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18. VP14 was expressed as a glutathione-S-transferase fusion protein (Pharmacia). Enzyme assays contained 100 mM bis-tris (pH 6.7), 0.05% Triton X-100, 10 mM ascorbate, 5 μM $FeSO_4$, catalase (1 mg/ml), and VP14 protein. Assays were incubated for 15 min at 22° to 24°C in a total volume of 100 μl. The appropriate substrate was added in 3 μl of ethanol. The enzyme assay and all subsequent procedures were performed under red light to minimize photooxidation of the precursors and products.
19. The cleavage products were analyzed on a JEOL AX 505 double-focusing mass spectrometer equipped with a Hewlett-Packard gas 5890 chromatograph, using electron ionization at 70 eV. The trimethylsilyl ether derivative of xanthoxin gave a spectrum that was similar to a previously reported spectrum (*28*). The C_{25} apo-aldehydes were introduced via a direct insertion probe heated from ambient temperature to 200°C at 64°C min^{-1}. The fragmentation patterns for the epoxy (5,6-epoxy-3-hydroxy-12′-apo-β-caroten-12′-al) and allenic-(3,5-dihydroxy-6,7-didehydro-12′-apo-β-caroten-12′-al) C_{25} cleavage products were nearly identical to published spectra for these compounds (*29*). Exact mass measurements were performed as described above, with the exception that the instrument's mass resolution was increased from 1000 to 7500. Perfluorokerosene was introduced simultaneously with the sample to provide reference ions for exact mass assignments. The theoretical mass of the isomeric C_{25} cleavage products from neoxanthin and violaxanthin ($C_{25}H_{34}O_3$) is 382.2508. The measured mass of the C_{25} product from neoxanthin was 382.2498, with an error of −2.6 parts per million (ppm) from the calculated mass. The measured mass of the C_{25} product from violaxanthin was 382.2501, with an error of −1.8 ppm.
20. S. H. Schwartz, B. C. Tan, D. A. Gage, J. A. D. Zeevaart, D. R. McCarty, data not shown.
21. Iron was chelated from the VP14 protein with 50 mM EDTA. The EDTA was subsequently removed on a G-25 Sephadex spin column equilibrated with 100 mM bis-tris and 0.05% Triton-X 100. VP14 protein (7 μg) was then added to reactions containing the indicated cofactors [n = 2; n.d., not detectable [treatment, xanthoxin (ng) ± SE]]: No iron, n.d.; 5 μM Fe^{2+}, 416.1 ± 8.1; 5 μM Fe^{3+}, n.d.; 5 μM Fe^{3+} + 10 mM ascorbate, 432 ± 8.4.
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23. The low- and high-resolution mass spectra of the C_{25} product from cleavage of 9-*cis*-zeaxanthin were obtained as described above (*19*). For C_{25} zeaxanthin apo-aldehyde (3-hydroxy-12′-apo-β-caroten-12′-al), the mass-to-charge ratio (m/z) and relative intensity (in parentheses) were as follows: 366 $[M]^+$ (100), 348 (6), 255 (4), 213 (8), 197 (8), 147 (17), 119 (20), 105 (15), 91 (15). The theoretical mass of the cleavage product ($C_{25}H_{34}O_2$) is 366.2559; and the experimentally determined mass was 366.2564, with an error of 1.5 ppm from the calculated mass. The C_{15} cleavage product was analyzed as described above, except that the probe was heated at 2°C min^{-1}. For C_{15} zeaxanthin apo-aldehyde (3-hydroxy-apo-β-caroten-11-al), m/z and relative intensity were as follows: 234 $[M]^+$ (67), 219 (17), 201 (48), 187 (35), 159 (34), 149 (79), 131 (43), 121 (52), 105 (52), 95 (100). The compound ($C_{15}H_{22}O_2$) has a theoretical mass of 234.1620; and the experimentally determined mass was 234.1611, with an error of −3.7 ppm.
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31. After the incubation, 1 ml of H_2O was added to the reactions. The reaction products were then partitioned three times into an equal volume of ethyl acetate with vigorous stirring on a vortex mixer. The ethyl acetate fractions were pooled and dried, and methyl ABA was added as an injection standard. The samples were stored under Ar at −80°C until analysis. Samples were injected on a μPorasil column (Waters) (30 by 0.4 cm) equilibrated with 90% hexane and 10% ethyl acetate. The column was eluted with a linear gradient to 20% hexane and 80% ethyl acetate over 17 min. A standard curve was constructed by injecting known quantities of the compounds analyzed and integrating the peak areas. The integrated peak area of methyl ABA was used to correct for variations in injections. The correlation coefficients were generally greater than 0.99.
32. The carotenoid substrates were isolated from spinach leaves (*30*). The *abal-5* mutant of *Arabidopsis thaliana* was used as a source of all-*trans*-zeaxanthin, which was isomerized with iodine (*22*) and rechromatographed to isolate 9-*cis*-zeaxanthin.
33. Supported by U.S. DOE grant DE-FGO2-91ER20021, NSF grant IBN-9118377 (J.A.D.Z.), NIH grant RR-00480 to the MSU Mass Spectrometry Facility (D.A.G), and NSF grant IBN-9406692 (D.R.M).

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Identification of a Chemokine Receptor Encoded by Human Cytomegalovirus as a Cofactor for HIV-1 Entry

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The human cytomegalovirus encodes a β-chemokine receptor (US28) that is distantly related to the human chemokine receptors CCR5 and CXCR4, which also serve as cofactors for the entry into cells of human immunodeficiency virus–type 1 (HIV-1). Like CCR5, US28 allowed infection of CD4-positive human cell lines by primary isolates of HIV-1 and HIV-2, as well as fusion of these cell lines with cells expressing the viral envelope proteins. In addition, US28 mediated infection by cell line–adapted HIV-1 for which CXCR4 was an entry cofactor.

Human immunodeficiency virus infects cells by a process of membrane fusion that is mediated by its envelope glycoproteins (gp120-gp41, or Env) and is generally triggered by the interaction of gp120 with two cellular components: CD4 and a coreceptor belonging to the chemokine receptor family (*1*). The coreceptor for HIV-1 strains adapted to replication in $CD4^+$ cell lines (TCLA strains) was identified by a genetic complementation approach and named fusin (*2*); however, it was later shown to be an α- (or CXC) chemokine receptor and designated CXCR4 (*3*). The isolation of fusin and the antiviral activity of certain β- (or CC) chemokines (*4*) led to the demonstration that the β-chemokine receptor CCR5 is the principal coreceptor for primary HIV-1 strains (*5–8*). In addition to CCR5, certain primary HIV-1 strains (dual tropic) use CXCR4 (*9*), or CXCR4 and CCR2b (*8*), as a coreceptor, whereas others (macrophage tropic) can use CCR3 (*7, 10*). The essential role of CCR5 is nevertheless indicated by the resistance to HIV-1 infection of individuals with defective *CCR5* alleles (*11*). The CCR5 and CXCR4 coreceptors are also used by HIV-2 and the related simian immunodeficiency viruses (*12, 13*).

Several homologs of chemokine receptors are encoded by herpesviruses (*14*); in particular, by the US27, US28, and UL33 open reading frames (ORFs) of the human cytomegalovirus (CMV) (*15*). In fibroblasts infected experimentally, these ORFs were transcribed at a high rate after viral DNA replication (*16*), but their pattern of expression in vivo and their role in the life cycle of CMV are unknown. The product

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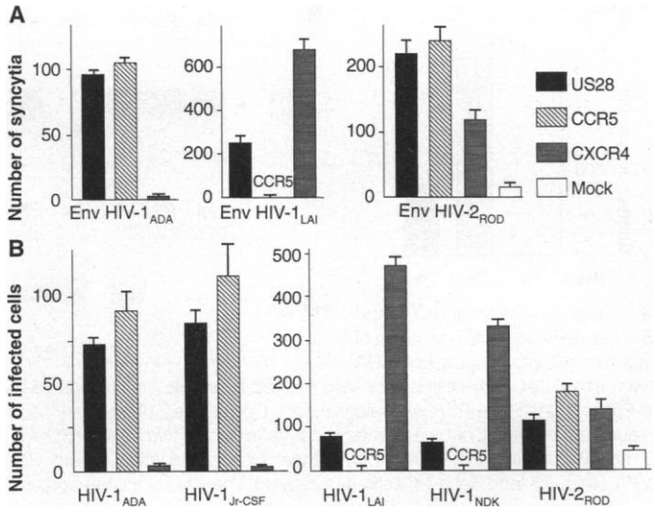


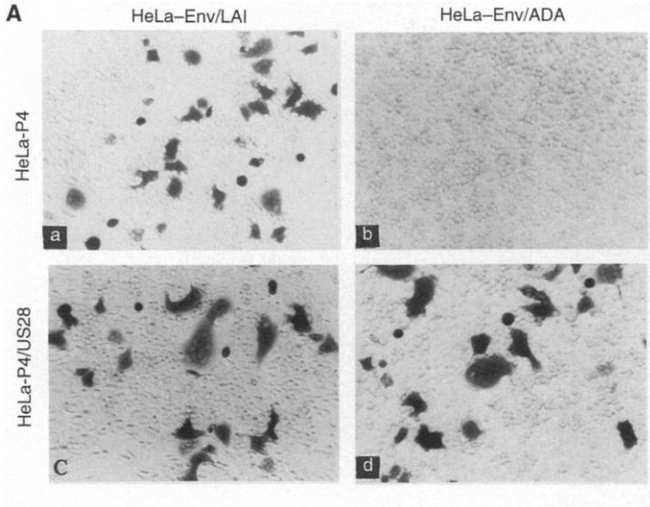
Fig. 1. HIV coreceptor activity of CXCR4, CCR5, and US28 expressed in the human cell line U373MG-CD4. U373MG-CD4 cells (LTR-*lacZ*$^+$) were harvested 24 hours after transfection and transferred to 24-well plates for coculture (1:1 ratio) with HeLa–Env/ADA, HeLa–Env/LAI, or HIV-2_{ROD}–infected cells (**A**), or for infection with the HIV-1 strains ADA, Jr-CSF, LAI, or NDK (same inoculums as in Table 1), or with HIV-2_{ROD} (50 ng of p24 per well) (**B**). Cells were fixed and stained with X-Gal 20 hours after initiation of coculture or 40 hours after infection. Data represent numbers of blue-stained foci per well (mean of two experiments, with independent transfections). Error bars represent the range between mean and maximal values.

of the US28 ORF (here referred to as US28) is a functional receptor for several β-chemokines, including the CCR5 ligands RANTES (regulated on activation, normal T expressed and secreted), macrophage inflammatory protein–1α (MIP-1α), and MIP-1β (*17*). We therefore investigated whether US28 exhibits HIV coreceptor activity.

The human glioma-derived cell line U373MG-CD4 is naturally resistant to HIV-1 entry and to fusion with Env$^+$ cells and is stably transfected with a long terminal repeat (LTR)–*lacZ* construct inducible by the HIV-1 transactivator Tat (*18*). Cells infected by HIV, or syncytia formed with Tat$^+$Env$^+$ cells, can therefore be detected with high specificity by an in situ β-galactosidase assay (blue staining with the X-Gal substrate), as described previously (*12*, *18*). U373MG-CD4 cells were transfected with expression vectors encoding US28, CCR5, or CXCR4 (*19*) and then tested for their ability to form syncytia with various Env$^+$ cells (Fig. 1A). As expected, U373MG-CD4 cells expressing CXCR4 formed syncytia with HeLa–Env/LAI cells (*20*) stably expressing Env from HIV-1_{LAI} (TCLA), whereas U373MG-CD4 cells expressing CCR5 formed syncytia with HeLa–Env/ADA cells stably expressing Env from the primary macrophage-tropic HIV-1_{ADA} (*21*). Both CXCR4 and CCR5 allowed the formation of syncytia with cells chronically infected with HIV-2_{ROD} (*22*). The expression of US28 allowed fusion of U373MG-CD4 cells with each of the three types of Env$^+$ cells. Similar results were obtained in infection assays (Fig. 1B). The macrophage-tropic HIV-1_{ADA} and HIV-$1_{Jr\text{-}CSF}$ (*23*) infected U373MG-CD4 cells expressing CCR5 or US28, but not those expressing CXCR4. The TCLA strains HIV-1_{LAI} and HIV-1_{NDK} (*24*) infected cells expressing CXCR4 or US28, but not those expressing CCR5. The U373MG-CD4 cells were not totally resistant to infection with HIV-2_{ROD}, but the efficiency of infection was markedly increased by expression of either US28, CXCR4, or CCR5. In these experiments, US28 behaved as a coreceptor for primary HIV-1 strains, and for HIV-2_{ROD}, with an efficiency similar to that of CCR5. In addition, US28 mediated infection by TCLA HIV-1 strains, although less efficiently than CXCR4.

The coreceptor activity of US28 was tested in another CD4$^+$ human cell line, HeLa-P4, also stably transfected with an LTR-*lacZ* construct (*25*). These cells are permissive to infection by TCLA and dual-tropic HIV-1 and HIV-2, but not to infection by primary strains with a macrophage-tropic or non–syncytium-inducing (NSI) phenotype. Accordingly, they formed syncytia, detectable by staining with X-Gal, on coculture with HeLa–Env/LAI but not with HeLa–Env/ADA cells (Fig. 2A). Fusion with HeLa–Env/ADA was readily observed when HeLa-P4 cells were transfected with the US28 expression vector (Fig. 2A), although a higher number of syncytia was generally apparent on expression of CCR5 (Fig. 2B). In contrast, fusion with HeLa–Env/ADA cells was not observed when HeLa-P4 cells were transfected with expression vectors encoding CXCR4 (Fig. 2B) or other chemokine receptors—in particular, CCR1 and the Duffy antigen—or with expression vectors containing the US27 or UL33 ORFs (*26*). Also, fusion with Env$^+$ cells was not detected when the US28 or CCR5 vectors were transfected into CD4-negative LTR-*lacZ* HeLa cells (*25*).

To facilitate detection of US28, CCR5, and CXCR4 at the cell surface, we engineered



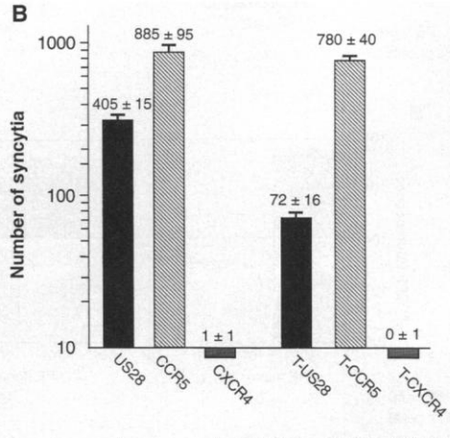


Fig. 2. HIV-1 coreceptor activity of CCR5 and US28 in HeLa-P4 cells (CD4$^+$ LTR-*lacZ*$^+$). (**A**) HeLa-P4 cells that had been mock-transfected (a and b) or transfected with the US28 vector (c and d) were cocultured with HeLa–Env/LAI cells (a and c) or HeLa–Env/ADA cells (b and d), and then stained with X-Gal, as described in Fig. 1A. (**B**) Syncytia formation in cocultures of HeLa–Env/ADA cells and HeLa-P4 cells transfected with expression vectors encoding CCR5, US28, CXCR4, or their epitope-tagged forms (T-US28, T-CCR5, and T-CXCR4). The experiment was performed as described in Fig. 1A. Data represent numbers of blue-stained foci per well.

their NH_2-termini to express a c-MYC epitope (27). Transfection of HeLa-P4 cells with vectors encoding the epitope-tagged forms of US28 or CCR5 allowed fusion with HeLa–Env/ADA cells (Fig. 2B). The epitope tag markedly reduced the activity of US28, but had a lesser effect on the activity of CCR5 (Fig. 2B). Flow cytometry of transfected HeLa-P4 cells (28) revealed that the surface expression of epitope-tagged US28 was reduced relative to that of the tagged forms of CCR5 or CXCR4 (Fig. 3). Differences in the surface expression of epitope-tagged chemokine receptors have been observed previously (7). Thus, modification of the NH_2-terminal domain of US28 may reduce HIV coreceptor activity or have an indirect effect on its transport to or stability at the cell surface.

HeLa-P4 cells were stably transfected with the CCR5 or US28 vectors (29), and clones were selected for their ability to form syncytia with HeLa–Env/ADA cells (Fig. 4A). Unlike the parental cells, HeLa-P5 (expressing CCR5) and HeLa-P6 (expressing US28) cells could be infected by HIV-1_{ADA}, HIV-$1_{Ir\text{-}CSF}$, and two primary NSI strains (VEN and BX01), as detected by staining cells with X-Gal (Table 1) or, in the case of HIV-1_{ADA}, by polymerase chain reaction (PCR) amplification of the viral DNA. The infection of HeLa-P5 cells was more efficient for all strains tested. However, Northern (RNA) blot analysis suggested that CCR5 is expressed at a higher level in this cell line relative to US28 in HeLa-P6 cells (Fig. 4B). The infection of HeLa-P5 and HeLa-P6 cells with HIV-1_{LAI} was less efficient compared with infection of HeLa-P4 cells (Table 1); this difference was apparently not due to a lower abundance of CXCR4 mRNA in the former cell types (Fig. 4B).

Deng *et al.* (5) did not detect infection with primary HIV-1 strains in a population of $CD4^+$ HeLa cells transduced with a US28 retroviral vector. The extent of US28 expression in these cells was probably lower than that in the HeLa-P6 clone or in transiently transfected cells. Our experiments with epitope-tagged chemokine receptors suggest that the surface expression of US28 is less efficient than that of CCR5. A threshold level of expression necessary to detect HIV coreceptor activity might therefore be more difficult to achieve for US28. Such an explanation also might underlie the apparent lack of HIV coreceptor activity of US28 when expressed with the vaccinia virus–T7

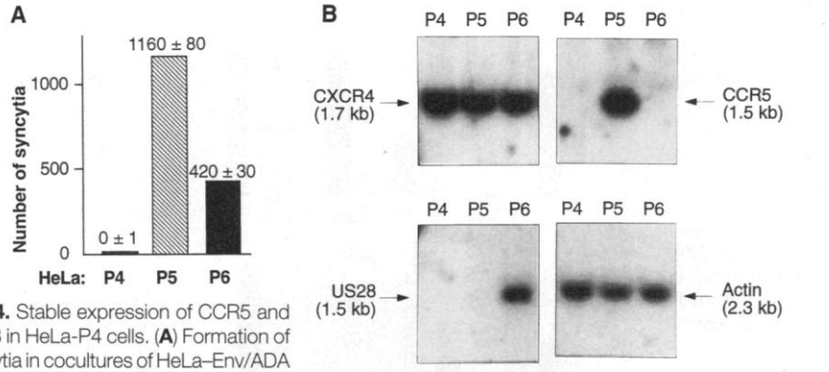


Fig. 4. Stable expression of CCR5 and US28 in HeLa-P4 cells. (**A**) Formation of syncytia in cocultures of HeLa–Env/ADA cells with either HeLa-P4 cells or cell lines derived by stable transfection of HeLa-P4 cells with CCR5 (HeLa-P5) or US28 (HeLa-P6) expression vectors. Cocultures (1 : 1) were stained with X-Gal after 20 hours. Data represent numbers of blue-stained syncytia (mean of two wells). (**B**) Detection of CXCR4, CCR5, US28, and β-actin (control) mRNA by Northern blot hybridization of total RNA (10 μg per lane) from HeLa-P4, HeLa-P5, and HeLa-P6 cells, as indicated. The sizes of the transcripts are indicated in kilobases.

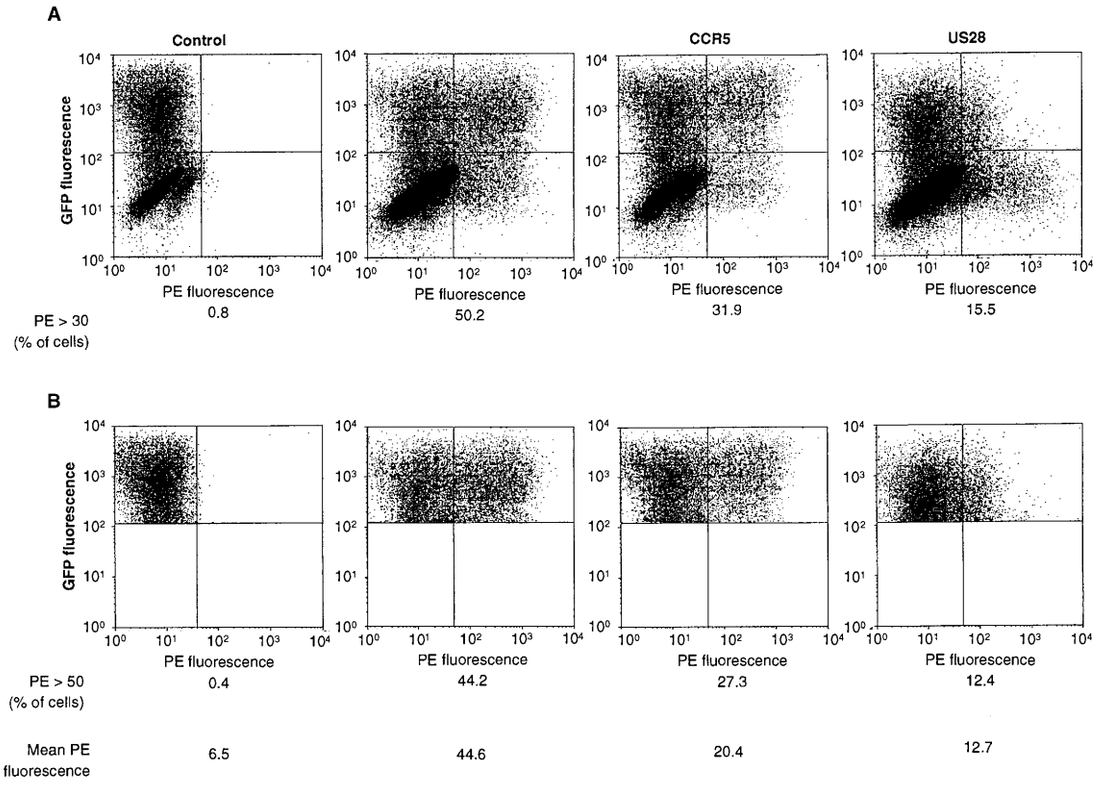


Fig. 3. Detection of epitope-tagged chemokine receptors by flow cytometry. HeLa-P4 cells were cotransfected with the EGFP-N1 vector encoding the green fluorescent protein (GFP) and either an expression vector encoding epitope-tagged CXCR4, CCR5, or US28 or the pCDNA-tag vector (control). Cells were detached 36 hours later and stained with the 9E10 tag-specific monoclonal antibody and phycoerythrin (PE)-conjugated secondary antibodies. (**A**) Analysis for red (*x* axis) and green (*y* axis) fluorescence. The percentage of PE-positive cells (PE fluorescence, >30 arbitrary units) is indicated. (**B**) Similar analysis for 10^4 cells selected for green fluorescence (>100 arbitrary units). The percentage of PE-stained cells (>50 arbitrary units) and mean PE fluorescence intensity are also indicated.

Table 1. HIV-1 infection of HeLa-P4 ($CD4^+$ LTR-*lacZ*) and derived cell lines stably transfected with CCR5 (HeLa-P5) or US28 (HeLa-P6) expression vectors. Data represent number of blue-stained cells per well (24-well plates after incubation with X-Gal 40 hours after infection).

HIV-1 strain	Phenotype	Inoculum (ng of p24)	Cell lines		
			HeLa-P4	HeLa-P5	HeLa-P6
LAI	TCLA	5	2700	520	870
VEN	Primary NSI	27	2	145	57
BXO1	Primary NSI	5	3	280	95
Jr-CSF	Primary MT*	180†	2	135	48
ADA	Primary MT	50†	2	275	75

*MT, macrophage tropic. †The p24 concentration–to-infectivity ratio was lower for these strains, produced by transfection of cloned proviruses into HeLa cells, than for VEN and BX01, which were produced from acutely infected lymphocytes.

RNA polymerase (vT7pol) system (*2*); CCR5 and CXCR4 were functional in parallel assays (*30*). The inhibition of protein synthesis by vaccinia virus infection may affect the transport of US28 or its turnover at the cell surface. Alternatively, it may block the expression of a cellular component required for the coreceptor activity of US28.

Infection by CMV is frequent among HIV-infected individuals and has been proposed to play a role in HIV pathogenesis (*31*), although this view is not supported by all epidemiological studies (*32*). The principal HIV-1 target cells, $CD4^+$ lymphocytes and monocytes-macrophages, support the replication of CMV in vivo (*33*). The abundance of CCR5 mRNA in these cells (*5*, *34*) appears markedly lower than that of US28 mRNA in fibroblasts infected with CMV (*16*). If US28 is expressed at a similar level in $CD4^+$ cells, CMV infection might therefore facilitate subsequent HIV-1 infection, explaining the frequent histological detection of dually infected cells in the brain, lung, or retina (*35*). In fibroblasts infected experimentally with CMV, US28 was apparently expressed as a late CMV gene (*16*). If the same pattern of expression occurs in vivo, CMV-infected cells should only express US28 transiently, before they are destroyed by cytopathic effects or by the immune system. However, some cells may survive for a sufficient period to support an HIV replication cycle. Also, US28 has been proposed to be associated with the CMV envelope and to be transferred to the membrane of target cells during virus entry (*16*). In this instance, cells could bear US28 at their surface before, or even in the absence of, CMV replication.

Several studies have demonstrated upregulation of HIV-1 replication by CMV infection in $CD4^+$ cell lines (*36*), macrophages (*37*), and syncytiotrophoblast cells (*38*). Various interpretations of these observations have been proposed, but possible effects on HIV-1 entry were not directly tested. However, CMV had no effect or down-regulated HIV-1 replication when cell lines were infected with phenotypically mixed HIV-1 particles via a CD4-independent pathway (*39*).

The coreceptor activity of CXCR4, CCR5, and US28 was observed for genetically divergent HIV-1 strains and for HIV-2, suggesting that these molecules interact with a conserved domain or conformational motif of gp120. The extracellular domains of these chemokine receptors, which are likely to mediate this interaction, differ from each other in primary structure. In contrast, receptors highly related to CCR5, such as CCR2a, do not mediate HIV-1 entry (*5*–*8*). The extracellular domain of US28 might coincidentally possess a conformation similar to that of CCR5 (and CXCR4), allowing interaction with gp120. It is possible that other chemokine receptors also interact with gp120 but lack a property required for HIV coreceptor function, such as the ability to colocalize with CD4. In addition to its possible role in the interactions of CMV and HIV, the US28 chemokine receptor may prove a useful tool with which to address the mechanism of action of HIV coreceptors.

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19. The CXCR4 cDNA (*12*) as well as the CCR5 and US28 ORFs were subcloned in the Rc/CMV vector (Invitrogen, La Jolla, CA), allowing expression from the CMV immediate-early promoter. The CCR5 and US28 ORFs were amplified by PCR from HeLa cells and from fibroblasts infected with CMV (AD169 strain), respectively. Their complete nucleotide sequences were determined and shown to be identical to those previously described. Consistent with previous studies (*17*), we found that the US28 ORF encodes 352 amino acids; the initial report predicted 323 amino acids (*15*). U373MG-CD4 cells were transfected in six-well plates (3 μg of DNA per well) by calcium phosphate precipitation. Cells were harvested 24 hours after transfection and seeded in 24-well plates for coculture with Env^+ cells, or for infection with HIV, as described (*12*).
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28. HeLa-P4 cells were cotransfected (6:1 ratio) with vectors encoding epitope-tagged receptors and EGFP-N1 (Clontech), which encodes the green fluorescent protein. Cells were detached with phosphate-buffered saline containing 1 mM EDTA 36 hours after transfection, and then stained with the

9E10 monoclonal antibody (4 μg/ml) (Boehringer, Mannheim, Germany) and phycoerythrin-conjugated goat antibodies to mouse immunoglobulin G (16 μg/ml) (Dako, Glostrup, Denmark). Fixed cells were analyzed for green and red fluorescence with an Epics Elite flow cytometer (Coultronics).
29. HeLa-P4 cells were cotransfected by calcium phosphate precipitation with a hygromycin-resistance vector (SV-hygro) and either Rc/CMV–CCR5 or Rc/CMV–US28 (1:20 ratio). Cell clones were selected in the presence of hygromycin B (150 μg/ml).
30. We performed the experiments essentially as described (*2*). Simian COS cells or HeLa cells were cotransfected with Rc/CMV vectors containing the CXCR4, CCR5, or US28 ORFs (cloned downstream of the T7 promoter) and Rc/CMV–CD4, and were infected with vT7pol. Cocultures were then performed with HeLa–Env/ADA or HeLa–Env/LAI cells transiently transfected with a T7-*lacZ* construct.
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Activation of the G Protein Gq/11 Through Tyrosine Phosphorylation of the α Subunit

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Various receptors coupled to the heterotrimeric guanine nucleotide-binding protein Gq/11 stimulate formation of inositol-1,4,5-trisphosphate (IP_3). Activation of these receptors also induces protein tyrosine phosphorylation. Formation of IP_3 in response to stimulated receptors that couple to Gq/11 was blocked by protein tyrosine kinase inhibitors. These inhibitors appeared to act before activation of Gq/11. Moreover, stimulation of receptors coupled to Gq/11 induced phosphorylation on a tyrosine residue (Tyr^{356}) of the $G\alpha_{q/11}$ subunit, and this tyrosine phosphorylation event was essential for Gq/11 activation. Tyrosine phosphorylation of $G\alpha_{q/11}$ induced changes in its interaction with receptors. Therefore, tyrosine phosphorylation of $G\alpha_{q/11}$ appears to regulate the activation of Gq/11 protein.

IP_3 is a second messenger that controls many cellular processes by causing release of Ca^{2+} from intracellular stores. Formation of IP_3 is stimulated by heterotrimeric guanine nucleotide-binding protein (G protein)–coupled receptors (GPCRs) and by receptors linked by tyrosine kinases either directly or indirectly. The formation of IP_3 activated by GPCRs is catalyzed by phospholipase Cβ (PLCβ) (*1*). G proteins are composed of three polypeptides denoted α, β, and γ. The α subunits, which bind and hydrolyze guanosine triphosphate (GTP) (*2*, *3*), are divided into four classes: $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and Gα12. The Gα subunits that regulate PLCβ belong to the Gq class ($G\alpha_q$, Gα11, Gα14, Gα15/16) (*4*). When an agonist binds to a GPCR, the receptor-linked G protein dissociates into a Gα subunit and a Gβγ dimer, each of which can activate target effectors. However, the precise in vivo mechanisms of receptor-mediated G protein activation remain to be elucidated (*2*, *3*). Several Gq or G11 (Gq/11)–coupled receptors induce tyrosine phosphorylation of cellular proteins (*5*). Here, we examined the role of tyrosine phosphorylation events in IP_3-Ca^{2+} signaling through Gq/11-coupled receptors.

The metabotropic glutamate receptor 1α (mGluR1α) is a Gq/11-coupled receptor (*6*). Application of glutamate to Chinese hamster ovary (CHO) cells expressing mGluR1α (*7*) increased tyrosine phosphorylation of cellular proteins within 1 min (Fig. 1A) (*8*). The tyrosine phosphorylation event was almost completely suppressed by genistein, a protein tyrosine kinase (PTK) inhibitor (*9*, *10*), and was enhanced by vanadate-H_2O_2, a protein tyrosine phosphatase (PTP) inhibitor (*10*) (Fig. 1B). The mGluR1α itself was immunoprecipitated with antibody to phosphotyrosine (anti-PY) only from cells treated with glutamate (Fig. 1C).

We examined the possible role of PTKs in Ca^{2+} mobilization (*11*). Addition of glutamate (100 μM) to the mGluR1α-expressing CHO cells increased the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$). However, when cells were incubated in the presence of genistein, no increase in $[Ca^{2+}]_i$ was observed [median inhibitory concentration (IC_{50}) ≈ 10 μM], even in cells treated with a high concentration of glutamate (1 mM; Fig. 2A). After genistein was washed out, the Ca^{2+} response was recovered. To demonstrate that the inhibition of Ca^{2+} release by PTK inhibitors is not a cell type–specific event, we recorded Ca^{2+}-activated Cl^- currents in *Xenopus* oocytes injected with mGluR1α mRNA (*12*). Current elicited by glutamate was blocked by any of three PTK inhibitors: genistein, tyrphostin AG213 (*9*), and AG60 (*13*) (Fig. 2B). The currents were restored after the drugs were removed. Thus, the stimulation-induced increase in $[Ca^{2+}]_i$ in these cells apparently requires PTK activity.

To determine whether the PTK inhibitors act before or after IP_3 formation, we examined the formation of IP_3 (*14*) in the presence or absence of PTK inhibitors. Genistein and tyrphostin AG213 almost completely abolished glutamate-stimulated IP_3 formation in mGluR1α-expressing CHO cells (Fig. 2C). The effect of genistein was concentration-dependent (IC_{50} ≈ 10 μM) (*15*). Daidzein, an analog of genistein that lacks PTK inhibitory activity (*10*), had no effect on IP_3 formation. A protein kinase C (PKC) inhibitor, H7,

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