Mechanism of Transdominant Inhibition of CCR5-mediated HIV-1 Infection by ccr5Δ32*

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Human chemokine receptor 5 (CCR5) functions as a co-receptor for Human immunodeficiency virus (HIV-1) infection. CCR5 is a seven-transmembrane cell surface receptor. Recently, a naturally occurring mutation of CCR5, ccr5Δ32, has been described. A small number of Caucasians are homozygously ccr5Δ32/ccr5Δ32, while a larger number of individuals are heterozygously CCR5/ ccr5Δ32. The ccr5Δ32/CCR5Δ32 genotype has been linked to a phenotype that is “highly” protected from HIV-1 infection. On the other hand, several studies have shown that the CCR5/ccr5Δ32 genotype confers “relatively” protection from AIDS with onset of disease being delayed by 2–4 years. Although it is known that peripheral blood lymphocytes from heterozygous individuals (CCR5/CCR5) are less infectible by M-tropic HIV-1s than CCR5/CCR5 cells, the molecular basis for this observation is unknown. Here we report on events that post-translationally modify CCR5. We show that CCR5 progresses through the endoplasmic reticulum prior to appearing on the cell surface. Mature CCR5 can be post-translationally modified by phosphorylation and/or co-translationally by multimerization. By contrast, mutant ccr5Δ32, although retaining the capacity for multimerization, was incapable of being phosphorylated. ccr5Δ32 heterocomplexes with CCR5, and this interaction retains CCR5 in the endoplasmic reticulum resulting in reduced cell surface expression. Thus, co-expression in cells of ccr5Δ32 with CCR5 produces a trans-inhibition by the former of ability by the latter to support HIV-1 infection. Taken together, our findings suggest CCR5/CCR5Δ32 heterodimerization as a molecular explanation for the delayed onset of AIDS in CCR5/ ccr5Δ32 individuals.

Human immunodeficiency virus (HIV-1) uses CD4 as the primary receptor and chemokine co-receptors to enter target cells (1). Chemokine receptors belong to the superfamily of G protein-coupled receptors that have seven transmembrane domains. Chemokines are a family of small proteins (7–16 kDa) that can be operationally divided in two subgroups. The α subfamily (CXC) is distinguished from the β subfamily (CC) by the insertion of a single amino acid between the first and the second cysteine residues. The binding of chemokines to their receptors induces a rapid calcium influx and inflammatory responses (2). The CXC chemokine receptor for stromal cell-derived factor-1 (CXCR4) was initially shown to be a co-receptor for T-cell-tropic (T-tropic) HIV-1s (5). Based on the finding that chemokines (CC-β) RANTES, MIP-1α, and MIP-1β inhibit infection by macrophage-tropic (M-tropic) HIV-1 isolates (6), subsequent studies revealed that CCR5 functions as a major co-receptor for M-tropic viruses (7–11). Accordingly, further investigations have demonstrated the existence, in some ethnic groups (e.g. Caucasians), of a natural genetic mutation in CCR5 (ccr5Δ32, an internal 32-nucleotide deletion in the CCR5 open reading frame). Homozygous ccr5Δ32/CCR5Δ32 genotype confers resistance to HIV-1 infection in vitro and in vivo (12). However, the incidence of homozygosity in Caucasians is low (1%), while heterozygous (CCR5/ ccr5Δ32) individuals exist more prevalently (up to 20% in some populations).

There is evidence that CCR5/ccr5Δ32 heterozygotes progress more slowly to AIDS (13–16). Currently, how heterozygosity (CCR5/ ccr5Δ32) mechanically impacts disease progression is unknown. It has, however, been observed that CCR5/ ccr5Δ32 PBMCs are less infectible in vitro by M-tropic HIV-1s than CCR5/CCR5 cells (12). Although the level of cell surface expression of CCR5 in the uninfected population is quite heterogeneous, varying up to 20-fold between individuals (17), one study has found that CCR5/ ccr5Δ32 T-cells are markedly reduced for surface expression of CCR5 compared with CCR5/CCR5 counterparts (18). Accordingly, a correlation between surface CCR5 expression and infectibility by M-tropic HIV-1s is suggested (18).

The mechanism through which ccr5Δ32 might affect CCR5-function remains to be clarified. To assess this issue we studied the effect of ccr5Δ32 on the processing, stability, and cell surface expression of CCR5. We found that 1) CCR5 is post-translationally phosphorylated upon MIP-1β stimulation of cells; 2) intracellularly, CCR5 exists as either CCR5/CCR5 or CCR5/ ccr5Δ32 multimers; 3) CCR5 and ccr5Δ32 are found in different cellular locales (the former is predominantly on the cell surface while the latter is retained in the ER); and 4) co-expression of ccr5Δ32 inhibited surface expression of CCR5 and CCR5-mediated infection by M-tropic HIV-1 isolates.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—All constructs were derived from pCCR5 (5). CCR5 mutants, cloned into pcDNA3 (Invitrogen), include pCMV/ CCR5Δcyt (aa 1–303), pCMV/CCR5/6TM (aa 1–270), pCMV/CCR5/5TM (aa 1–235), and pCMV/CCR5/Δ32 (aa 1–187). Each mutant was gener-

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ated by polymerase chain reaction with the HA epitope fused to each cDNA at the 3’ terminus. pCMVCCR5-Flag was a gift from Ron Willey (National Institutes of Health).

**Cell Culture, Infection, and Transfection**—HeLa cells were propagated in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum. PBMCs were activated for 3 days in RPMI with 10% fetal bovine serum containing PHA and then washed and resuspended into the same medium without PHA and with 100 units/ml interleukin-2 (Boehringer Mannheim). PBMCs were exposed to M-tropic HIV-1 isolate AD8 (500 units of TCID50) for 1 h at 37 °C, washed, and then resuspended into fresh medium. Virus replication was monitored by reverse transcriptase assay as described previously (19). Transfection of HeLa cells was performed using calcium phosphate.

**Pulse-Chase Analysis**—24 h after transfection, cells were washed twice in DMEM without methionine and cysteine starved for 30 min in the same medium at 37 °C. [35S]Methionine + cysteine (translabel ICN) at 1 mCi/ml final concentration was added to the cells. The cells were then incubated at 37 °C for the indicated amount of pulse time and then washed and resuspended in DMEM with methionine and cysteine for the indicated chase times.

**Protein Analysis**—For immunoprecipitation, identical protein amounts were suspended into 1 ml of Triton lysis buffer (0.5% Triton X-100, 300 mM NaCl, 50 mM Tris, pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride) and incubated for 2 h at 4 °C with either anti-Flag M2 (Eastman/Kingsey Co.) or anti-HA (12CA5, Boehringer Mannheim). A mixture of protein A- and protein G-Sepharose (Pharmacia Biotech Inc.) was added to each sample followed by a 1-h incubation at 4 °C. Three washes were performed in Triton wash buffer (0.1% Triton X-100, 300 mM NaCl, 50 mM Tris, pH 7.4, 0.2 mM phenylmethylsulfonyl fluoride) and a final wash in SDS/deoxycholate buffer (300 mM NaCl, 50 mM Tris, pH 7.4, 0.1% SDS, 0.1% deoxycholate). The immunoprecipitated products were solubilized in 1 × loading buffer (125 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.01% bromphenol blue) and resolved by SDS-PAGE.

**Confocal Microscopy**—HeLa cells were seeded onto coverslips and transfected. 24 h later, cells were fixed with fresh 4% paraformaldehyde, pH 7.0, for 10 min at room temperature. Fixed cells were permeabilized with a 2-min wash in 100% methanol at room temperature followed by several washes in PBS with 4% bovine serum albumin (PBS/BSA). Appropriately diluted primary antibody was incubated with coverslips overnight at 4 °C. Excess antibody was removed with four washes in PBS/BSA. Species-specific second antibody conjugated to Texas Red (Cappel) was then reacted with the coverslips for 1 h at room temperature followed by four washes in PBS/BSA. The final samples were mounted onto slides and visualized using a Zeiss Axiopt photonic confocal microscope.

**Yeast Two-hybrid Assay**—Two-hybrid assays were performed according to manufacturer’s protocols (CLONTECH).

**RESULTS AND DISCUSSION**

To ask whether CCR5 forms an oligomer, HeLa cells were transfected with pCMV/CCR5-Flag, which contains the Flag epitope fused to the C terminus of CCR5. 24 h later, cells were pulsed with [35S]methionine + cysteine for 15 min, washed, and then chased in complete medium for 1 h. Extracts prepared from pulsed and chased cells were immunoprecipitated using anti-Flag M2. The immunoprecipitates were resuspended into either 1 × loading buffer (125 mM Tris, pH 6.8, 20% glycerol, 2% SDS, 2% β-mercaptoethanol, 0.01% bromphenol blue) and resolved by SDS-PAGE.

The reduced mobility of CCR5 compared with the pulsed sample (Fig. 1, A, lane 1) is consistent with a post-translational modification. When the same analysis was repeated using native loading buffer (Fig. 1A, lanes 3 and 4), the mobility difference observed for pulsed (lane 3) and pulsed + chased (lane 4) samples was replicated. The native buffer-PAGE revealed additional CCR5-specific bands with sizes consistent with dimeric moieties (d; Fig. 1A, lanes 3 and 4).

To characterize better multimerization potentials, we analyzed CCR5 using the yeast two-hybrid approach (Table I). In this analysis, C-terminal truncation mutants of CCR5 were found to interact with wild type CCR5. However, a mutant deleted in the first 58 amino acids failed to interact with intact CCR5. Thus, in yeasts, the CCR5-CCR5 interactive domain resides in the N-terminal portion of the protein, which encompasses the first transmembrane region.

The biological implications of dimerization were further examined. We co-expressed CCR5 and ccr5Δ32 in HeLa CD4+ LTR-βgal cells and assayed for cell fusion engendered by an M-tropic HIV-1 isolate, HIV-1ΔD8s (20). A prediction of protein dimerization is that ccr5Δ32 could be a dominant negative inhibitor of CCR5 function. We found that transfection of HeLa CD4+ LTR-CAT with wild type CCR5 (Fig. 2A) did render, as expected, HeLa cells susceptible to HIV-1ΔD8s infection (Fig. 2A, compare lane 8 with 9). Co-transfection of CCR5 with ccr5Δ32 (CCR5 lacking the cytoplasmic tail) did not affect HIV-1ΔD8s infection (Fig. 2A, lane 10). However, co-expression of CCR5 with either ccr5Δ6TM, or ccr5Δ3TM, or ccr5Δ4TM (ccr5Δ32) or ccr5Δ3TM (i.e. C-terminal mutants deleted for six, five, four, or three transmembrane regions, respectively) dramatically reduced the ability of cells to support HIV-1ΔD8s-induced fusion. No effect was seen when pNL4–3 (T-tropic HIV-1) infections were conducted under parallel conditions (Fig. 2A, lanes 1–7), confirming M-tropic specificity of CCR5 in the above experiments.

Co-expression of ccr5Δ32 and CCR5 in monolayer cells can be regarded as an artificial approach modeling in vivo infection. To construct a more physiological test, we conducted infections comparing PBMCs from one CCR5/CCR5 and two CCR5/ ccr5Δ32 individuals. These results (Fig. 2B) are consistent with the above findings (Fig. 2A) and previously published results (12), confirming that simultaneous presence of ccr5Δ32 with

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2 R. Willey, unpublished observation.
CCR5 in the same cell reduces susceptibility to infection by M-tropic HIV-1.

The infection results led us to consider how ccr5 D32 might affect the in vivo presentation of CCR5. To address this question, we used confocal immunofluorescence to visualize potential intracellular influences of ccr5 D32 on CCR5. Upon staining with specific antiserum, steady-state CCR5 and ccr5 D32 were found in distinctly different subcellular locales. While CCR5 was found on cell surfaces (Fig. 3A), ccr5 D32 stained in the ER (Fig. 3B). ER localization was observed for all ccr5 mutants (ccr5/6TM, ccr5/5TM, ccr5/4TM, and ccr5/3TM), except ccr5 cyt, which was found on the cell surface (data not shown).

When CCR5 and ccr5Δ32 were co-expressed in the same cell, significantly reduced surface staining of the former was seen (Fig. 3C). The above findings are consistent with CCR5-CCR5 dimers. The biochemical evidence provided by mobility shifts in PAGE (Fig. 1, compare lanes 1 and 2) could also be explained by other modifying events such as phosphorylation. Because the C terminus of CCR5 is rich in serine and threonine residues, which are potential substrate sites for G protein-coupled receptor-kinase(s), and because phosphorylation could conceivably influence overall receptor function, we queried for this possibility. We, thus, transfected HeLa cells with pCMV/CCR5-Flag and labeled the cells in parallel with either [35S]methionine (Fig. 4A, lanes 1–4) or [32P]orthophosphate (Fig. 4A, lanes 5–8). Cell extracts were prepared and immunoprecipitated using anti-Flag antibody (A) or anti-HA antibody (B).
infection would be difficult (23). We note additionally that a protective effect to a CCR2 mutation has been recently described (24). Whether the CCR2 mutation shares functional similarities with the CCR5 Δ32 mutation (e.g. producing non-productive transdominant multimerization or affecting phosphorylation) remains to be explored. However, the natural existence of transdominant negative chemokine receptor mutants might be a general principle that explains differential resistance of certain subpopulations for AIDS and that might help guide future intervention strategies for HIV-1.

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