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Multiple Residues Contribute to the Inability of Murine CCR-5 To Function as a Coreceptor for Macrophage-Tropic Human Immunodeficiency Virus Type 1 Isolates

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Infection of CD4-positive cells by human immunodeficiency virus type 1 (HIV-1) requires functional interaction of the viral envelope protein with a coreceptor belonging to the chemokine receptor family of seven-membrane-spanning receptors. For the majority of macrophage-tropic HIV-1 isolates, the physiologically relevant coreceptor is the human CCR-5 (hCCR-5) receptor. Although the murine homolog of CCR-5 (mCCR-5) is unable to mediate HIV-1 infection, chimeric hCCR-5/mCCR-5 molecules containing single extracellular domains derived from hCCR-5 are effective coreceptors for certain macrophage-tropic HIV-1 isolates. Here, we have sought to identify residues in hCCR-5 critical for HIV-1 infection by substitution of mCCR-5-derived residues into the context of functional chimeric hCCR-5/mCCR-5 receptor molecules. Using this strategy, we demonstrate that residues 7, 13, and 15 in the first extracellular domain and residue 180 in the third extracellular domain of CCR-5 are important for HIV-1 envelope-mediated membrane fusion. Of interest, certain substitutions, for example, at residues 184 and 185 in the third extracellular domain, have no phenotype when introduced individually but strongly inhibit hCCR-5 coreceptor function when present together. We hypothesize that these changes, which do not preclude chemokine receptor function, may inhibit a conformational transition in hCCR-5 that contributes to HIV-1 infection. Finally, we report that substitution of glycine for valine at residue 5 in CCR-5 can significantly enhance the level of envelope-dependent cell fusion by expressing cells. The diversity of the mutant phenotypes observed in this mutational analysis, combined with their wide distribution across the extracellular regions of CCR-5, emphasizes the complexity of the interaction between HIV-1 envelope and coreceptor.

Infection of cells by human immunodeficiency virus type 1 (HIV-1) requires interaction of the viral envelope protein with not only CD4 but also a second cell surface molecule, termed a coreceptor (reviewed in reference 19). Coreceptor usage varies significantly among different HIV-1 isolates, although all known coreceptors are members of the G-protein-coupled chemokine receptor family of seven-membrane-spanning receptors. The primary coreceptor used by non-syncytium-inducing, macrophage-tropic (M-tropic) HIV-1 isolates, which constitute the majority of primary isolates, is CCR-5 (1, 6, 8, 12, 27). In contrast, syncytium-inducing, T-cell-line-adapted (T-tropic) HIV-1 isolates predominantly use CXCR-4 as a coreceptor (13). Other chemokine receptors utilized by a small percentage of generally dualtropic HIV-1 isolates include CCR-2b and CCR-3 (6, 11). The importance of two orphan chemokine receptors, termed Bonzo/STRL33 and BOB/GPR15, in infection by HIV-1 remains to be established, although these proteins were recently shown to serve as coreceptors for several simian immunodeficiency virus and HIV-2 isolates (2, 9). The critical importance of CCR-5 for infection by primary, M-tropic HIV-1 isolates, however, has been highlighted by the finding that a small percentage of humans lack a functional *CCR-5* gene and as a result appear highly, although not completely, resistant to infection by HIV-1 (17, 22). Importantly, primary T cells derived from such individuals are refractory to infection by M-tropic HIV-1 isolates in vitro (17, 22, 27), thus demonstrating that CCR-5 is the physiologically relevant coreceptor for the majority of primary isolates.

At present, relatively little is known about how the viral envelope and coreceptor interact, although it appears clear that interaction is dependent upon a prior conformational shift induced by binding of the envelope gp120 subunit to CD4 (24, 26). This in turn is believed to lead to the formation of a ternary complex, consisting of gp120, coreceptor, and CD4, on the surface of the target cell (15, 24, 26). It is unknown how this protein complex then induces the fusion of the viral and host cell membranes, although the envelope gp41 subunit is believed to play a critical role at this stage.

An important unresolved question is the identity of the amino acid residues in gp120 and the coreceptor that interact during infection. However, it is well established that HIV-1 tropism, and hence coreceptor usage, is largely controlled by a small number of residues located in the envelope V3 loop (6, 14, 23, 25). Efforts to identify residues in the CCR-5 coreceptor involved in mediating infection have thus far largely focused on the functional analysis of chimeric receptors generated with human CCR-5 (hCCR-5) and a chemokine receptor lacking coreceptor function, such as the murine CCR-5 homolog (mCCR-5) (3, 5, 20, 21). These studies have led to three major conclusions. Firstly, the residues in hCCR-5 involved in mediating HIV-1 infection are diffuse, being located on at least three of the four extracellular domains of CCR-5. Secondly, these residues are functionally redundant, so that several distinct regions of hCCR-5 can suffice independently to confer coreceptor function when substituted into mCCR-5. Lastly, different HIV-1 envelope proteins interact differently with CCR-5, such that CCR-5 residues important for mediating

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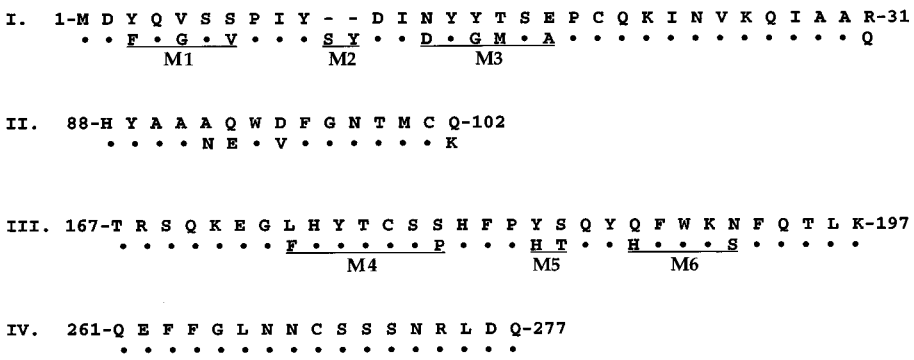

FIG. 1. Predicted amino acid sequence of the four extracellular domains of hCCR-5 (upper lines) and mCCR-5 (lower lines). Identity is indicated by dots, and deletions are indicated by dashes. The HMMM chimera substitutes residues 1 to 30 of hCCR-5 into mCCR-5 and therefore excludes divergent residue 31. The MHMM and MMHM chimeras represent precise substitutions of the second and third extracellular domain, respectively, of hCCR-5 into mCCR-5. The location and identity of the six clustered substitution mutations introduced into the HMMM (M1 to M3) or MMHM (M4 to M6) chimera are indicated.

fusion by one envelope protein may be largely irrelevant to the interaction of CCR-5 with a second envelope protein. Overall, these data demonstrate that the envelope–CCR-5 interaction is likely to be highly complex and to involve the interaction of multiple residues in both proteins.

As noted above, the mCCR-5 chemokine receptor, despite extensive sequence similarity to hCCR-5, fails to function as an HIV-1 coreceptor (3, 5, 20). Therefore, it is apparent that one or more of the 20 extracellular residues that differ between mCCR-5 and hCCR-5 must contribute to the interaction with the HIV-1 envelope protein. Using mutational analysis in the context of chimeric mCCR-5/hCCR-5 receptors, we have now identified several residues, located in three of the four extracellular domains of hCCR-5, that play roles in mediating infection by HIV-1.

MATERIALS AND METHODS

Construction of molecular clones and mutants. Mammalian expression plasmids encoding hCCR-5, human CXCR-4 (hCXCR-4), and mCCR-5 have been described previously (5). The HMMM chimera contains hCCR-5 residues 1 through 30, encoding most of the first extracellular domain, substituted into mCCR-5 (see Fig. 1) (5). Similarly, the MMHM chimera contains the entire third extracellular domain of hCCR-5 (residues 167 to 197) in place of the equivalent sequence in mCCR-5 (5). The MHMM chimera was generated by recombinant PCR with oligonucleotide primers that precisely substituted the second extracellular domain of hCCR-5 into the mCCR-5 context (see Fig. 1). It is important to note that this MHMM chimera is distinct from the one previously described by Bieniasz et al. (5) and also termed MHMM. The latter chimera, which substituted residues 35 to 167 of hCCR-5 into mCCR-5, was not expressed on the surfaces of transfected cells and was therefore uninformative. All wild-type and mutant chimeras were expressed by using the pBC12/CMV mammalian expression vector (7), and all contained hemagglutinin (HA) epitope tags at their amino termini. Missense mutations were introduced into the coreceptor chimeras by using synthetic oligonucleotide primers and the Quick Change mutagenesis kit (Stratagene) and were verified by using an ABI DNA sequencing kit (Applied Biosystems). Point mutants are named according to the nature and location of the mutation; thus, the S7V mutation encodes valine in place of serine at residue 7 of the HMMM chimera. The expression plasmids pBC12/CMV/CD4 and pBC12/HIV/SEAP, and the HIV-1 proviral clones containing the ADA, BaL, 89.6, and IIIB envelope genes, have been described previously (4, 5, 23). An additional HIV-1 proviral clone, containing the *env* gene of the M-tropic YU-2 isolate (16), was generated as previously described (5) by substitution of the appropriate PCR-generated YU-2 *Sal*I-*Bam*HI DNA fragment into the context of the pIIIB proviral clone.

Cell fusion assay for coreceptor function. COS and 293T cells were maintained as previously described (5). Fusion indicator cells were generated by cotransfection of COS cells (35-mm cultures) with Lipofectamine and 800 ng of BC12/CMV/CD4, 800 ng of BC12/HIV/SEAP, and 800 ng of pBC12/CMV-based plasmids expressing one of the parental, chimeric, or mutant chimeric receptors. Where necessary, the total amount of plasmid DNA in each transfection was maintained at 2.4 μg by inclusion of the requisite quantity of the parental pBC12/CMV plasmid. Simultaneously, virus-producing cells were generated by transfection of COS cells with 2 μg of the relevant HIV-1 proviral construct. At 48 h posttransfection, producer and indicator cells were harvested by trypsinization, and equal numbers (5×10^4) were cocultivated in 48-well plates. After 48 h, culture supernatants were harvested and secreted alkaline phosphatase (SEAP) activity was determined as described previously (4).

Analysis of CCR-5 expression levels. 293T cells were transfected with 2 μg of the coreceptor expression plasmids, and 72 h later unfixed cells were stained with a murine monoclonal antibody (12CA5; Boehringer Mannheim) specific for the HA epitope tag (10 μg/ml), followed by fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin G (Sigma) (1:200). Surface expression of the chimeric receptors was then quantified by fluorescence-activated cell sorting (FACS).

RESULTS

Single extracellular domains from hCCR-5 can confer HIV-1 coreceptor function on mCCR-5. Comparison of the predicted amino acid sequences of the hCCR-5 and mCCR-5 chemokine receptors shows that the sequences are ~82% identical, with differences scattered throughout these related molecules (5, 17, 19). Examination of the extracellular domains, which are presumably the most relevant to HIV-1 coreceptor function, reveals that the amino terminal tail of mCCR-5 has eight substitutions and a 2-amino-acid insertion relative to the same sequence in hCCR-5 (Fig. 1). Similarly, the 15-amino-acid second extracellular domain of hCCR-5 differs at 4 residues, while the 31-amino-acid third extracellular domain differs at 6 residues, from mCCR-5. The fourth extracellular domain is identical in both molecules. Despite this extensive similarity, and despite the fact that hCCR-5 and mCCR-5 are both able to functionally interact with the human chemokines MIP-1β and RANTES, mCCR-5 has failed to act as a functional coreceptor for any HIV-1 isolate tested thus far (3, 5, 18, 20).

Previously, we have described a quantitative, linear assay for the measurement of coreceptor-dependent, HIV-1 envelope-induced cell fusion (5). In this assay, indicator cells are generated by transfection of, in this case, COS cells with expression vectors encoding human CD4, a candidate coreceptor, and an indicator construct consisting of the HIV-1 long terminal repeat linked to the SEAP indicator gene. In the absence of the HIV-1 Tat *trans*-activator, this construct produces only a minimal level of SEAP. A second batch of COS cells are simultaneously transfected with a full-length HIV-1 proviral clone. At 48 h after transfection, these two cultures are harvested, mixed, and then cocultivated for a further 48-h period. Fusion of cells expressing the HIV-1 envelope protein with cells expressing CD4 and a functional coreceptor allows the HIV-1 Tat protein to activate transcription of the SEAP gene linked to the HIV-1 long terminal repeat and results in a large increase in SEAP activity in the supernatant media.

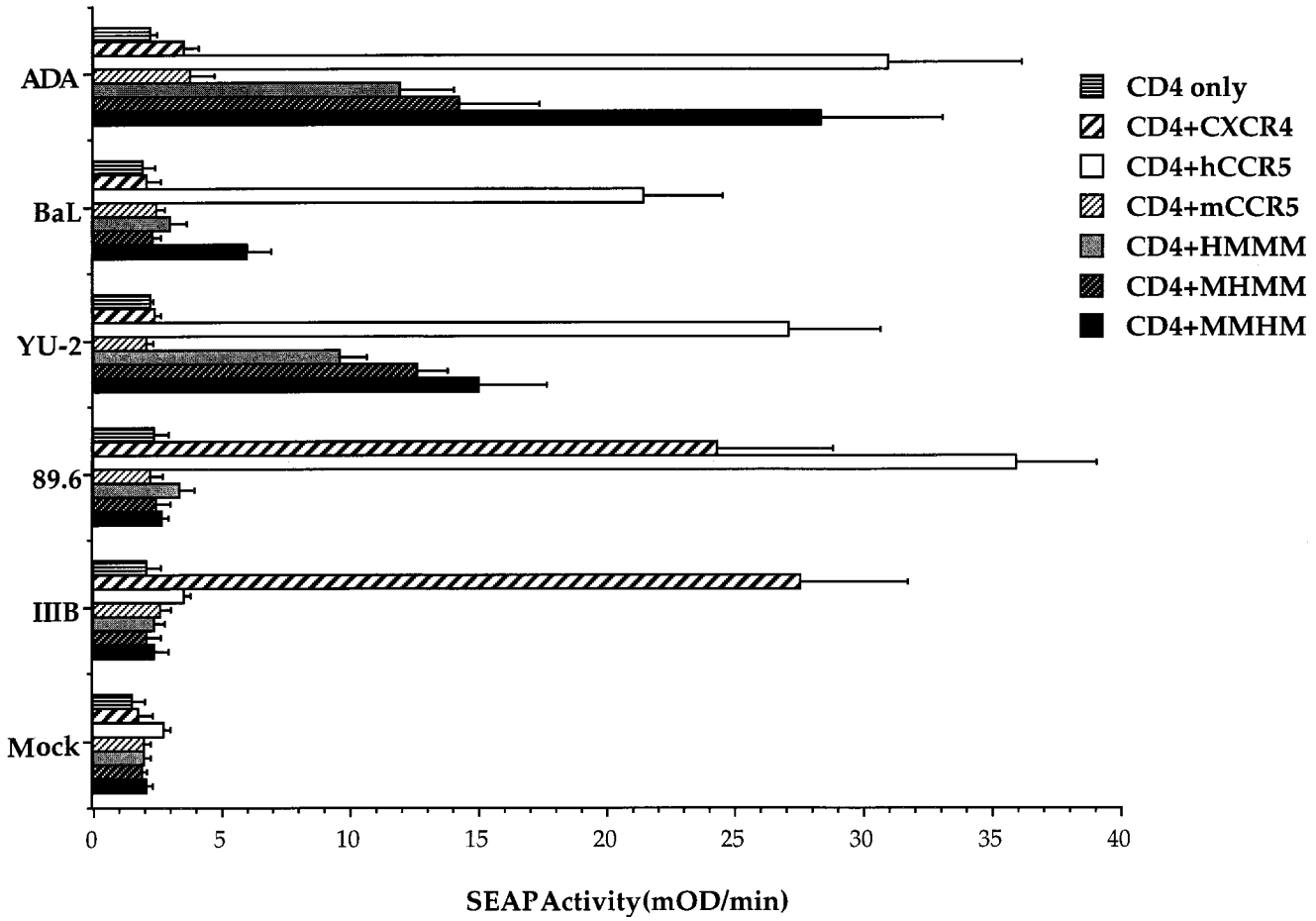

FIG. 2. Analysis of the coreceptor activities of hCCR-5/mCCR-5 chimeras. One set of COS cell cultures was transfected with one of the indicated HIV-1 proviral expression plasmids or with a control (mock) vector. A second set was transfected with the pBC12/HIV/SEAP indicator construct and plasmids expressing CD4 and one of the indicated amino-terminally HA-tagged chemokine receptors. Cells derived from each set were then cocultivated starting 48 h posttransfection, and fusion efficiency was assessed by measurement of supernatant SEAP activity 48 h later. The data shown represent averages of three independent experiments, with standard deviations indicated.

In the experiment represented in Fig. 2, we have used this assay to compare the abilities of three M-tropic HIV-1 isolates (ADA, BaL, and YU2), one dualtropic HIV-1 isolate (89.6), and one T-tropic HIV-1 isolate (IIIB) to utilize wild-type forms of hCCR-5, mCCR-5, and hCXCR-4 as coreceptors. In addition, we have also tested the abilities of these viruses to use hCCR-5/mCCR-5 chimeras containing precise substitutions of the first (HMMM), second (MHMM), or third (MMHM) extracellular domain of hCCR-5 into an otherwise entirely mCCR-5 context. As expected, these data demonstrate that the three M-tropic HIV-1 isolates and the dualtropic isolate can utilize hCCR-5 effectively, while the T-tropic isolate IIIB is inactive. In contrast, while both 89.6 and IIIB can utilize hCXCR-4 efficiently, none of the three M-tropic isolates gives any detectable activity. In addition, none of the tested viruses are able to utilize mCCR-5 appreciably (Fig. 2).

As previously reported by ourselves and others (5, 20), viruses able to utilize hCCR-5 can differ widely in the ability to utilize specific hCCR-5/mCCR-5 chimeras. Thus, the ADA isolate utilizes the MMHM chimera as effectively as wild-type hCCR-5 and gives readily detectable activity with both HMMM and MHMM. In contrast, both the dualtropic 89.6 and the M-tropic BaL isolates give little or no activity with any tested hCCR-5/mCCR-5 chimera (Fig. 2). Finally, the YU-2 M-tropic isolate is similar to ADA in that it can also utilize all three hCCR-5/mCCR-5 chimeras reasonably well, although YU-2 does differ from ADA in that YU-2 utilizes the MMHM chimera significantly less effectively than wild-type hCCR-5. Based on these data, it is apparent that viruses tropic for hCCR-5 can nevertheless differ significantly from one another in how they interact with at least three of the four extracellular domains of CCR-5. In addition, it is also apparent that for at least some HIV-1 isolates, the first three extracellular domains of hCCR-5 can act as functionally redundant envelope binding sites. Thus, for the M-tropic ADA isolate of HIV-1, each of the first three extracellular domains of hCCR-5 is sufficient to mediate HIV-1 infection when inserted into the otherwise nonpermissive mCCR-5 context (Fig. 2). These data, therefore, demonstrate that although mCCR-5 and MHMM differ at only four residues, the latter can nevertheless function as an HIV-1 coreceptor while the former does not. Similarly, HMMM and MMHM differ by only 9 and 6 residues, respectively, from mCCR-5 yet are nevertheless able to function as coreceptors for the ADA strain HIV-1 envelope. Therefore, it is apparent that several of the amino acid residues that differ between hCCR-5 and mCCR-5 must make important contributions to the interaction between CCR-5 and the ADA envelope.

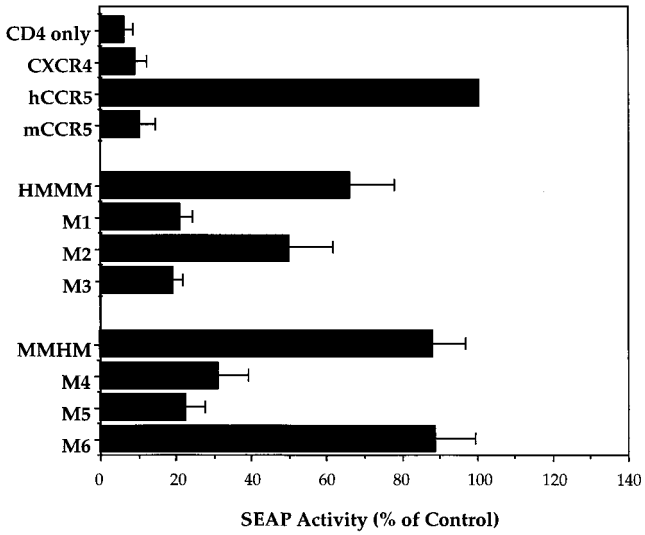

FIG. 3. Analysis of the levels of HIV-1 coreceptor activities displayed by mutants of HMMM and MMHM. Coreceptor activity was assayed in COS cells as described in the text by using the ADA viral envelope protein. All cultures were also transfected with a plasmid expressing human CD4. These data represent averages of three independent experiments, with standard deviations indicated. Observed SEAP activities are given relative to that of the parental hCCR-5 coreceptor, which was arbitrarily set at 100%.

Mutational analysis of hCCR-5/mCCR-5 receptor chimeras. Because the extracellular domains of hCCR-5 are at least in part functionally redundant in the ability to mediate M-tropic HIV-1 coreceptor function (3, 5, 19), it appears possible that mutational analysis of wild-type hCCR-5 might be relatively uninformative, as loss of coreceptor function in one extracellular domain would be compensated for by the remaining intact domains. In contrast, the HMMM, MHMM, and MMHM chimeras represent appropriate contexts in which to map individual residues that mediate M-tropic HIV-1 coreceptor function, in that fusion is then dependent on a single, nonredundant hCCR-5-derived extracellular sequence.

To map hCCR-5 residues important for HIV-1 ADA fusion, we first constructed a series of clustered point mutants in the context of HMMM (M1, M2, and M3) and MMHM (M4, M5, and M6). As shown in Fig. 1, these CCR-5 mutants bear substitutions of the appropriate mouse sequences in place of the human sequences in clusters of from two to four residues in the first and third extracellular domains. As shown in Fig. 3, the M1 mutant of HMMM (Y3F, V5G, and S7V), as well as the M3 mutant (N13D, Y15G, T16M, and E18A), were both only minimally active as coreceptors for HIV-1 when tested in COS cells. In contrast, the M2 mutant, bearing a 2-residue insertion between hCCR-5 residues 10 and 11, was not significantly less active than the parental HMMM chimera. Similarly, both the M4 (L174F and S180P) and M5 (Y184H and S185T) mutants of MMHM showed poor coreceptor activities, while the M6 mutant (Q188H and N192S) was as active as the MMHM parent. Similar data for each of these wild-type and mutant chimeric receptors were obtained for both COS cells (Fig. 3) and transfected 293T cells (data not shown).

Point mutants of CCR-5 display negative synergy in their effects on coreceptor function. The data presented in Fig. 3 suggest that the 2-amino-acid insertion in the mCCR-5 first extracellular domain (mutant M2) and the Q188H and N192S substitutions in the third extracellular domain (mutant M6) are unlikely to contribute significantly to the inability of mCCR-5

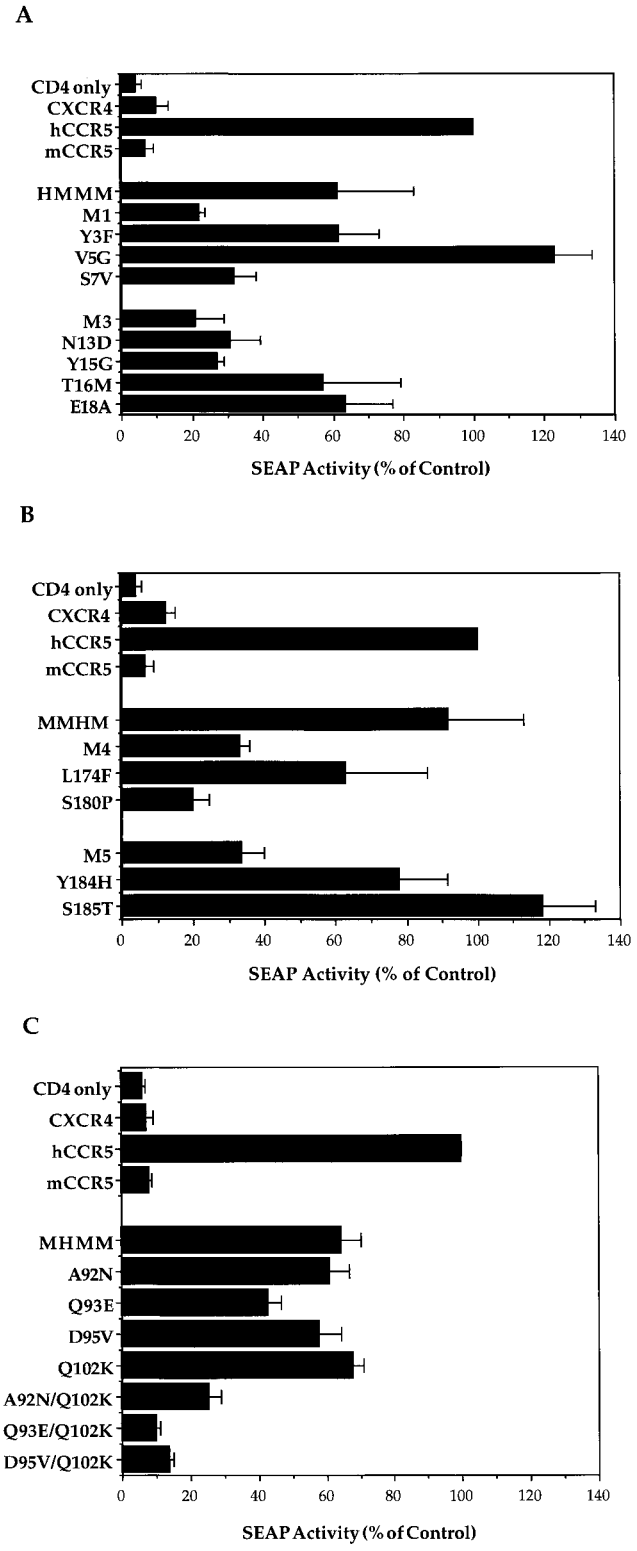

FIG. 4. Functional analysis of substitution mutants of hCCR-5/mCCR-5 chimeras. The indicated missense mutants of HMMM (A), MMHM (B), and MHMM (C) were analyzed for coreceptor function as described in the legend to Fig. 3.

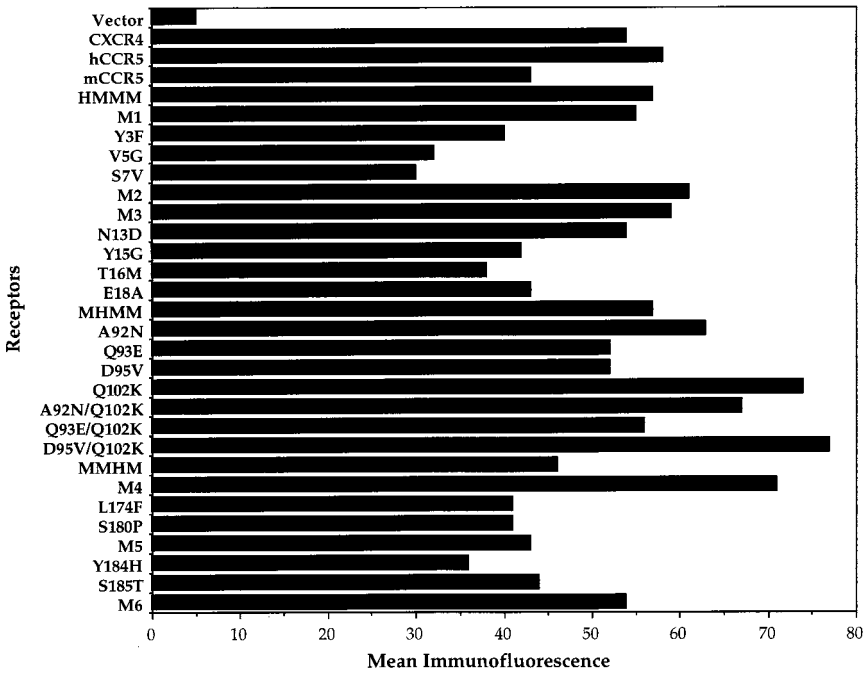

FIG. 5. Relative cell surface expression of wild-type and mutant hCCR-5/mCCR-5 chimeras. FACS analysis of 293T cells transfected with HA-tagged forms of the indicated parental and chimeric receptors is shown. Data are given as mean fluorescence intensities of total cell populations and are representative of two independent experiments.

to function as an HIV-1 coreceptor. However, these data also suggest that at least two distinct sequence differences in both the first and third extracellular domains, defined here by mutants M1 and M3 and mutants M4 and M5, respectively, must contribute to this negative phenotype.

To determine if individual residues caused the observed phenotypes, or whether they instead resulted from a particular combination of substitutions, we next tested all of the residues implicated by the analysis presented in Fig. 3 as individual missense mutations of either HMMM (Fig. 4A) or MMHM (Fig. 4B). Individual analysis of the three residues encompassed by the M1 mutation of HMMM revealed that substitution of phenylalanine for tyrosine at residue three (Y3F) had no significant effect on the activity of HMMM (Fig. 4A). Surprisingly, the V5G mutant of HMMM displayed significantly enhanced coreceptor function, giving rise to a level of cell fusion that was clearly higher than that seen with the parental HMMM chimera and at least as high as that seen with the wild-type hCCR-5 coreceptor. However, substitution of valine for serine at residue seven (S7V) did produce a significant drop in activity, relative to the HMMM parent, to a level comparable to that seen with the M1 triple mutant. Similarly, for the M3 quadruple mutant, the individual substitution mutants T16M and E18A had no detectable effect on the activity of the HMMM parent (Fig. 4A). In contrast, both the conservative N13D and the less conservative Y15G mutations exerted marked inhibitory effects. Overall, this analysis identifies serine 7, asparagine 13, and tyrosine 15 in the first extracellular domain as contributing to hCCR-5 coreceptor function. Similar coreceptor activity data were obtained for each of these receptor mutants in both COS cells (Fig. 4A) and 293T cells (data not shown).

We next wished to address the individual contributions of each of the two residues substituted in the M4 and M5 mutants of the MMHM chimera to the minimal coreceptor function of these molecules (Fig. 3). As shown in Fig. 4B, the L174F mutation had only a limited effect on the biological activity of the MMHM chimera, while the S180P mutation produced dramatic inhibition, apparently accounting for the entire phenotype of the M4 double mutant. In contrast, neither the Y184H nor the S185T mutation exerted a significant phenotype when tested alone, although the M5 double mutant encompassing both of these changes is clearly highly compromised in coreceptor function (Fig. 4B). In the latter case, it is therefore apparent that these two substitutions, while individually innocuous, are able to exert a synergistic inhibitory effect when introduced together. This analysis was repeated in 293T cells, with very similar results (data not shown).

Finally, we wished to examine the effects of the four single-residue differences in the second extracellular domain of hCCR-5/mCCR-5 on coreceptor function. Surprisingly, as shown in Fig. 4C, none of these four residues had any marked effect on the substantial coreceptor activity of the MHMM chimera when introduced individually, although these four residues represent the only differences between the active MHMM chimera and the nonfunctional mCCR-5 molecule. To examine whether this result might again represent an example of negative synergy between individually innocuous mutations, we generated three double missense mutations in the MHMM context, i.e., A92N/Q102K, Q93E/Q102K, and D95V/Q102K. As shown in Fig. 4C, each of these double mutants was in fact highly compromised in coreceptor function.

Cell surface expression of chimeric CCR-5 receptors. A critical concern in measurement of the ability of mutant molecules to serve as cell surface receptors is that expression levels must be comparable. We therefore analyzed cell surface expression levels of all of the described wild-type and mutant chimeric receptors by FACS of transfected cells with a monoclonal antibody specific for the HA epitope tag introduced at the amino termini of these proteins. As shown in Fig. 5, this

analysis demonstrated comparable levels of cell surface expression for all tested CCR-5 derivatives (≥60% of wild-type hCCR-5). The slight variability that was observed is unlikely to significantly contribute to the lower level of coreceptor function observed with some CCR-5 mutants, in that the V5G mutant of HMMM, which displayed a very high level of coreceptor activity (Fig. 4A), appeared to display one of the lowest levels of cell surface expression (Fig. 5).

DISCUSSION

Analysis of the abilities of chimeric receptors derived from hCCR-5 and mCCR-5 to function as HIV-1 coreceptors has revealed that HIV-1 isolates can differ greatly in the ability to functionally interact with specific chimeras (Fig. 2). Thus, certain M-tropic HIV-1 isolates, including ADA, YU-2, and SF162, are able to interact effectively with hCCR-5/mCCR-5 chimeras in which only a single hCCR-5 extracellular domain is present (5, 20). In contrast, other M-tropic isolates, including BaL, M23, and E80, fail to interact effectively with the same HMMM, MHMM, and MMHM chimeras but will efficiently fuse to cells expressing hCCR-5/mCCR-5 chimeras in which any single extracellular domain in hCCR-5 is replaced by the equivalent mCCR-5 sequence (5, 20). Finally, certain dualtropic HIV-1 isolates, including 89.6 and GUN-1, are highly sensitive to any perturbation of hCCR-5 and interact poorly with almost all hCCR-5/mCCR-5 chimeras (5, 20). From these data we can conclude that the extracellular sequences in hCCR-5 involved in M-tropic HIV-1 infection are, depending on the particular isolate tested, either highly (ADA, YU-2, and SF162) or substantially (BaL, M23, and E80) redundant. In contrast, for dualtropic isolates such as 89.6 and GUN-1, this redundancy is quite limited. Therefore, one would predict that mutation of individual residues in hCCR-5 would be unlikely to significantly affect infection by M-tropic HIV-1 isolates unless (i) the mutant receptor was no longer expressed normally on the cell surface or (ii) the introduced mutation affected the conformation of the CCR-5 receptor such that envelope binding was sterically hindered. In contrast, such hCCR-5 mutations would be predicted to be far more likely to exert a significant inhibitory effect on dualtropic HIV-1 infection. In fact, a recently published mutational analysis of hCCR-5 (10) reported no significant effect by a series of tested mutations on infection by the M-tropic isolate JR-FL, although substitution of alanine at residues 11, 197, or 276 did inhibit infection by the dualtropic 89.6 isolate (10).

Because M-tropic isolates of HIV-1 are by far the most common form seen in vivo (19, 27), it is important to understand how M-tropic, as opposed to dualtropic, isolates interact with the dominant coreceptor for M-tropic HIV-1, i.e., the hCCR-5 chemokine receptor. However, because of the functional redundancy described above, mutational analysis of hCCR-5 itself might be relatively uninformative. For this reason, we have instead chosen to perform such a mutational analysis using a set of three chimeric hCCR-5/mCCR-5 receptor molecules (HMMM, MHMM, and MMHM) that each contain a single hCCR-5-derived extracellular domain yet that retain the ability to function as effective coreceptors for M-tropic HIV-1 isolates such as ADA (Fig. 2). In this way, we could feel confident that the particular hCCR-5-derived extracellular domain being subjected to mutational analysis was indeed playing a critical role in the HIV-1 envelope-mediated fusion process.

In the analysis presented here, we have focused on the small number of residues which differ between hCCR-5 and mCCR-5, in the expectation that while at least some of these must contribute to hCCR-5 coreceptor function, these changes would be very unlikely to affect either cell surface receptor expression or overall receptor conformation. This expectation arises from the fact that both hCCR-5 and mCCR-5 are functional receptors for the human chemokines MIP-1β and RANTES even though only the former can mediate M-tropic HIV-1 infection (5, 18, 20). This mutational analysis has identified two types of inhibitory effects mediated by substitution of mCCR-5 residues into hCCR-5. Thus, several individual changes in the first extracellular domain (S7V, N13D, and Y15G) and one substitution in the third extracellular domain (S180P) were found to exert marked inhibitory effects on ADA envelope-mediated cell fusion. Of these, the most interesting may be N13D, in that this is a relatively conservative mutation that is perhaps unlikely to significantly affect the global conformation of the first extracellular domain of hCCR-5. Instead, these data suggest that asparagine 13 may represent a contact point for infection by ADA. In contrast, the other three mutations (S7V, Y15G, and S180P) are less conservative and may therefore act by modifying the conformation of other, flanking residues. Clearly, distinguishing these possibilities would require, at minimum, a more intense mutational analysis or, preferably, knowledge of the molecular structure of hCCR-5.

An interesting phenomenon uncovered during this analysis is the existence of marked negative synergy between mutations that individually are phenotypically silent. Thus, the Y184H and S185T mutants of the MMHM chimera have little effect individually, but when present together in the M5 mutant they exert a marked inhibitory effect on coreceptor function (Fig. 4B). Similarly, the four residues that differ between hCCR-5 and mCCR-5 in the second extracellular domain (Fig. 1) have no effect on HIV-1 coreceptor function by the MHMM chimera when introduced individually (Fig. 4C) but effectively inhibit activity when present simultaneously or in certain groups of two residues. The molecular basis for this effect, of course, is at present unclear, although it might suggest that multiple substitutions can influence a segment of hCCR-5 to adopt a different, less favorable conformation. Alternatively, these mutations may collectively block a conformational shift in hCCR-5 required for the infection process but irrelevant to chemokine receptor function. Finally, the possibility that these residues directly contact the envelope but are highly functionally redundant, while in our view less probable, cannot be eliminated at this time. Certainly, it would be of interest to know whether these mutations block envelope binding by hCCR-5 or, instead, affect steps subsequent to binding. In either event, these data demonstrate that certain residues in hCCR-5 play important roles in HIV-1 infection that would be missed by mutational strategies such as alanine scanning.

A final, unexpected observation is the finding that the V5G mutant of the HMMM chimera is a significantly more active coreceptor than HMMM and is perhaps even more active than hCCR-5 itself (Fig. 4A). The fact that even the limited mutational analysis reported here can identify gain-of-function mutants (e.g., V5G), inhibitory point mutants (e.g., S180P), and mutants that exert their effects via some form of negative synergy (e.g., Y184H and S185T) scattered over three of the four extracellular domains of hCCR-5 emphasizes the remarkable complexity and plasticity of the functional interaction between hCCR-5 and the HIV-1 envelope protein.

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