A Tyrosine-sulfated Peptide Derived from the Heavy-chain CDR3 Region of an HIV-1-neutralizing Antibody Binds gp120 and Inhibits HIV-1 Infection*

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Sulfated tyrosines at the amino terminus of the principal HIV-1 coreceptor CCR5 play a critical role in its ability to bind the HIV-1 envelope glycoprotein gp120 and mediate HIV-1 entry. Human antibodies that recognize the CCR5-binding region of gp120 are also modified by tyrosine sulfation, which is necessary for their ability to neutralize HIV-1. Here we demonstrate that a sulfated peptide derived from the CDR3 region of one of these antibodies, E51, can efficiently bind gp120. Association of this peptide, pE51, with gp120 requires tyrosine sulfation and is enhanced by, but not dependent on, CD4. Alteration of any of four pE51 tyrosines, or alteration of gp120 residues 420, 421, or 422, critical for association with CCR5, prevents gp120 association with pE51. pE51 neutralizes HIV-1 more effectively than peptides based on the CCR5 amino terminus and may be useful as a fusion partner with other protein inhibitors of HIV-1 entry. Our data provide further insight into the association of the CCR5 amino terminus with gp120, show that a conserved, sulfate-binding region of gp120 is accessible to inhibitors in the absence of CD4, and suggest that soluble mimetics of CCR5 can be more effective than previously appreciated.

The entry of HIV-1 into target cells requires expression of the cellular receptor CD4 and a coreceptor, principally the chemokine receptors CCR5 or CXCR4 (1–7). Virus association with the primary receptor, CD4, triggers conformational changes in the envelope glycoprotein, gp120, that allow high affinity binding to the coreceptor (8, 9). HIV-1 variants that utilize CCR5 as a coreceptor, R5 isolates, mediate transmission and predominate throughout most of the asymptomatic phase of infection. In many cases, coincident with the decline in immune function, variants emerge that can utilize a range of coreceptors in addition to CCR5, in particular CXCR4 (10, 11). Some of these variants (R5X4 isolates) are dual tropic and utilize both principal coreceptors. Other variants (X4 isolates), including those that have been extensively passaged on tissue-culture cell lines, lose the ability to use CCR5 and enter cells primarily through CXCR4 (7, 12).

The ectodomains of CCR5 and CXCR4 consist of an amino terminus and three extracellular loops held in position by seven transmembrane helices (13). Entry of R5 HIV-1 isolates depends largely on the amino terminus and second extracellular loop of CCR5 (14–17). An acidic, tyrosine-rich sequence in the CCR5 amino terminus (residues 10–18) is especially important for virus entry (18–22). The tyrosines of this region are post-translationally modified by the addition of sulfate (23), and all R5 isolates examined to date are sensitive to the loss of one or more of these sulfates. Sulfated peptides corresponding in sequence to the CCR5 amino terminus can slow infection of R5 isolates (24, 25) and also can reconstitute the ability of a CCR5 variant lacking this region to support infection (26). In contrast, most X4 isolates are substantially less dependent on the amino terminus of CXCR4 or on the tyrosine sulfate moieties present there (27–29).

The HIV-1 envelope glycoprotein is composed of a surface (gp120) and a transmembrane (gp41) protein, processed from a single precursor (gp160) (30). Several groups of antibodies neutralize HIV-1 infection by binding gp120 or gp41 (31–34). These include antibodies whose association with gp120 is enhanced by CD4 (35). The epitopes of these CD4-induced (CD4i) antibodies overlap the coreceptor-binding region of gp120 (8, 9). The prototypes of these antibodies, 17b and 48d, are only weakly neutralizing (35, 36) and neutralize T-cell line-adapted X4 isolates more potently than primary R5 isolates (31, 35). However, the heavy-chain complementarity-determining region 3 (CDR3) of a subset of CD4i antibodies are tyrosine-rich and tyrosine-sulfated. Like the CCR5 amino terminus, sulfation of these CDR3 regions is critical for the ability of these antibodies to bind gp120 and neutralize infection. Unlike most anti-gp120-neutralizing antibodies, these tyrosine-sulfated antibodies preferentially neutralize R5 rather than X4 isolates.

Here we demonstrate that the CDR3 region of one such antibody, expressed as peptide, is capable of binding and precipitating HIV-1 gp120 and of inhibiting HIV-1 infection more efficiently than peptides based on the amino terminus of CCR5.
This antibody-derived peptide is sensitive to alterations in gp120 that disrupt association with CCR5. Association of this peptide with gp120 is sulfate-dependent and enhanced by, but not dependent on, the presence of CD4. Our data provide further structural insight into the association of gp120 with the CCR5 amino terminus and raise the possibility that the tyrosine sulfate-binding domain of HIV-1 gp120 can be a useful target of anti-viral therapies.

**EXPERIMENTAL PROCEDURES**

*Plasmids and Cells*—The CDR3 regions of the sulfated CD4i antibodies 412d, C12, 47e, E51, the unsulfated CD4i antibody 17b, and amino-terminal domains of CCR5 were generated by overlapping PCR and ligated between the NheI and BamH I restriction sites of a previously described pcDM8-derived plasmid expressing the signal sequence of CD5 and the Fc domain of human IgG1 (23). These plasmids express proteins described in the text as p412d-Ig, p17b-Ig, pC12-Ig, p47e-Ig, pE51-Ig, and pR5-Ig. Plasmids expressing α-defensin 1 or the first two immunoglobulin domains of human CD4 (defensin-Ig and CD4-Ig, respectively), fused to the Fc domain of human IgG1 were generated by PCR amplification and ligated into the same pcDM8-derived plasmid. Plasmids encoding tyrosylprotein sulfotransferase 2 (TPST-2) and single-chain variants of the plasmid expressing TPST-2, were metabolically labeled with [35S]methionine and -cysteine. Labeled supernatants were harvested 2 days post-transfection. In parallel, 293T cells were transfected with a plasmid expressing human TPST-2. We also fused to the same Fc region (pR5-Ig), were cotransfected into 293T cells with a plasmid expressing human TPST-2. We have previously shown (37) that expression of exogenous CCR5 also fused to the same Fc region (pR5-Ig), were cotransfected into 293T cells with a plasmid expressing human TPST-2. We have previously shown (37) that expression of exogenous CCR5 also fused to the same Fc region (pR5-Ig), were cotransfected into 293T cells with a plasmid expressing human TPST-2. We have previously shown (37) that expression of exogenous CCR5 also fused to the same Fc region (pR5-Ig), were cotransfected into 293T cells with a plasmid expressing human TPST-2. We have previously shown (37) that expression of exogenous CCR5 also fused to the same Fc region (pR5-Ig), were cotransfected into 293T cells with a plasmid expressing human TPST-2. We have previously shown (37) that expression of exogenous CCR5 also fused to the same Fc region (pR5-Ig), were cotransfected into 293T cells with a plasmid expressing human TPST-2. We have previously shown (37) that expression of exogenous CCR5 also fused to the same Fc region (pR5-Ig), were cotransfected into 293T cells with a plasmid expressing human TPST-2.

**RESULTS**

The amino terminus of CCR5 includes several sulfated tyrosines that are critical to its role as an HIV-1 coreceptor (23). Tyrosine-sulfated peptides based on sequences from the CCR5 amino terminus bind HIV-1 gp120 and inhibit HIV-1 infection (24, 25, 39). However, although specific, these peptides inhibit infection at concentrations of 50 to 100 μM (24, 25). A subset of Cd4-inducible antibodies also utilize tyrosine sulfate to recognize the CCR5-binding region of the HIV-1 envelope glycoprotein (37). We therefore sought to determine whether peptides derived from the sulfated heavy-chain CDR3 regions of these antibodies bound gp120 and slowed infection more efficiently than peptides based on CCR5 sequences.

A series of plasmids was generated that express the heavy-chain CDR3 regions of the CD4-inducible antibodies 412d, 17b, C12, 47e, and E51 fused to the Fc region of human IgG1 (p412d-Ig, p17b-Ig, pC12-Ig, p47e-Ig, and pE51-Ig; see Table 1). These plasmids, as well as one expressing the amino terminus of CCR5 also fused to the same Fc region (pR5-Ig), were cotransfected into 293T cells with a plasmid expressing human TPST-2. We have previously shown (37) that expression of exogenous TPST-2 enhances the sulfation of overexpressed single-chain antibody (scFv; all subsequent scFv and Fc-fusion proteins were generated in the presence of exogenous TPST-2 unless otherwise indicated). Transfected cells were metabolically labeled with [35S]methionine and -cysteine. Cell supernatants, or sera from HIV-1-infected patients, were incubated with metabolically

Infection Assay—293T cells were transfected with a plasmid encoding the genome of the NL4-3 HIV-1 isolate, modified to express the R5X4 envelope glycoprotein of the 89.6 isolate and GFP, or with an NL4-3 variant with a deletion in the env gene and cotransfected with the envelope glycoproteins of the ADA, YU2, NL4-3, JR-FL, SG3, or HXB2 isolates, or with the G protein of the vesicular stomatitis virus (18, 38). Supernatants of transfected cells were harvested, and reverse transcriptase activity was measured, as described previously (3). These supernatants, diluted to 20,000 reverse transcriptase counts per ml, were added to PM1, GHOST-CXCR4, or GHOST-CCR5 cells in the presence of the indicated concentrations of p17b-Ig, nCCR5-Ig, pΔE51-Ig, and CD4-Ig. Media were changed the following day and GFP expression in infected cells was measured 2 days later by flow cytometry.
TABLE 1
Peptide sequences characterized in this study

| Peptide origin | Sequence |
|----------------|----------|
| CCR5 N terminus | HHQVSPYIYTDYVYQFSQK |
| E51 CDR3 | NSIAQVAAADYDGGYMD |
| 412d CDR3 | PYYDYNYADYDGGYFD |
| 47e CDR3 | GGQYPDLYSPFDYNYQMD |
| C12 CDR3 | DYGPDWNYDYYDRGSGYF |
| 17b CDR3 | YYGEADKDYYGNFLK |

FIGURE 1. The CDR3 region of antibody E51 precipitates HIV-1 gp120. A, radiolabeled HIV-1 gp120 (ADA isolate) was incubated with radiolabeled supernatants from 293T cells transfected with plasmid expressing the indicated peptide-Ig fusion proteins or with patient sera. Mixtures were precipitated with protein A-Sepharose and analyzed by SDS-PAGE. C, bands that migrate with the 67-kDa marker indicate residual dimeric forms of the peptide-Ig proteins. Left, the presence and absence of radiolabeled gp120 (indicated) and radiolabeled gp120 was used to precipitate various CDR3-Ig and single-chain antibody proteins (Fig. 1B). Radiolabeled pE51-Ig, p412d-Ig, p17b-Ig, and single-chain variants of the sulfated antibodies E51 and 412d (E51 scFv, 412d scFv) were incubated with Sepharose beads conjugated to CD4-Ig and radiolabeled gp120. CD4-bound gp120 precipitated both single-chain antibodies. However, CD4-bound gp120 precipitated only pE51-Ig but not p412d-Ig or p17b-Ig. Together with the data of Fig. 1A, these data demonstrate a specific association between gp120 and pE51-Ig.

We also assayed two variants of pE51-Ig for their ability to bind HIV-1 gp120. In one, pΔE51-Ig, the first nine uncharged residues of pE51-Ig were deleted. In a second, the final three (of five) tyrosines were altered to glutamic acid. This pE51-Ig variant is described as YYEEE-Ig to indicate the amino acids present at the five tyrosines of unmodified pE51-Ig (see Table 2 for pE51-Ig variant names and peptide sequences). As shown in Fig. 1C, pΔE51-Ig immunoprecipitated gp120 with an efficiency comparable with pE51-Ig, whereas YYEEE-Ig could not detectably bind gp120.

We next assayed the ability of pE51-Ig, pΔE51-Ig, YYEEE-Ig, and patient sera to immunoprecipitate the gp120 proteins of clade B isolates YU2, JR-FL, ADA, and HXBc2. YU2, JR-FL, and ADA envelope glycoproteins utilize CCR5 to infect...
cells, whereas HXBc2 utilizes the coreceptor CXCR4. YU2 gp120 bound pE51-Ig and pH9004 E51-Ig as efficiently as ADA gp120, whereas that of JR-FL and HXBc2 bound pE51-Ig and pH9004 E51-Ig detectably, but less efficiently. In each case the addition of soluble CD4 enhanced association. YYEEE-Ig did not precipitate the gp120 of any isolate assayed. These data indicate some variation in affinity of the gp120 of clade B isolates for pE51-Ig and pH9004 E51-Ig and suggest that, like the E51 antibody, these fusion proteins bind a CD4-inducible epitope on gp120.

We further defined the determinants on pH9004 E51-Ig essential for association with gp120 (Fig. 3). We characterized a series of pH9004 E51-Ig variants, in which each of the five tyrosines were individually altered to phenylalanine or aspartic acid. Alteration of any of the five tyrosines to phenylalanine abolished detectable gp120 association. Alteration of the first four tyrosines to aspartic acid similarly abolished gp120 binding, whereas alteration of the fifth tyrosine diminished, but did not abolish, gp120 association. In this experiment we also assayed the contribution of pH9004 E51-Ig sulfation to its association with gp120 by expressing pH9004 E51-Ig in the absence of exogenous TPST-2. We have previously shown that protein overexpressed in 293T cells is not efficiently sulfated without the coexpression of exogenous TPST-2 (37). pH9004 E51-Ig expressed in the absence of exogenous TPST-2 could not detectably precipitate gp120 (Fig. 3). These data suggest a critical role for tyrosines, and for sulfate groups on these tyrosines, in the ability of pH9004 E51-Ig to immunoprecipitate gp120.

To identify the region of gp120 bound by pE51-Ig, gp120 variants were generated in which each of three gp120 residues (residues 420, 421, and 422), previously demonstrated to be critical for association of gp120 to CCR5, were altered to alanine (40). As described, each of these variants bound CD4-Ig and was recognized by patient sera with comparable efficiency (Fig. 4A). However, none of these gp120 variants detectably bound pE51-Ig (Fig. 4B). These data suggest that pE51-Ig specifically binds gp120 in a region critical for its association with CCR5. If so, they also localize the tyrosine sulfate-binding region of gp120 to residues 420 through 422.
Sulfated peptides based on the amino terminus of CCR5 specifically block HIV-1 infection, but only at 50–100 μM concentrations (24, 41). The ability of pΔE51-Ig, but not pR5-Ig, to precipitate gp120 in the presence and absence of CD4 suggested that pΔE51-Ig may be more effective at inhibiting HIV-1 infection. Accordingly, CD4-Ig, pΔE51-Ig, pR5-Ig, and p17b-Ig were compared for their ability to inhibit infection of an HIV-1 variant expressing GFP and the envelope glycoprotein of the R5X4 isolate 89.6 (Fig. 5A). Virus was incubated with the CD4-positive T-cell line PM1 and the indicated fusion proteins for 1 h and then washed. As we and others have previously reported for sulfated CCR5-derived peptides (24, 41), pR5-Ig partially inhibited infection at a concentration of ~40 μM, whereas p17b-Ig had no detectable effect on HIV-1 entry. Consistent with its ability to bind gp120, pΔE51-Ig inhibited infection markedly more efficiently than did pR5-Ig, with 50% inhibition observed at ~2 μM. Expectedly, CD4-Ig blocked infection in the nanomolar range. Similar results were obtained using a broader range of envelope glycoproteins of clade B isolates (Fig. 5, B and C). Infection mediated by the envelope glycoproteins of the R5 isolates ADA, YU2, JR-FL, and of the X4 isolates HXB2 and NL4-3 was inhibited by pΔE51-Ig, comparably that of the 89.6 isolate, and for all isolates pΔE51 inhibited entry more efficiently than pR5-Ig. However, the clade C isolate SG3 was only modestly inhibited by pΔE51. In addition, HIV-1 pseudotyped with the vesicular stomatitis virus G protein was not substantially inhibited by pΔE51 (Fig. 5C). These data indicate that pΔE51-Ig neutralizes a broad range of HIV-1 isolates more efficiently than does pR5-Ig.

**DISCUSSION**

Here we demonstrate that a peptide based on the tyrosine-sulfated CDR3 region of the antibody E51, fused to the Fc region of human IgG1, could immunoprecipitate gp120 of several clade B HIV-1 isolates. In contrast, an analogous fusion protein based on the tyrosine-sulfated amino terminus of CCR5 could not detectable precipitate gp120. This latter construct inhibited HIV-1 infection at approximately the same high concentration required for synthetic sulfated peptides, whereas the IC50 (50% inhibitory concentration) of pΔE51-Ig was at least 10-fold lower. pΔE51-Ig has several notable properties. First, although its binding to gp120 appears to be enhanced by the presence of CD4, its association did not require CD4, suggesting that a tyrosine sulfate-binding pocket on gp120 is exposed in the absence of CD4. Second, most of its five tyrosines contribute to gp120 association. Third, as is the case with CCR5 and the E51 antibody, association of pΔE51-Ig with gp120 depends on sulfation of one or more tyrosines. Fourth, binding of pΔE51-Ig was abolished by gp120 alterations that most potently interfered with CCR5 association, localizing the interaction of this peptide, and presumably the sulfated region of CCR5, to this conserved region of gp120.

The conservation of a tyrosine-sulfate-binding pocket, and its exposure in the absence of CD4, suggests that this region of pΔE51-Ig neutralizes HIV-1 infection more efficiently than a CCR5-derived peptide-Ig variant. A, PM1 cells were incubated with an HIV-1 variant expressing the envelope glycoprotein of the R5X4 89.6 isolate and GFP in the presence of the indicated concentrations of purified peptide-Ig variants. Viral entry, as measured by flow cytometry, was determined 2 days post-infection. B, HIV-1 viruses pseudotyped with the envelope glycoproteins of ADA, JR-FL, or HXB2 HIV-1 isolates were incubated with GHOST-CCR5 (ADA, JR-FL) or GHOST-CXCR4 (HXB2) cells in the presence of the indicated concentrations of the purified peptide-Ig variants. Viral entry, measured by flow cytometry, was determined 2 days post-infection. C, an experiment similar to that in B except that viruses were pseudotyped with the vesicular stomatitis virus G protein (VSV-G). Viruses pseudotyped with the YU2, ADA, and JR-FL envelope glycoproteins were incubated with GHOST-CCR5 cells in the presence of a 3 μM concentration of the indicated peptide-Ig variants. All other pseudotyped viruses were incubated with GHOST-CXCR4 cells, again with a 3 μM concentration of the indicated peptide-Ig variants.
gp120 may be a useful therapeutic target. The present study shows that it is possible to enhance the antiviral activities of peptides that are similar to the CCR5 amino terminus. The basis of this enhancement is unclear, but replacement of hydrophobic residues, which may point into the body of CCR5, would be likely to increase solubility and flexibility of the peptide. The elimination of additional residues separating tyrosine 3 and tyrosine 10 of CCR5 may permit binding of an additional sulfated tyrosine to gp120 without the entropic penalty of folding the small intervening loop.

Although more efficient than CCR5-based peptides in inhibiting HIV-1 infection, pΔE51-lg is still not sufficiently potent for therapeutic use. These studies nonetheless suggest that the sulfate-binding pocket, which is conserved both in R5 and X4 isolates, may be an important therapeutic target. Peptides like pΔE51 may also be useful when fused to soluble CD4 or other protein inhibitors of HIV-1 entry. Such fusion proteins would likely exhibit higher affinity than their unmodified counterparts and may limit viral escape. Finally, these studies suggest that work identifying additional sulfated antibodies may yield more potent entry inhibitors and provide additional insight into the CCR5 determinants necessary for its association with gp120.

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