Selective Employment of Chemokine Receptors as Human Immunodeficiency Virus Type 1 Coreceptors Determined by Individual Amino Acids within the Envelope V3 Loop

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The chemokine receptor CCR5 acts as an essential cofactor for cell entry by macrophage-tropic human immunodeficiency virus type 1 (HIV-1) strains, whereas CXCR4 acts as an essential cofactor for T-cell-line-adapted strains. We demonstrated that the specific amino acids in the V3 loop of the HIV-1 envelope protein that determine cellular tropism also regulate chemokine coreceptor preference for cell entry by the virus. Further, a strong correlation was found between HIV-1 strains classified as syncytium inducing in standard assays and those using CXCR4 as a coreceptor. These data support the hypothesis that progressive adaptation to additional coreceptors is a key molecular basis for HIV-1 phenotypic evolution in vivo.

Recent discoveries have revealed that chemokine receptors act as essential cofactors for the entry of human immunodeficiency virus type 1 (HIV-1) into cells. CCR5 mediates viral entry into macrophages, whereas CXCR4 mediates entry into many CD4-positive transformed T-cell lines (2, 12, 16, 19–21). Although virtually all primary HIV-1 isolates replicate in primary CD4-positive T lymphocytes, certain variants (macrophage-tropic) fail to infect transformed T-cell lines, whereas other strains (T cell-tropic) replicate well in these cell lines but not in macrophages. Changes in cellular tropism by HIV-1 strains seem to be a key event in the pathogenesis of HIV-1 disease (24, 28, 31, 32, 35, 36); for example, the transition from macrophage tropism to T-cell tropism in vivo and the emergence of viruses that induce syncytia in T-cell lines are associated with more rapid progression of HIV-1 disease. Amino acids in the hypervariable V3 region of the HIV-1 envelope glycoprotein 120 (gp120) have been shown to influence these effects (6, 11, 15, 22, 23, 26, 34).

In this study, we used a transient transfection-infection system to investigate the potential structure-function relationship between distinct amino acids in the V3 loop and chemokine coreceptor use by HIV-1. In this assay, human CD4 and either CCR5 or CXCR4 are expressed in COS-7 cells (3). The transfected cells are then exposed to individual HIV-1 clones, and cell entry is quantitated by fluorescence-activated cell sorting to measure the intracellular expression of the viral capsid protein p24.

To demonstrate the usefulness of this assay for evaluating the coreceptor preferences of different HIV-1 strains, we studied the reciprocal employment of CCR5 and CXCR4 by JR-CSF, a macrophage-tropic strain, and NL4-3, a virus that infects T-cell lines (1, 6, 9). JR-CSF infected nearly 2% of CCR5-

expressing cells but only 0.1% of CXCR4-expressing cells, whereas NL4-3 infected approximately 7% of CXCR4-expressing cells but only 0.2% of CCR5-expressing cells (Fig. 1). These findings show that this assay can qualitatively distinguish coreceptor preferences of HIV-1 strains. Since several variables, such as viral titer, can confound the absolute levels of infectivity by a strain, this assay is most informative for elucidating relative coreceptor preferences within a strain rather than direct quantitative differences between strains.

Two recent reports indicated that the gp120 V3 loop can influence the ability of HIV-1 variants to use different chemokine receptors (12, 13); both groups used chimeric viruses containing the V3 regions derived from different macrophage-tropic viruses (i.e., ADA, YU2, BAL) in the genomic backbone of HXB2. To evaluate whether this observation also applies to the viruses and the assay system used in this study, a chimeric virus containing the V1-V3 region derived from NL4-3 in the backbone of JR-CSF [JR-CSF(NL4-3)] and a chimeric virus containing the V3 loop of JR-CSF in the backbone of NL4-3 [NL4-3(JR-CSF)] were studied in our transfection-infection system (Fig. 1). Both chimeric viruses selectively used the chemokine receptor for cell entry according to the origin of their V3 loop inserts: JR-CSF(NL4-3) preferentially used CXCR4 rather than CCR5, whereas NL4-3(JR-CSF) preferentially used CCR5 and not CXCR4. Hence, these data substantiate the important role of the V3 loop in determining coreceptor use by a given HIV-1 strain.

Recent findings suggested that the intracellular processing of chemokine receptors varies within different cell types (29). Moreover, monkey CV-1 or COS-7 cells apparently have an intracellular restriction that reduces the efficiency of their infection by HIV-1 (7, 30). Therefore, to corroborate our results obtained by the transient transfection-infection assay in monkey cells, we studied the infectivity of the same recombinant HIV-1 variants in human HeLa-CD4 cells and in HeLa-CD4 cells stably expressing CCR5. In contrast to phytohemaggluti-

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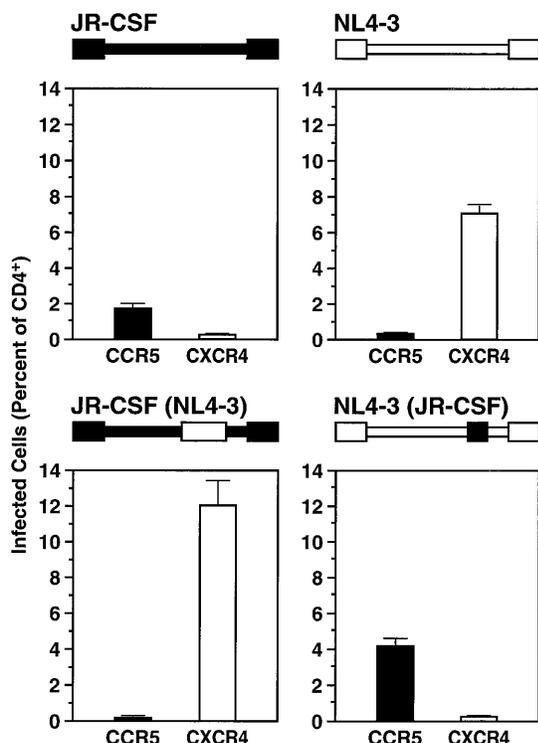
FIG. 1. The V3 region of HIV-1 gp120 determines coreceptor utilization. The infectivity (p24-positive cells as a percentage of CD4$^+$ cells) of parental and chimeric virus clones was quantitated using dual-color flow cytometry as described in the text. JR-CSF(NL4-3) has the JR-CSF backbone with substitution of the V1-V3 region derived from NL4-3. NL4-3(JR-CSF) (identical to clone 10-26 [11]) has the NL4-3 backbone with selective substitution of the V3 region derived from NL4-3. Plasmid DNA from the molecular clones was used to transfect HeLa cells or 1022 cells to produce infectious HIV virions. These virions were expanded by one cell-free passage on blasted human PBMC. The results represent the means and standard errors of data from three experiments.

nin-stimulated human peripheral blood mononuclear cells (PBMC) that are permissive for HIV-1 strains requiring either the CCR5 or the CXCR4 coreceptor, HeLa cells inherently express CXCR4 but not CCR5. Thus, HeLa-CD4 cells permit the assessment of the infectivity of HIV-1 strains exclusively using the CXCR4 chemokine coreceptor and show no background foci when infected with macrophage-tropic strains. In order to permit the detection of macrophage-tropic strains requiring the CCR5 coreceptor, HeLa-CD4/CCR5 cells were generated by transducing HeLa-CD4/CXCR4 cells with the pSFF retroviral vector (4, 27), which contained the human CCR5 coding sequence inserted into the expression site (30). Infectivity was quantitated by a focal immunoassay as described previously (11). In this setting, the T-cell-tropic variants, NL4-3 and JR-CSF(NL4-3), infected both cell lines whereas the macrophage-tropic strains, JR-CSF and NL4-3(JR-CSF), infected only HeLa-CD4/CCR5 cells (Fig. 2). These results support those obtained in the transient assay.

To investigate the role of individual amino acids within the V3 loop, we studied the infectivity pattern of a panel of recombinant HIV-1 variants containing highly related V3 loop sequences from two primary isolates with either T-cell (123) or macrophage (JR-CSF) tropism. These sequences were created by site-directed mutagenesis of individual amino acids in the V3 loop within the genomic background of NL4-3. As shown by Chesebro et al. (11), these variants differ in their abilities to infect primary monocyte-derived macrophages and HeLa cells

stably expressing CD4 and in their capacities to induce syncytia upon infection of MT-2 cells (Fig. 3).

This analysis substantially extended the earlier findings (11) by revealing that all of the macrophage-tropic variants (JR-CSF, 242, and 126) used CCR5, whereas the T-cell-tropic variants (123, 255, and 134) used CXCR4, and the dual-tropic variants (241, 254, and 256) effectively utilized either receptor (Fig. 3). Thus, the cellular tropism of these HIV-1 variants with an isogenic background other than the V3 region is clearly linked to selective use of coreceptors. This analysis also demonstrated that the same V3 amino acids dictating cellular tropism of these HIV-1 variants also control the engagement of either CXCR4 or CCR5. For example, comparison of variants 241 and 242 showed that the selection of either Gln or Glu at position 25 in the V3 loop regulated the ability to utilize CXCR4 (but not CCR5) for cell entry (Fig. 3). Comparison of variants 123 and 256 as well as variants 254 and 255 demonstrated that use of CCR5 (but not CXCR4) is influenced by position 30. Furthermore, in variants 126 and 134, position 13 regulated the reciprocal use of these two coreceptors, thereby determining whether the variant targeted macrophages or T cells. Thus, individual residues that regulate functional interaction with these coreceptors are identifiable. In addition, these data show that both the coreceptor preferences and the cellular tropisms of HIV-1 are linked to the same positions in the V3 loops of the mutants examined. These conclusions are also supported by assays performed using the stable HeLa transfectants (Fig. 2). In this study the results obtained were all in the context of only a single genetic background (i.e., other Env segments). In other Env contexts residues at other positions may well be important for cellular tropism (5, 14, 18, 25, 39) and, presumably, for coreceptor use. Indeed, we have observed that various positions in different primary isolates affect
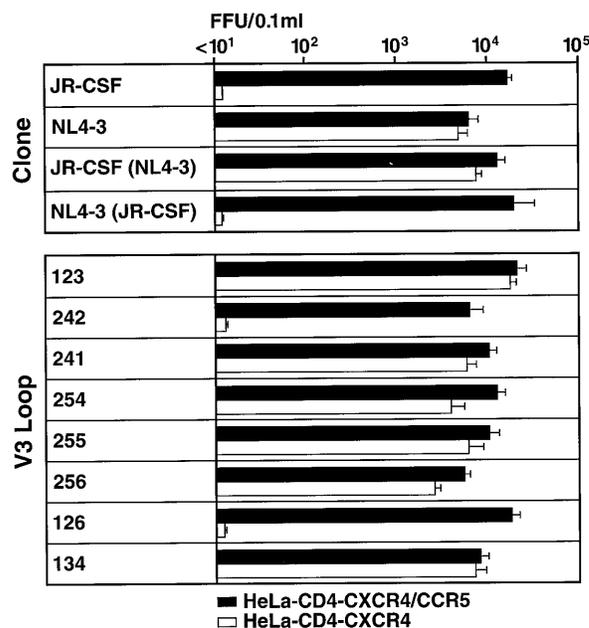
FIG. 2. Stable HeLa transfectants expressing CXCR4 or both CXCR4 and CCR5 define the coreceptor preferences of recombinant viruses with variations in the V3 loop. Reciprocal chimeric viruses (top) or matched chimeric viruses with similar V3 regions in the backbone of NL4-3 (bottom) were used. The results represent the means and standard errors of data from three experiments. Although no foci of macrophage-tropic infection occur in the HeLa-CD4 cells that lack CCR5, the backgrounds are indicated as less than 10 focus-forming units (FFU)/0.1 ml because diluted virus stocks were used in these assays.

FIG. 3. Distinct coreceptor utilization maps to individual amino acids. Coreceptor utilization as assessed by the transfection-infection assay was determined for a panel of matched chimeric HIV-1 variants with similar V3 regions in the backbone of NL4-3. Cellular tropism (monocyte-derived macrophages and HeLa cells) and SI versus non-SI (NSI) phenotype as determined by MT-2 cocultivation assay are described in detail elsewhere (11) and are summarized here for reference. All HIV-1 variants grew well in PBMC.

cellular tropism (10) and coreceptor utilization (data not shown) in a context-specific manner. The great sensitivity of these relationships to subtle sequence changes undoubtedly contributes to viral evolution and pathogenesis in vivo. The recently reported discordance of some HIV-1 strains that use CCR5 but are not able to infect macrophages (8, 17) and the discovery of additional chemokine receptors as HIV-1 coreceptors are likely to add to the complexity of the specific cellular entry mechanisms of HIV-1.

Further investigation of the recombinant HIV-1 variants showed a perfect correlation between the syncytium-inducing (SI) phenotype, as assessed with an MT-2 cocultivation assay (11), and the ability to infect cells through CXCR4 (Fig. 2 and 3). This SI phenotype was independent of functional association with CCR5. Elsewhere we show that macrophage-tropic isolates of HIV-1 cause abundant syncytia when they are assayed in various cells that contain large amounts of CCR5 (30, 33, 37). Thus, the distinction of viruses based on syncytium formation is dependent on the cells employed. Hence, because the chemokine receptors are essential coreceptors for HIV-1 cell entry and because coreceptor utilization can now be defined, we propose that the most useful nomenclature for HIV-1 isolates would specify their coreceptor designation(s) rather than their syncytium-inducing properties.

These findings indicate that distinct amino acids in the V3 loop regulate the use of chemokine coreceptors, although the specific amino acid positions may vary in different Env contexts. They further support the hypothesis that adaptation to additional coreceptors may be responsible for the phenotypic evolution of HIV-1 in vivo associated with progressive diversification of HIV-1 strains (or quasispecies) (38). By this mechanism, the evolution in vivo of multiple quasispecies that use different chemokine receptors for cell entry potentially leads to infection of other cell types and the concomitant acceleration of HIV-1 disease. Furthermore, the functional redundancy of regulatory sites in the V3 loop may relate to our recent observation of functional redundancy among various domains of the

coreceptors themselves (3). These findings deepen our understanding of the molecular mechanism for the so-called "switch" from macrophage to T-cell tropism during progression of HIV-1 disease, which may be an important factor in the pathogenesis of AIDS.

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