methionine (lanes 5–8), lysed, and immunoprecipitated with the anti-CCR5 antibody 5C7. Immunoprecipitates were treated with enzyme buffer alone (lanes 1, 4, 5, and 8), neuraminidase (lanes 2 and 6), or a cocktail of four O-glycosidases (lanes 3 and 7). Lanes 1–4 represent a 36 hr film exposure, whereas lanes 5–8 derive from the same gel exposed for 12 hr. Numbers on the left indicate the positions of molecular weight markers.

(B) Thin layer chromatography of tyrosine sulfate derived from full-length CCR5. [35S]sulfate-labeled CCR5 was excised from the polyacrylamide gel exposed for Figure 1, lane 6, and digested at 110°C for 24 hr in $Ba(OH)_2$ in the presence of unlabeled tyrosine sulfate. Digests were pH neutralized with sulfuric acid in phenol red, dried and resuspended in TLC electrophoresis buffer, spotted on a TLC plate, and run for 20 min at 1000 V (lanes 2 and 4) next to unlabeled tyrosine and serine sulfate (lanes 1 and 3). The TLC plate was stained with ninhydrin to visualize amino acids (lanes 1 and 2), and an autoradiograph was made using the same TLC plate (lanes 3 and 4).

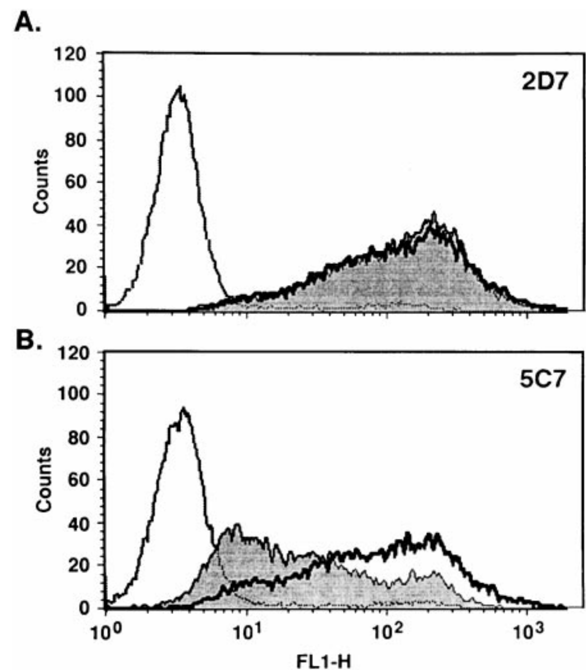

Figure 3. FACS Analysis of Chlorate-Treated Cells with the Anti-CCR5 Antibodies 5C7 and 2D7

Cf2Th-CCR5 or Cf2Th cells were incubated in sulfate-free media in the presence of 10 mM sodium chlorate or 10 mM magnesium sulfate for 16 hr. Cells were washed twice and stained with the second extracellular loop anti-CCR5 antibody 2D7 (A) or the N-terminal anti-CCR5 antibody 5C7 (B). The FACS profiles of chlorate-treated Cf2Th-CCR5 cells are indicated in filled gray, and that of sulfate-treated Cf2Th-CCR5 cells are indicated by a thick black line. The FACS profile of sulfate-treated Cf2Th control cells is indicated with a thinner black line. Mean fluorescence measured by 2D7 was 185.2, 182.5, and 9.4 for sulfate-treated Cf2Th-CCR5, chlorate-treated Cf2Th-CCR5, and control Cf2Th cells, respectively. Mean fluorescence measured by 5C7 was 143.6, 56.1, and 8.6 for sulfate-treated Cf2Th-CCR5, chlorate-treated Cf2Th-CCR5, and control Cf2Th cells, respectively. The figure shows a representative experiment of five independent FACS analyses performed in parallel with the experiments of Figures 4, in which 2D7 staining of chlorate-treated Cf2Th-CCR5 cells and Cf2Th control cells was 103% ± 8.1% and 8.9% ± 5.4%, respectively, of Cf2Th-CCR5 cells treated with sulfate. 5C7 staining of chlorate-treated Cf2Th-CCR5 cells and Cf2Th control cells was 37.3% ± 11.1% and 11.6% ± 5.7%, respectively, of Cf2Th-CCR5 cells treated with sulfate.

Pouyani and Seed, 1995). We first analyzed chlorate- and sulfate-treated cells by FACS with two CCR5 antibodies, 5C7 and 2D7, raised against human CCR5 expressed in murine cells (Wu et al., 1997). 5C7 recognizes a linear epitope at the N terminus of CCR5, whereas 2D7 recognizes a conformation-dependent epitope that maps largely to the second extracellular loop of CCR5 (Wu et al., 1997). FACS analysis of the Cf2-CCR5-CD4 cells showed that the N-terminal CCR5 antibody 5C7 is sensitive to chlorate treatment (Figure 3B), whereas the FACS profiles of the same cells treated with the 2D7 antibody could not distinguish between chlorate- and sulfate-treated cells (Figure 3A). We conclude that chlorate does not interfere with the expression of CCR5 or with its conformation insofar as the latter can be assessed by the integrity of the 2D7 epitope. However, chlorate treatment does affect 5C7 binding, indicating that in chlorate-treated cells, the CCR5 N terminus is specifically altered. 5C7 also failed to recognize a CCR5 variant in which the N-terminal tyrosines had been altered to phenylalanine (data not shown), supporting the notion that the lower recognition of CCR5 by 5C7 following chlorate treatment is due to the loss of sulfate, rather than a nonspecific effect of chlorate. The broad distribution of the 5C7 FACS profile of the chlorate-treated cells may indicate that some sulfated CCR5 is still present on these cells.

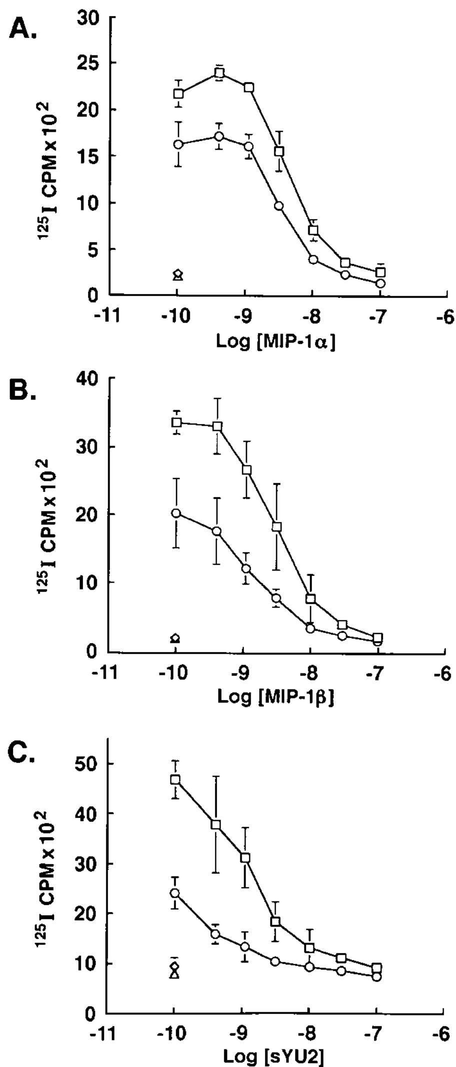

Figure 4. Association of Chemokines and gp120/CD4 Complexes with Cf2Th-CCR5 Cells Is Inhibited by Chlorate

(A) Cf2Th-CCR5 cells (0.5×10^6) were treated with sulfate or chlorate as described in Figure 6, washed twice, and incubated for 1 hr at 25°C with 0.1 nM ^{125}I-labeled MIP-1α and increasing amounts of cold MIP-1α competitor. Sulfate-treated cells are indicated with squares; chlorate-treated cells, with circles; sulfate-treated cells in the presence of 4 μg 2D7 antibody are indicated with a diamond, and Cf2Th control cells are indicated with a triangle. FACS measurements performed on the same cells in parallel with the anti-CCR5 antibodies 2D7 and 5C7 gave mean fluorescence values for chlorate-treated cells that were 106.5% ± 8.4% and 38.4% ± 0.4%, respectively, of those for sulfate-treated cells.

(B) The same experiment as described in (A), except that 0.1 nM ^{125}I-labeled MIP-1β is incubated with cold MIP-1β competitor. FACS measurements performed on the same cells in parallel with the anti-CCR5 antibodies 2D7 and 5C7 gave mean fluorescence values for chlorate treated-cells that were 103% ± 5.1% and 31.1% ± 7.0%, respectively, of those for sulfate-treated cells.

(C) Cf2Th-CCR5 cells (0.5×10^6) were treated with sulfate or chlorate as described above, washed and incubated for 1 hr at 25°C with 0.1 nM ^{125}I-YU2 gp120 and unlabeled 100 nM soluble CD4 with increasing amounts of unlabeled YU2 gp120 competitor. FACS measurements performed on the same cells in parallel with the anti-CCR5 antibodies 2D7 and 5C7 gave mean fluorescence values for chlorate-treated cells that were 97.5% ± 5.5% and 42.6% ± 5.3%, respectively, of those for sulfate-treated cells. Figures represents an average and range of two (A and B) or three (C) independent experiments.

The abilities of the CCR5 ligands MIP-1α and MIP-1β to bind to these same cells were analyzed. Cf2Th-CCR5 cells treated with chlorate (circles) bound ^{125}I-MIP-1α and ^{125}I-MIP-1β less efficiently than sulfate-treated cells (squares) (Figures 4A and 4B, respectively). FACS analysis with the 2D7 antibody run in parallel with these experiments showed no decrease in CCR5 expression in chlorate-treated cells relative to sulfate-treated cells (Figure 4 legend). CCR5-negative Cf2Th cells (triangle) and Cf2Th-CCR5 cells treated with 4 μg of the anti-CCR5 antibody 2D7 (diamond) showed little ability to bind ^{125}I MIP-1α or ^{125}I MIP-1β, demonstrating that the observed interactions were specific for CCR5. Background values of chemokine binding to both CCR5-negative cells and 2D7-treated CCR5-Cf2Th cells were unchanged in the presence or absence of chlorate (data not shown).

Previous studies showed that the gp120 envelope glycoprotein of CCR5-using HIV-1 isolates, when complexed with soluble CD4 (sCD4), bound CCR5-expressing cells with high affinity (Trkola et al., 1996; Wu et al., 1996). As shown in Figure 4C, complexes of sCD4 and soluble ^{125}I-labeled gp120 from the CCR5-using HIV-1 isolate YU2 bound chlorate-treated Cf2Th-CCR5 cells (circles) less efficiently than those treated with sulfate (squares). Again, FACS analysis run in parallel demonstrated that the anti-CCR5 antibody 2D7 stained chlorate-treated Cf2Th-CCR5 cells as efficiently as cells treated with sulfate (Figure 4C legend). In the presence of 4 μg of 2D7, ^{125}I-labeled gp120/CD4 complexes bound chlorate-treated Cf2Th cells (diamond) with the same low efficiency as Cf2Th control cells lacking CCR5 (triangles), or cells treated with 100 nM cold gp120 competitor (rightmost square and circle). These results demonstrate that chlorate interferes with the ability of gp120/CD4 complexes to bind CCR5 specifically. We conclude that chlorate treatment of Cf2Th-CCR5 cells does not interfere with the expression of CCR5 or its recognition by the conformation-dependent 2D7 antibody, but it does interfere with the ability of HIV-1 gp120 and CCR5's natural ligands to bind CCR5, consistent with an influence of sulfated tyrosines on the efficiency of these associations.

Sulfation of Specific N-Terminal Tyrosines

We next sought to determine which of the four N-terminal tyrosines of CCR5 are sulfated. To enhance expression of CCR5 variants, a codon-optimized CCR5 construct tagged at its C terminus with nine amino acids of rhodopsin (T. M. and J. S., unpublished data) was used for these studies. This construct, and variants thereof, could be efficiently precipitated with the anti-rhodopsin antibody 1D4 (Figure 5).

Initially, a series of codon-optimized CCR5 variants in which some or all of its N-terminal tyrosines had been altered to phenylalanine were assayed for their ability to incorporate sulfate. HeLa cells were transfected with codon-optimized wild-type CCR5, a construct in which all four of the N-terminal tyrosines had been altered to phenylalanine (FFFF), or constructs in which three out of four tyrosines had been altered to phenylalanine (constructs YFFF, FYFF, FFYF, and FFFY, with each letter representing the amino acid residue at position 3, 10,

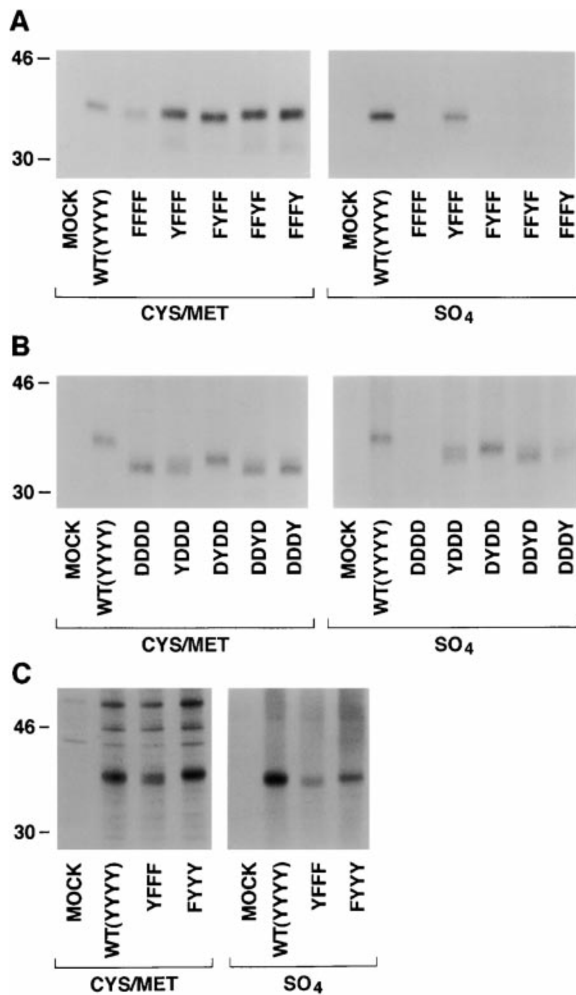

Figure 5. Sulfation of Specific CCR5 Tyrosines Is Dependent N-Terminal Residues

(A) Plasmids encoding codon-optimized CCR5 constructs containing a C-terminal tag in which some or all of the N-terminal tyrosines have been modified to phenylalanine were transiently transfected into HeLa cells, labeled with [^{35}S]cysteine and methionine or [^{35}S]sulfate, as indicated, and precipitated with the C-terminal tag antibody 1D4. Cells were transfected with vector alone, wild-type codon-optimized CCR5, a codon-optimized CCR5 construct in which all four tyrosines had been altered to phenylalanine (construct FFFF), or constructs in which three of four tyrosines had been altered to phenylalanine (constructs YFFF, FYFF, FFYF, and FFFY, which have intact tyrosines at positions 3, 10, 14 and 15, respectively).

(B) Same experiment as in (A) except that every combination of three or all four tyrosines has been altered to aspartic acids.

(C) Same experiment as in (A) except that wild-type CCR5 (construct YYYY) and constructs YFFF and FYYY are compared.

14, and 15, respectively). Cells labeled with either [^{35}S]cysteine and methionine or [^{35}S]sulfate were lysed, and the CCR5 variants were immunoprecipitated with the antibody 1D4. Figure 5A shows that wild-type CCR5 could incorporate sulfate and that construct FFFF failed to incorporate any detectable sulfate, excluding the presence of sulfate on regions other than CCR5's N terminus. Of the remaining constructs, only YFFF could efficiently incorporate sulfate, although very faint bands were visible in lanes of immunoprecipitated FYFF, FFYF, and FFFY after extended exposure (data not shown). The efficiency of the sulfate incorporation into YFFF was significantly lower than wild-type CCR5 when compared with their cysteine- and methionine-labeled counterparts. Additionally, a construct expressing the last three tyrosines in the N terminus of CCR5 (FYYY) is sulfated far more efficiently than the sum of FYFF, FFYF, and FFFY (Figures 5A and 5C). These data imply that tyrosine 3 and at least one additional tyrosine in the CCR5 N terminus are sulfated, but that the presence of phenylalanines or the absence of proximal tyrosines retards the sulfation of constructs FYFF, FFYF, and FFFY.

To examine the presence of sulfation in a different context, an additional panel of CCR5 mutants was made and tested for their ability to incorporate sulfate. A CCR5-derived construct (DDDD) in which each of the tyrosines was mutated to aspartic acid, and those in which three of four tyrosines were altered to aspartic acid (YDDD, DYDD, DDYD, and DDDY), were assayed. As shown in Figure 5B, the DDDD construct could not incorporate any detectable sulfate. Each of the constructs containing a single tyrosine could efficiently incorporate sulfate. The efficient sulfation of these constructs contrasts with the low levels of sulfation of the FYFF, FFYF, and FFFY variants. Together, these results suggest the possibility that the negative charges of the sulfate group itself contribute to the subsequent sulfation of nearby tyrosines.

Sulfated Tyrosines Contribute to the Efficiency of HIV-1 Entry

To determine a role for the CCR5 tyrosine sulfates in HIV-1 entry, we compared the ability to support HIV-1 infection of CCR5 variants shown to be sulfated (Figure 5) with analogous variants in which the sulfated tyrosine was altered to phenylalanine. Figure 6A shows that viruses pseudotyped with the envelope glycoprotein of the YU2 HIV-1 isolate were sensitive to alteration of all of the N-terminal tyrosines to phenylalanine (construct FFFF, circles in Figure 6A), at comparable expression levels of mutant and wild-type receptors. Both of the viruses pseudotyped with the envelope glycoproteins of the primary HIV-1 isolates ADA, and 89.6 exhibited greater sensitivity to the loss of the hydroxyl and/or sulfate moieties at the N terminus, with 89.6 entry values into cells expressing the FFFF construct at levels equivalent to that of CCR5-negative Cf2Th cells (circles in Figures 6B and 6C). Entry of all three viruses was at or near background when each of the N-terminal tyrosines was altered to aspartic acid (construct DDDD, triangles in Figures 6A–6C), indicating some role in HIV-1 entry for the phenyl group of the tyrosines as well.

We then compared the efficiency of virus entry associated with the DDDD construct to that of constructs in which one of the four aspartic acids present in DDDD was restored to a tyrosine. Each of these restored tyrosines is modified by sulfate (Figure 5B). The 89.6 virus exhibited background entry levels in cells expressing the FFFF, DDDD, YDDD, DYDD, DDYD, and DDDY constructs (Figure 6C and data not shown). However, cells expressing the DYDD, DDYD, and DDDY variants showed a significant increase in their ability to support infection

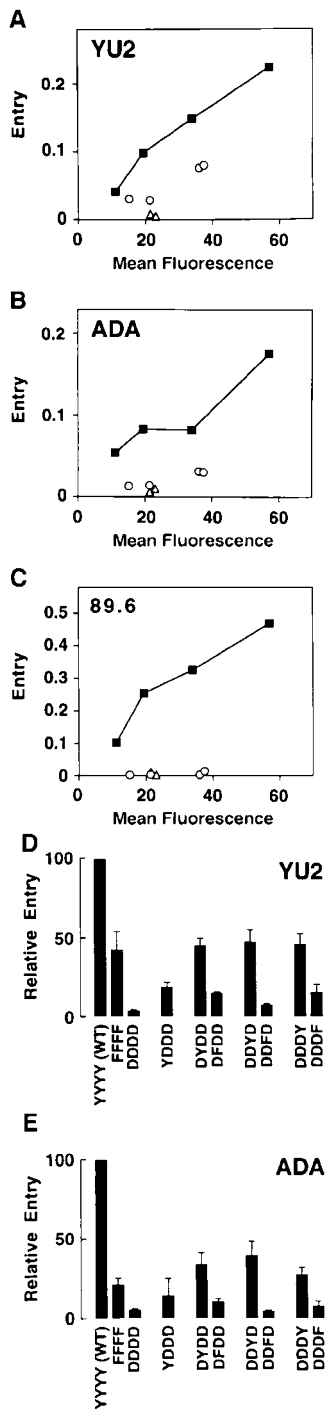

Figure 6. Sulfate on Tyrosines Contributes to the Efficiency of HIV-1 Entry

Varying amounts of plasmid DNA encoding wild-type codon-optimized CCR5 or codon-optimized CCR5 variants expressing alterations in their N-terminal tyrosines were cotransfected with a plasmid expressing CD4 into Cf2Th cells. Transfected cells were incubated for 1 hr with HIV-1 virus containing a CAT reporter gene and pseudotyped with envelope glycoproteins of the ADA, YU2, or 89.6 primary HIV-1 isolates. In parallel, an aliquot of the same cells was analyzed for variant-CCR5 receptor surface expression by FACS using the 2D7 antibody directed against the second extracellular loop of CCR5. A representative example of three experiments with similar outcomes is shown. HIV-1 entry, as measured by CAT activity, is plotted against the expression level for wild-type CCR5 (squares), for a construct in which each of the N-terminal tyrosines is changed to phenylalanine (construct FFFF, circles), and for a construct in which the same tyrosines are changed to aspartic acid (construct DDDD, triangles). The entry of viruses pseudotyped with the YU2 envelope glycoproteins is shown in (A), with the ADA envelope glycoproteins in (B), and with the 89.6 envelope glycoproteins in (C). The entry of viruses pseudotyped with the YU2 or ADA envelope glycoproteins is shown in (D) and (E), respectively, using a larger panel of CCR5 variants. Figures (D) and (E) represent an average and range of two or three experiments similar to that in (A)–(C), representing a total of at least six data points for each CCR5 construct assayed. Entry is quantified as the percent entry on equivalently expressed wild-type CCR5, a value determined from a standard curve of virus entry versus surface expression of wild-type CCR5.

by ADA and YU2 pseudotyped viruses, as compared with cells expressing the DDDD protein. The entry of the ADA and YU2 viruses into cells expressing the YDDD construct was close to, but still detectably greater than, entry into cells expressing the DDDD protein (Figures 6D and 6E). These data demonstrate that the presence of sulfated tyrosines at positions 10, 14, and 15, and to a lesser extent at position 3, contributes substantially to CCR5's ability to support infection by two primary HIV-1 viruses.

To distinguish the role of the sulfate group from that of the phenyl ring of the sulfated tyrosine, we compared directly the coreceptor function of the DYDD, DDYD, and DDDY constructs with their unsulfated analogs (DFDD, DDFD, and DDDF, respectively). As summarized in Figures 6D and 6E, the efficiency with which viruses pseudotyped with the ADA and YU2 envelope glycoproteins infected cells expressing each construct lacking a sulfate group was substantially less than that of cells expressing CCR5 variants in which the sulfate was present. Specifically, the DFDD variant supported entry of YU2 and ADA viruses at 33% and 30%, respectively, of the infection levels supported by the DYDD construct. Similarly, the DDFD protein supported YU2 and ADA virus entry at 15% and 10%, respectively, of that observed for the DDYD variant, and the DDDF protein supported YU2 and ADA virus entry at 34% and 10%, respectively, of that observed for the DDDY construct. We conclude that the presence of a sulfate moiety on tyrosines 10, 14, and 15 of CCR5 substantially enhances the entry of at least two primary HIV-1 viruses.

Sulfation of CXCR4

Because the N termini of many chemokine receptors and other 7TMS receptors that serve as primate immunodeficiency virus coreceptors contain regions that resemble those of molecules known to be tyrosine sulfated (Rosenquist and Nicholas, 1993; Bundgaard et al., 1997), we examined whether these N termini could be sulfated. Chimeric molecules consisting of a signal sequence, the N termini of several 7TMS receptors, and the hinge and final two constant domains of the human immunoglobulin IgG1 heavy chain were expressed in HeLa cells, which were radiolabeled with [^{35}S]sulfate. Cell supernatants were immunoprecipitated with protein A-Sepharose and analyzed by SDS-PAGE. The N termini of each of the

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Receptor	N-terminal sequence
CCR5	M**DY**QVSSPI**YD**IN**YY**TS**E**PCQKINVK
CCR2b	MLSTSRSRFIRNTNESGEEVTTFF**DYDY**GAPCHKFDVK
CCR3	MTTSLDTVETFGTTS**YYDD**VGLLCEKADTR
CCR8	M**DY**TL**D**LSVTTVT**DYYY**P**D**IFSSPCDAELIQ
d6	MAATASPQPLATEDADAENSSF**YYYDY**L**DE**VAFMLCRKDAVV
CXCR3	MVLEVSDHQVLNDAEVAALLENFSSS**YDY**G**E**N**E**S**D**SCCTSPPCPQDFSL
CXCR4	MEGISI**Y**TS**D**N**Y**T**EE**MGSG**DYD**SMK**E**PCFREENA
strl33	MA**E**H**DY**H**EDY**GFSSFNDSSQEEHQDFL
dez	MRM**EDEDY**NTSIS**Y**G**DEY**P**DY**L**D**SIVVLEDLSPLEA
gpr1	MEDLEETLF**EE**F**E**N**Y**S**YD**L**DYY**SL**E**SDLEEKVQL
gpr15	MDP**EE**TSV**Y**L**DYYY**ATSPNSDIRETHSHV
apj	MEEGG**D**F**D**N**YY**GA**D**NQS**E**C**EY**TDW
c5aR	MNSFN**Y**TTP**DY**GH**YDD**K**D**TLDLNTPVDKTSNTL

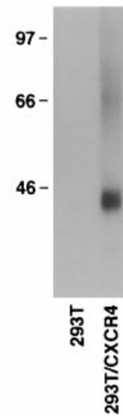

Figure 7. CXCR4 Can Incorporate [^{35}S]Sulfate

(A) The amino acid sequences of the N termini of the receptors that when fused to the Fc domains of IgG1 incorporate [^{35}S]sulfate. Tyrosines and proximal acidic amino acids are shown in bold. All receptors listed except C5aR and CXCR3 have been reported to function as HIV-1 and/or SIV coreceptors.

(B) 293T cells and 293T cells stably transfected with CXCR4 containing a C-terminal rhodopsin tag, as indicated, were labeled with [^{35}S]sulfate, immunoprecipitated with the anti-rhodopsin antibody 1D4, and analyzed by SDS-PAGE.

receptors shown in Figure 7A could efficiently incorporate [^{35}S]sulfate in these fusion constructs (data not shown). Neither the construct containing the IgG1-derived domains alone nor one containing the second extracellular loop of CCR5 could incorporate sulfate, although both were efficiently expressed (data not shown). These data raise the possibility that many 7TMS receptors, and in particular most or all of those receptors reported to serve as HIV-1 or SIV coreceptors, contain N termini that can be modified by sulfation.

To further substantiate the hypothesis that sulfation is a common modification of chemokine receptors and of HIV-1 coreceptors, we investigated the sulfation of one additional HIV-1 coreceptor, the chemokine receptor CXCR4. 293T cells or 293T cells expressing CXCR4 containing a C-terminal tag recognized by the antibody 1D4 were labeled with [^{35}S]sulfate, lysed, and protein was precipitated with 1D4 and protein A-Sepharose beads. As shown in Figure 7B, a sulfated protein of a size appropriate for CXCR4 was observed in cells expressing CXCR4, but not in the parental 293T cells. These data indicate that native CXCR4 is efficiently sulfated.

Discussion

The N terminus of CCR5 contains four tyrosines at positions 3, 10, 14, and 15 (Raport et al., 1996; Samson et al., 1996). Alteration of any one of these tyrosines interferes with the ability of CCR5-using HIV-1 viruses to enter cells expressing mutant receptors (Farzan et al., 1998; Rabut et al., 1998; Ross et al., 1998). We have shown here that two or more of the tyrosines in this region are sulfated and that chlorate, an inhibitor of this sulfation, did not affect CCR5 surface expression but interfered with the ability of gp120/CD4 complexes to bind CCR5. Moreover, we have shown that CCR5-derived variants in which sulfate was documented to be present on a single N-terminal tyrosine supported more efficient entry of HIV-1 than analogous constructs lacking only the sulfate moiety. It is therefore likely that at least one sulfated tyrosine in the N terminus of CCR5 contributes to the formation of a surface important for the association of CCR5 with the gp120 envelope glycoprotein.

A number of diverse 7TMS receptors have been shown to serve as HIV-1 or SIV coreceptors (Doms and Peiper, 1997; Choe et al., 1998b; Littman, 1998). Despite the fact that each of these receptors belongs to the G protein–coupled family of 7TMS receptors and that many of them are chemokine receptors, there is very little obvious similarity among these receptors in their external domains. One commonality is the presence of an N-terminal region rich in tyrosines and acidic amino acids. This motif has been useful in the identification of new receptors for HIV-1 and SIV (Farzan et al., 1997a; Choe et al., 1998a). The N termini of most of these receptors can be sulfated when expressed in a secreted fusion construct, and full-length CXCR4 can also incorporate sulfate. These observations suggest that HIV and perhaps SIV envelope glycoproteins conserve their ability to interact with tyrosine sulfate, implying that the region of gp120 which interacts with sulfated regions of the coreceptor is itself conserved. A strongly positive region of gp120, conserved among HIV-1 viruses of different clades and tropisms, has been shown to be important for the association of gp120 with CCR5 (Kwong et al., 1998; Rizzuto et al., 1998; Wyatt et al., 1998). Additionally, a conserved arginine in the third variable loop of gp120 has been shown to be important for entry into CXCR4- and CCR5-expressing cells (Wang et al., 1998). Some of these positively charged residues may directly interact with the large and highly polar sulfate group on the primate immunodeficiency coreceptors.

While it is probable that tyrosine sulfates in the N terminus of 7TMS molecules contribute to HIV coreceptor function, it is unlikely that they are sufficient. 7TMS molecules that have not been shown to function as HIV-1 coreceptors, like CXCR3 and the C5a receptor, nonetheless have N termini that may be tyrosine sulfated, and not every coreceptor supports the entry of every HIV-1 variant. Also, other regions of CCR5 have been shown to be important in HIV-1 entry (Doms and Peiper, 1997), notably the second extracellular loop. Although the CCR5 second extracellular loop contains three tyrosine residues, our data suggest that these are not sulfated. Exactly why HIV-1 and SIV appear to adapt only to coreceptors that contain one or more sulfated tyrosines remains to be explored further.

Glycosylation of CCR5 on serines and/or threonines

may also play some role in modulating the efficiency with which HIV-1 enters specific target cells. It has been shown that proteins expressed in resting T cells, activated T cells, and macrophages have different O-linked glycosylation patterns (Carlsson et al., 1986; Fukuda et al., 1986). A closer examination of the differential regulation of O-linked glycosylation and tyrosine sulfation in different cell types, in different individuals, and in different species may contribute to an understanding of variability in viral tropism and disease progression.

Inhibition of sulfation also interfered with the ability of MIP-1α and MIP-1β to bind cells expressing CCR5. Tyrosine sulfation of CCR5 thus appears to contribute to the binding of its natural ligands to this receptor. This result is consistent with a two-step model of chemokine binding, in which the initial association is mediated by the N terminus of the receptor, whereas greater specificity of the interaction is mediated by the remaining extracellular domains (Gerard and Gerard, 1994; Monteclaro and Charo, 1996). Such a model may explain the disruption of MIP-1α binding by alteration of CCR5 N-terminal tyrosine residues (Farzan et al., 1998), whereas substitution of the N terminus of CCR5 with that of CCR2, which is likely also sulfated, results in a receptor that retains a high affinity for MIP-1α (Farzan et al., 1997b; Samson et al., 1997).

Chemokines, including MIP-1α and MIP-1β, have been shown to bind to sulfated glycosaminoglycans (GAGs) on cells and in the extracellular matrix, and expression of GAGs on the surface of cells expressing chemokine receptors enhances the activity of the chemokine, perhaps by tethering the chemokine for interaction with multiple receptors (Tanaka et al., 1993; Webb et al., 1993; Luster et al., 1995). It is possible that sulfated N termini serve an analogous function in contexts where GAGs are not present or that they compete with GAGs for association with the chemokines to facilitate chemokine internalization. Finally, it is possible that chemokines, and perhaps HIV-1, make use of the fact that their receptors are unsulfated in the endoplasmic reticulum and Golgi apparatus, reducing the likelihood of ligand binding in these compartments in cells that express both receptor and ligand.

Our results suggest that the N termini of many 7TMS receptors, including many chemokine receptors and the C5a receptor may be tyrosine sulfated. This sulfation may contribute to the association of these negatively charged receptor N termini with their positively charged ligands. A number of other 7TMS receptors including the Duffy antigen, CCR1, CCR4, CCR6, CXCR1, C3XCR1/V28, the cytomegalovirus receptor US28, and the FMLP-like receptor 1 also contain N-terminal motifs that suggest the possible presence of sulfated tyrosines. Although a more careful examination of the function of tyrosine sulfation in each of these systems is required, this posttranslational modification may play a wider role in receptor-mediated biological processes than previously appreciated.

Experimental Procedures

Cells and Antibodies

HEK293T, HeLa, and Cf2Th cells were obtained from American Type Culture Collection (ATCC CRL 11554, ATCC CCL 2, and ATCC CRL 1430, respectively). Stable Cf2Th-CCR5 cell lines were made by transfection of Cf2Th cells with a pcDNA3 plasmid Invitrogen encoding CCR5 and selection with 0.8 mg/ml Neomycin. Stable Cf2Th-CD4/CCR5 cell lines were made by transfection of Cf2Th cells with the same pcDNA3 plasmid encoding CCR5 and a pCEP4 plasmid encoding CD4 and selected with 0.8 mg/ml Neomycin and 0.1 mg/ml Hygromycin. Stable CXCR4–293T cells were made by transfection of a pcDNA3 plasmid encoding CXCR4, and selection with 0.8 mg/ml Neomycin. Anti-CCR5 antibodies 2D7 and 5C7 were a generous gift of LeukoSite, Inc. (Wu et al., 1997). Anti-rhodopsin antibody 1D4 was a generous gift of the National Cell Culture Center.

Plasmid Constructions

Plasmids expressing CCR5 or CD4 were previously described (Choe et al., 1996). Constructs expressing fusion proteins containing fragments of CCR5 or other 7TMS receptors and the Fc domains of human IgGI were made by cloning the appropriate PCR fragments into a pCDM8 plasmid, generously provided by Dr. Brian Seed (Pouyani and Seed, 1995). DNA encoding a codon-optimized CCR5, which contains nine C-terminal residues derived from the C terminus of bovine rhodopsin, was generated by PCR and ligated into the vector pcDNA3.1 (T. M. and J. S., unpublished data).

Labeling and Immunoprecipitation of CCR5, CXCR4, and CD4

Cf2Th or Cf2Th-CD4/CCR5 cells (5×10^6) were washed three times in PBS and incubated for 24 hr at 37°C in DMEM without cysteine and methionine with 200 μCi each [^{35}S]cysteine and [^{35}S]methionine, or in sulfate-free media (ICN) with 500 μCi of [^{35}S]sulfate (Dupont NEN). Cells were lysed in 0.5% NP40-PBS and immunoprecipitated with the anti-CCR5 antibody 5C7 or the anti-CD4 antibody OKT4a and protein A-Sepharose beads. Full-length codon-optimized CCR5 variants containing a C-terminal rhodopsin tag were similarly precipitated from transiently transfected HeLa cell lysates with the anti-rhodopsin antibody 1D4. Full-length CXCR4 with the C-terminal rhodopsin tag was similarly immunoprecipitated from stably transfected 293T cells by the 1D4 antibody.

Detection of N- or O-Glycosylation on CCR5 and CD4

CCR5 or CD4 was immunoprecipitated as above. Protein-A beads and precipitates were incubated at 37°C for 2 hr in a total volume of 30 μl with neuraminidase buffer alone (50 mM sodium acetate [pH 5], 150 mM NaCl, 9 mM $CaCl_2$), with endoglycosidase F and endo F buffer (50 mM potassium phosphate [pH 6.8], 25 mM EDTA, 0.5 NP40, 0.1% SDS), with 50 mU neuraminidase in neuraminidase buffer, or with a cocktail of glycosidases containing 50 mU neuraminidase, 3 mU O-glycosidase, 25 mU β-galactosidase, and 100 mU N-aceytl-β-D-glucosaminidase in O-glycosidase buffer (PBS, 1% Triton ×100, 0.5% SDS). All enzymes were obtained from Boehringer Mannheim. Digested samples were analyzed by SDS-PAGE.

Alkaline Hydrolysis and Amino Acid Analysis of Tyrosine Sulfate from CCR5

A [^{35}S]sulfate-labeled band corresponding to CCR5 was excised from a dried polyacrylamide gel and digested in 1 N $Ba(OH)_2$ in the presence of unlabeled tyrosine sulfate (Bachem) at 110°C for 24 hr and then neutralized with sulfuric acid using phenol red as an indicator, as described (Huttner, 1984). The sample was dried and resuspended in electrophoresis buffer (5% acetic acid, 0.5% pyridine) and spotted on a cellulose-TLC plate (Macherey-Nagel) adjacent to 2 ng unlabeled tyrosine sulfate and 2 ng unlabeled serine sulfate (Bachem). Electrophoresis was performed at 1000 V for 20 min in electrophoresis buffer. The TLC plate was dried and placed on film for autoradiography, following visualization of unradiolabeled sulfated amino acids with 1% ninhydrin.

Binding of HIV-1 gp120 Envelope Glycoprotein and Chemokines to Cf2Th-CCR5 Cells

Cf2Th-CCR5 cells were washed three times in PBS and incubated 16 hr in sulfate-free media (ICN) in which either magnesium sulfate or sodium chlorate was added to a final concentration of 10 mM. Cells were detached with PBS containing 5 mM EDTA. EDTA was chelated with the same volume of PBS containing 5 mM magnesium chloride, cells were washed 2 times in PBS, and resuspended in

binding buffer (50 mM HEPES [pH 7.5], 1 mM $CaCl_2$, 5 mM $MgCl_2$, 0.5% BSA, 0.02% sodium azide) at 10^7 cells per milliliter. Fifty milliliters (0.5×10^6 cells) was then incubated for 1 hr at 25°C with 50 μl binding buffer containing 0.1 nM ^{125}I-labeled MIP-1α or MIP-1β (Dupont NEN) or ^{125}I-labeled HIV-1 YU2 envelope glycoprotein gp120 (Wu et al., 1996; Farzan et al., 1997b) and the indicated concentration of unlabeled competitor. In experiments using gp120, 100 nM unlabeled soluble CD4 was also included. Cells were washed twice in binding buffer containing 500 mM NaCl, and bound ^{125}I was counted in a gamma counter. Aliquots of the same cells used for binding experiments were also analyzed for CCR5 expression by FACS.

Assaying the Ability of CCR5 Variants to Support HIV-1 Infection

HIV entry into Cf2Th cells expressing CD4 and wild-type or mutant CCR5 proteins was measured as previously described (Choe et al., 1996). Briefly, HIV-1 proviral DNA lacking a functional envelope gene and encoding chloramphenicol acetyltransferase (CAT) as a reporter was cotransfected into HeLa cells with plasmids encoding the envelope glycoproteins of the ADA, YU2, or 89.6 primary HIV-1 isolates. Viruses harvested from cell supernatants corresponding to 2,000 to 10,000 cpm reverse transcriptase activity were incubated for 1 hr at 37°C with Cf2Th cells transfected with CD4 and with varying (1–15 μg) amounts of the coreceptor-expressing plasmid. In all transfections, the total amount of DNA was kept constant by including a plasmid encoding the pcDNA3.1 vector alone. Sixty hours postinfection cells were lysed and CAT activity was measured. In parallel with the infection experiments, coreceptor expression was quantitated by FACS analysis with the antibody 2D7. Entry values for variant CCR5 receptors are expressed as a percentage of wild-type CCR5 entry at the same cell surface expression level, calculated from a standard curve of entry versus cell surface expression of wild-type, codon-optimized CCR5.

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