HIV-1 preferentially binds receptors copatched with cell-surface elastase

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Human leukocyte elastase (HLE) interacts with HIV-1 glycoprotein (gp)41, suggesting a nonenzymatic receptor function for HLE in the context of HIV-1. HLE is found localized to the cell surface, but not granules in HIV permissive clones, and to granules, but not the cell surface of HIV nonpermissive clones. Inducing cell-surface HLE expression on HLE null, HIV nonpermissive clones permits HIV infectivity. HIV binding and infectivity diminish in proportion to HLE RNA subtraction. HIV binding and infectivity show dose dependence for the natural HLE ligand α1-proteinase inhibitor (α1-antitrypsin, α1-PI). Chemokines prevent, whereas α1-PI promotes, copatching of HLE with the canonical HIV receptors. Recent demonstration that decreased viral RNA is significantly correlated with decreased circulating α1-PI in HIV seropositive individuals is consistent with a model in which HLE and α1-PI can serve as HIV coreceptor and cofactor, respectively, and potentially participate in the pathophysiology of HIV disease progression. (Blood. 2003;102:4479-4486)

Introduction

HIV-1 entry and fusion are thought to involve an initial interaction between the envelope glycoprotein, gp120, and cell-surface CD4. This interaction exposes a site within gp120 that then interacts with a coreceptor (ie, CCR5 or CXCR4), inducing a conformational change within the gp41 portion of the viral envelope; this set of events results in insertion of the fusion domain of gp41 into the cell membrane. Cell-surface expression of specific chemokine receptors and CD4 is necessary, but not sufficient, to confer HIV-1 permissivity.1-2 Cellular resistance to HIV-1 infectivity can be due to fusion/entry failure, suggesting this differentiation-associated restriction is due to a positive factor or negative factor.

Comparison of HIV-1 permissive and permissive U937 subclones revealed relatively equivalent cell-surface CD4 and CXCR4 and a lack of CCR5.1 A notable difference was that nonpermissive, but not permissive, subclones expressed detectable granule-associated proteinases, cathepsin G (CatG) and human leukocyte elastase (HLE).3 These same proteinases are known to be cell-surface-associated in certain situations and to bind HIV-1 envelope proteins,4,5 but in association with the lipid bilayer, enzymatic activity and antigen detection are absent or compromised.6 This suggests that plasma membrane–associated proteinases may exhibit nonenzymatic receptor functions and that location, rather than gene expression, might impact HIV-1 permissivity.

Traditionally, the proteinase activity of HLE has been characterized in aqueous environments, and cell-surface lipids are known to negatively influence its catalytic activity7 further supporting a nonenzymatic function for plasma membrane HLE.7 In fact, the primary actions of cell-surface HLE and CatG involve adhesion, chemotaxis, and stem-cell mobilization.8-10 Granule-associated HLE rapidly translocates to the cell surface in response to many agonists including the bacterial endotoxin lipopolysaccharide (LPS),11 suggesting these activation signals rapidly mobilize HLE to the cell surface in the absence of protein synthesis. The precise domains in HLE that allow its association with the plasma membrane are not completely known; however, the α1-antitrypsin (α1-PI) domain that initiates chemotaxis has been identified as a hydrophobic pentapeptide concealed within its C-terminal region.9 The corresponding hydrophobic pentapeptides found in various other serine proteinase inhibitors contain a pair of phenylalanine residues that share the motif FXXFX or FXXFX, where X represents the hydrophobic amino acids V, L, I, or M.9 The identification of a pentapeptide having a similar motif within the fusion domain of HIV-1 gp41 (FLGFL) and other human viruses led to the discovery of a ligand-receptor interaction between gp41 and the α1-PI receptor HLE.5

In HIV-1–seropositive patients, viral load is correlated with circulating levels of α1-PI.12 In this study, 100% of patients in the asymptomatic category of disease manifested deficient levels of α1-PI. Further, in vitro infectivity was shown to be highly correlated with cell-surface HLE (r2 = 0.81, P = .01).13 Individuals with the inherited form of α1-PI deficiency, especially males, are notably susceptible to respiratory infections.14 Thus, α1-PI deficiency acquired during HIV-1 infection may be a mechanism responsible in part for the attendant emphysema, pulmonary pathology, and onset of opportunistic infections as HIV-1 disease progresses. The discovery that defective chemokine expression corresponded to HIV-1 resistance15 precipitated the identification of chemokine receptors during HIV-1 entry.16 The relationship between α1-PI and HIV-1 disease suggested the potential participation of an additional coreceptor cognate for α1-PI. The physical relationship between proteinases, proteinase inhibitors, and chemokine receptors has not previously been examined.
**Materials and methods**

**Cells and reagents**

U937 subclones were generously provided by the Laboratory of Immunoregulation, National Institute for Allergy and Infectious Diseases, National Institutes of Health (NIH), and maintained using RPMI-1640 containing 10% fetal bovine serum (FBS) determined to have endotoxin levels less than 0.3 endotoxin units (EU)/mL. Cells were harvested during exponential growth and were more than 95% viable as determined by trypan blue exclusion. Endotoxin stimulation was performed by incubation with *Escherichia coli* strain O26:B6 LPS (Sigma Chemical, St Louis, MO) at a concentration of 5 μg/mL/2 x 10^6 cells in the presence or absence of 0.5 μg recombinant LPS-binding proteins (LBPs; Xoma, Berkeley, CA) in AIM V (R) serum-free media (Gibco Invitrogen, Carlsbad, CA) for 60 minutes at 37°C in a humidified chamber containing 5% CO₂. Cells in AIM V (R) were examined by flow cytometry to exhibit CD4 and CXCR4 levels different from cells in RPMI-1640, 10% FBS (data not shown). The functionally active concentrations of 2 preparations of different from cells in RPMI-1640, 10% FBS (data not shown). The functionally active concentrations of 2 preparations of different from cells in RPMI-1640, 10% FBS (data not shown). The functionally active concentrations of 2 preparations of different from cells in RPMI-1640, 10% FBS (data not shown).

**Receptor/ligand binding analysis**

Preparation of solubilized clone 10 membrane extract, the synthetic pentapeptide FLGFL representative of the HIV-1 fusion domain, and stromal-derived factor 1 (SDF-1) were counted. Specific binding (B) was determined as the difference between binding to coated and uncoated wells.

**Immunocytochemical transmission electron microscopy (TEM)**

TEM was performed by Wallace Ambrose, Microscopy Laboratory, Dental Research Center, University of North Carolina-Chapel Hill. Grids were stained using rabbit anti-HLE (Biodesign, Kennebunkport, ME) or anti-CatG (Biodesign) at concentrations of 1 mg/mL in 0.01 M phosphate, 0.15 M NaCl, pH 7.2 (PBS). Antibody binding was detected using Protein A polygold (0.23 μg Protein A/mL; Sigma) and photographed using a Philips CM/12 TEM/STEM transmission electron microscope (FEI, Hillsboro, OR). Positive-control grids were stained with rabbit anti-nuclear factor kappa-B (NFκB) p50 (Biodesign) and negative-control grids were stained without primary antibody.

**Receptor/ligand binding analysis**

Preparation of solubilized clone 10 membrane extract, the synthetic pentapeptide FLGFL representative of the HIV-1 fusion domain, and receptor/ligand binding analysis was performed as previously described. Binding by 125I-U937 clone 10–solubilized membranes to FLGFL was performed in hypertonic phosphate buffer, pH 7.4, 0.5 M NaCl, 0.05% tween-20 (HPSB) containing 2.5% lipid-free bovine serum albumin (BSA) to limit nonspecific ionic binding. The bound and unbound (U) fractions were counted. Specific binding (B) was determined as the difference between binding to coated and uncoated wells.

**Immunofluorescent receptor staining and flow cytometric analysis**

Three-parameter flow cytometric analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA). Cells were washed twice in PBS and resuspended at 1 x 10⁶ cells/mL. Monoclonal antibodies included anti–CXCR4–phycoerythrin (PE) (PharMingen, San Diego, CA), anti–CD4–peridinin chlorophyll–alpha protein (PerCP) (clone SK3; Becton Dickinson), and isotype-matched controls. To detect cell-surface HLE, cells were incubated with polyclonal sheep anti-HLE–fluorescein isothiocyanate (FITC) (Biodesign, Kennebunkport, ME) or negative-control sheep immunoglobulin G (IgG)–FITC (Cappel, Aurora, OH). Alternatively, cell-surface HLE was detected using polyclonal rabbit anti-HLE (Biodesign) or negative-control rabbit IgG (Chemicon, Temecula, CA), and binding was detected using biotinylated antirabbit IgG (Amersham, Amersham, United Kingdom) followed by streptavidin–FITC (PharMingen). To investigate the influence of alternate receptor ligation, whole blood was stained by incubating 100 μL cells with 10 μL of each antibody stepwise for 15 minutes each at 20°C in the order anti–HLE–FITC, anti–CXCR4–PE, anti–CD4–PerCP, or in the order anti–CD4–PerCP, anti–HLE–FITC, anti–CXCR4–PE. Cells were washed in 2 mL PBS between each staining step. After the final wash, the cell pellet was resuspended and fixed in 0.5 mL 1% paraformaldehyde in PBS. For each analysis, at least 10 000 to 30 000 events were acquired. List mode multiparameter data files were analyzed using CellQuest Software (Becton Dickinson). Median fluorescence intensity (MFI) for each coreceptor was detected, and relative fluorescence intensity (RFI) was determined as MFI of coreceptor minus MFI of isotype control.

**Receptor patching and colocalization**

U937 cells (1 x 10⁶ cells/mL) were incubated with various receptor ligands for 60 minutes in humidified 5% CO₂ at 37°C. Ligands included polyclonal rabbit anti-HLE (1.4 mg/mL; Biodesign), monoclonal murine anti-HLE (65 μg/mL; Dako, Carpinteria, CA), 1.25 nM α1PI (A9024), 1.25 nM stromal-derived factor 1α (SDF-1α; Peprotech, Rocky Hill, NJ), 10 μM FLGFL solubilized in 9.5% ethanol vehicle (0.95% ethanol final concentration), vehicle only, or buffer only (Hanks balanced salt solution [HBSS]). To examine receptor localization, cells were incubated at 37°C, 5% CO₂, with each ligand to determine optimal ligand concentrations, and cells were observed at 60-minute intervals for 24 hours to determine optimal detection time (data not shown). To demonstrate colocalization induced by receptor ligation, 1 x 10⁶ cells/mL HBSS were incubated at 37°C, 5% CO₂, first with 1 ligand at its optimal concentration for 15 minutes and subsequently with a second ligand at its optimal concentration for 60 minutes in sterile polypropylene microfuge tubes with caps loosened.

After washing 3 times in HBSS, aliquots (30 μL) were applied to the sample chambers of a cytophase apparatus (ThermoShandon, Pittsburgh, PA), and slides were centrifuged at 850 rpm for 3 minutes. Slides were fixed by application of 50 μL 10% formalin in PBS to the sample chambers of the cytophase apparatus followed by an additional centrifugation at 850 rpm for 5 minutes. Slides were sequentially incubated for 90 minutes at 20°C with a monoclonal antibody having specificity for human α1PI (5 μg/mL; Zymed Laboratories, South San Francisco, CA), biotinylated sheep antimouse immunoglobulin (100 μg/mL; Amersham, Arlington Heights, IL), FITC-conjugated streptavidin (80 μg/mL; Jackson Laboratories, West Grove, PA), rabbit antihuman SDF-1α (3 μg/mL; Peprotech), biotinylated donkey antirabbit immunoglobulin (100 μg/mL; Amersham, Arlington Heights, IL), and Texas Red–conjugated streptavidin (80 μg/mL; Jackson Laboratories). Slides were washed once between each incubation.

To demonstrate receptor copatching induced by HIV, an inoculum of 1 x 10⁶ infectious units of HIV-1 Tropic strain NL4-3 (HIVNL4-3, a generous contribution from Dr W. Resch, Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill) was incubated with 1.5 x 10⁷ cells in 1 mL RPMI-1640 containing 10% FBS at 37°C in humidified 5% CO₂. Aliquots of 250 μL were removed at 15-minute intervals for 90 minutes. Cells were washed 3 times in fresh medium and fixed using 1% paraformaldehyde. Fixed cells were sequentially incubated for 60 minutes at 20°C with rabbit polyclonal anti-HLE (1 mg/mL; Biodesign), biotinylated donkey antirabbit immunoglobulin (100 μg/mL; Amersham, Arlington Heights, IL), FITC-conjugated streptavidin (50 μg/mL; Becton Dickinson), monoclonal anti-gp120 (approximate epitope V3 loop, 10 μg/mL; Research Diagnostics, Flanders, NJ), biotinylated sheep antimouse immunoglobulin (100 μg/mL; Amersham), gold-conjugated streptavidin (1/50; Nanoprobes, Yaphank, NY), and either PerCP-conjugated monoclonal anti–CD4 (clone SK3; Becton Dickinson) or Per-CP–conjugated CXCR4 (Becton Dickinson). Bound anti-gp120 was detected using silver enhancement as recommended by the manufacturer (LI Silver; Nanoprobes). Antibody controls were purified rabbit IgG (1 mg/mL; Chemicon) and murine IgG (10 μg/mL; Jackson Laboratories). Cells were washed once between each staining step.

Slides were examined by epi-illumination UV microscopy on a Zeiss Axioskop (Thornwood, NY) equipped with filters for excitation/emission wavelengths (nm) 546/590 and 450/520 to 490/520. Optical sections of intact cells were examined by confocal laser scanning microscopy (CLSM).
performed by Dr C. R. Bagnell Jr, Confocal Microscopy Facility, Department of Pathology and Laboratory Medicine, UNC-Chapel Hill. Digital images were artificially colorized using Adobe Photoshop software (San Jose, CA).

**Antisense oligomers**

Morpholino antisense oligomers (Gene-Tools, Corvallis, OR) were 22- or 25-mers, targeted respectively to the HLE start codon (5'-CCAGGAGTTATGTCAGGTCAGG-3') or to the 3' splice site of HLE exon 4 (5'-CGACCGGTGAGCTTGACCGCTTG-3'). Anti–β-globin oligonucleotides were used as negative controls as described previously. Cellular delivery was achieved by passing 2.5 × 10^6 cells suspended in 250 µL culture media containing 10µM individual oligonucleotides through a syringe fitted with a 25-gauge needle. Oligonucleotide-loaded cells were incubated for 60 minutes at 37°C in wells of a tissue-culture plate followed by addition of 250 µL fresh media. Cells were maintained in culture for 36 hours, 48 hours, or 66 hours, monitored for viability, and resuspended at 5 × 10⁶ cells/200 µL for flow cytometry or HIV-1 infectivity assays. Optimal mRNA inhibition was determined to occur at 48 hours. All treatments yielded more than 99% viability.

**HIV-1 infectivity**

To demonstrate the influence of αPI on infectivity, clone 10 cells were resuspended at 2 × 10^5 cells/mL in AIM V(R) serum-free medium (Gibco) containing varying concentrations of αPI (Sigma; cat. no. A9024) either in 96-well tissue-culture plates (Costar, Cambridge, MA) or in microfuge tubes (Sarstedt, Newton, NC). Cells infected in tissue-culture plates were directly washed free of virus with PBS without transferring from wells and resuspended in AIM V(R) serum-free medium containing the original concentration of αPI present during infection. Cells infected in microfuge tubes were washed free of virus with PBS, resuspended at 1 × 10^6 cells/mL in AIM V(R) serum-free medium containing the original concentration of αPI present during infection, and 0.2 mL (2 × 10⁵ cells) were transferred to wells in tissue-culture plates. To demonstrate the influence of nonpermissive clones, LPS-stimulated clone 17 cells in the presence or absence of LBP were resuspended in serum-free medium with or without 30 µM αPI. HIV NL4-3 viral stocks were propagated as previously described, and incubated with cells at a multiplicity of infection (MOI) of approximately 1 × 10⁻², 1 × 10⁻³, and 1 × 10⁻⁴ for 3.5 hours at 37°C in a humidified chamber containing 5% CO₂. Culture supernatants were collected and replaced with the corresponding medium every other day for 2 weeks. Culture supernatants were stored at −20°C for analysis of reverse transcriptase (RT) activity. RT activity was detected as previously described.

To demonstrate the influence of antisense oligonucleotides, antisense-treated clone 10 cells were enumerated and resuspended at 5 × 10⁵ cells/200 µL well in 96-well tissue-culture plates in RPMI-1640 containing 10% FBS. HIV NL4-3 (4.9 × 10⁵ IU/mL MAGI cells) was incubated at a concentration of 1 × 10⁵ IU/well for 2 hours at 37°C in a humidified chamber containing 5% CO₂. Cells were washed free of virus and resuspended in RPMI-1640 containing 10% FBS. Cells were harvested at 48 hours and analyzed by flow cytometry for cell-surface expression of HLE and by polymerase chain reaction (PCR) for the presence of HIV-1 minus-strand, strong-stop DNA. Enumeration of viable cells in suspension at 48 hours revealed 1.7 × 10⁶ cells/well to 3.2 × 10⁶ cells/well that were 99% to 100% viable.

**HIV-1 DNA PCR**

An equivalent number of cells from each treatment were harvested from tissue culture and washed once in PBS. Lysis, PCR amplification, and γ³²P-labeled R/U5 primer pairs for HIV-1 minus-strand, strong-stop DNA were as previously described. β-actin primer pairs were 5'-GGGCGAAC-AAGCGATGACT-3' and 5'-GAAGTCACGGCGACGAGC-3'. Amplified products were identified and pixelated for quantification using 6% nondenaturing polyacrylamide gel electrophoresis.

**Results**

**Different subcellular compartments occupied by HLE in HIV-1 permissive and nonpermissive U937 subclones**

Immunocytochemical TEM examination of U937 subclones revealed HLE associated with the plasma membrane in HIV-1 permissive clone 10 (Figure 1A). Plasma membrane–associated HLE was never seen in unstimulated HIV-1 nonpermissive clone 17 (data not shown). Instead, HLE was found in clusters in clone 17 associated with intracytoplasmic, electron-dense, irregular-shaped bodies resembling granules (Figure 1B). Localization of HLE to an internal compartment in clone 17 was confirmed by CLSM (data not shown). Intracytoplasmic clusters of HLE were never seen in permissive clone 10. Positive-control protein CatG was found to localize to the same structures as HLE in clones 10 and 17, whereas NFκB was found to localize to the cytoplasm and mitochondria in both clones equivalently (data not shown). That HLE was found to

![Image](https://www.bloodjournal.org)
reside in granule-like structures in nonpermissive cells, but on the cell surface in permissive cells suggested these clones had become arrested at disparate stages of differentiation.

The endotoxin LPS, a glycolipid found on Gram-negative bacteria, produces multiple effects on cells including release of granule-associated HLE, which becomes bound to the plasma membrane.11 Granule release is mediated by the interaction of LPS with 2 different signaling receptors including CD14 and β2-integrins CD11a/CD18, CD11b/CD18, and CD11c/CD18. In plasma, LBP facilitates receptor binding to LPS monomers by its action as a lipid transfer protein. When nonpermissive clone 17 was stimulated in the presence of LPS and LBP, HLE was visualized by TEM to translocate from granules to the cell surface, the cytoplasm, and the extracyttoplasmic space (Figure 1C). These results suggest that in response to LPS, granule release allows plasma membrane association of HLE in the absence of protein synthesis.

Analysis of HIV-1 coreceptors by flow cytometry in the presence or absence of LPS stimulation was compared using 3-color fluorescence. Because HLE density was expected to be low or not detectable, HIV-1 permissive clone 10 was examined with or without anti-CD4 as a staining control. Surprisingly, HLE was undetectable when the order of addition was reversed (Figure 1D). The explanation for differences in detection of surface HLE on fixed cells in the presence or absence of anti-CD4 is not known, but evidence suggests a potential role for membrane remodeling or autodegradation.21 In contrast, the order of coreceptor ligation had negligible influence on the fluorescence intensity of CD4 or CXCR4. LPS stimulation of HIV-1 nonpermissive clone 17 was found to have little or no influence on CD4 or CXCR4 density; however, HLE expression was found to increase. Further, as with clone 10, the order of receptor ligation influenced detection of HLE, but not detection of CD4 or CXCR4. The relatively identical HLE expression induced by LPS with or without LBP is interpreted to result from factors in the serum-containing tissue-culture medium. Results suggest a dynamic functional association between membrane-associated HLE and the HIV-1 coreceptor CD4.

HLE binding to the HIV-1 fusion domain

Association constants for soluble proteins are conventionally measured in 0.15 M NaCl, pH 7.2, to accommodate comparisons between proteins competing for the same soluble ligand and yield values in the range of 10^6 M^-1. To optimize binding of nonsoluble proteins, association constants may be determined by modifying salt, pH, or temperature. Although a constant determined in 0.5 M NaCl at 0°C is not directly comparable to a constant determined in 0.15 M NaCl at 25°C, each constant is valid and definitive for the conditions under which affinity is measured. We have previously found that hypertonic saline (0.5 M NaCl) can be used to limit nonspecific ionic interactions between cell-surface receptors and the hydrophobic pentapeptide FLGFL, representative of the HIV-1 fusion domain. Using these conditions for Scatchard analysis, we demonstrated the existence of a single receptor (Kassoc = 1 × 10^5 M^-1) on CEM T-lymphoblastoid cells for FLGFL. By competitive inhibition, the receptor was identified as HLE (HLE IC50 = 8.0 mM, α5PI IC50 = 8.1 mM with a ratio of HLE/α5PI = 0.99 at IC100). These results can be interpreted to mean that HLE and α5PI competitively inhibit the same molecular interaction involving FLGFL, but by different mechanisms. FLGFL was further shown to bind purified HLE, further supporting the identity of the FLGFL receptor as HLE. To compare whether membrane-associated HLE might also act as a receptor for the fusion domain on U937 cells, clone 10 plasma membranes were isolated, solubilized, and iodinated (125I-U937) as previously described.5 Saturable binding of 125I-U937 to immobilized FLGFL was detected (Figure 2A), and Scatchard analysis at saturation revealed linearity (Figure 2B). As we found using CEM cells, these data suggest the existence of a single proteinaceous receptor.24 Competitive inhibition in hypertonic saline using FLGFL solubilized in 9.5% ethanol revealed 100% inhibition when the peptide was more than 1.25 mM (data not shown). Competitive inhibition using HLE (IC100 = 77 mM) and α5PI (IC100 = 91 mM) under these conditions (data not shown) revealed virtually identical affinities (Kassoc = 1 × 10^5 M^-1) and competitive binding ratios (HLE/α5PI = 0.85) as determined using CEM, and this strongly suggests that during unfolding of the HIV-1 envelope and exposure of the hydrophobic fusion domain, the HIV-1 fusion domain and HLE participate in a single molecular interaction.

In support of this hypothesis, HLE visualized by CLSM using a monoclonal antibody was found to patch in response to soluble FLGFL or α5PI (Figure 2C-D), but not in response to SDF-1α or vehicle (data not shown). These results support the specificity of binding of the fusion peptide to HLE and support a role for HLE during HIV-1 binding to cells of either monocytic and lymphocytic lineage. Receptor patching is known to be induced by crosslinking. Since receptor crosslinking by the HIV-1 fusion pentapeptide seems unlikely to have occurred, anti-HLE–induced patching was examined by CLSM for comparison. HLE was found to copatch with CXCR4 on uropods in response to crosslinking by anti-HLE (Figure 2E-F). Nystatin is a cholesterol-binding antibiotic that selectively disrupts HIV-1 receptors residing on cholesterol-containing platforms and has been demonstrated to interfere with vitro HIV-1 infectivity (IC50 = 4 μM) in H9, HUT-78, and U937 cells, but has little effect in vivo except against candidiasis.26 HLE and CXCR4 copatching stimulated by α5PI was found to be completely abrogated by pretreating for 60 minutes with 50 μM nystatin (data not shown).

Immunolocalization of receptors on cells sequentially incubated with α5PI and SDF-1α was also examined by CLSM. When clone 10 was stimulated first by α5PI and second by SDF-1α, α5PI and SDF-1α were found copatched on the cell surface (Figure 2G). Copatching appeared not to resemble classic capping induced by crosslinking, but appeared as protrusions of the plasma membrane. When clone 10 was stimulated first by SDF-1α and second by α5PI, α5PI was not detectable, and SDF-1α was detected in an internal section of the cell but not on the cell surface (Figure 2H). These results support the cell-surface coassociation of receptors for SDF-1α and α5PI in HIV-1 permissive clones. Each preparation of proteinase inhibitor is composed of a unique ratio of active and inactive protein. To determine whether receptors recognize active or inactive forms of α5PI, 2 preparations of active-site standardized α5PI were compared which were 32.7% and 8.3% active. When clone 10 was stimulated by these 2 preparations at equivalent protein concentrations, a greater concentration of the 8.3% active preparation was required than the 32.7% active preparation (data not shown) suggesting the active fraction, and not the inactive fraction, of α5PI was recognized by the receptor. That ligation of cell-surface HLE is sufficient to induce colocalization of HLE with CXCR4 suggests that receptor copatching may be integral to HIV-1 entry.

Enhanced HIV-1 infectivity in the presence of exogenous α5PI

It has previously been shown that HIV-1 gp120 induces colocalization of CD4 and CXCR4.28 That α5PI induced copatching of HLE
Homogeneous receptor density at equilibrium was achieved by exposing cells to photomicrographs were magnified to the same scale; bar represents 25 μm. Homogeneous receptor density at equilibrium was achieved by exposing cells to α1PI by culturing in medium containing 10% FBS prior to staining. and CXCR4 suggested that α1PI might also influence infectivity. HIV-1 permissive clone 10 and nonpermissive clone 17 were infected with HIV-1NL4-3 using varying MOI in the absence or presence of various physiologic concentrations of α1PI in serum-free medium. It is known that α1PI is a chemoattractant that induces adherence. To examine the influence of α1PI without biasing the outcome against adherent cells, cells were infected and cultured in the same wells of a 96-well tissue-culture plate. For comparison, cells were infected in microfuge tubes and transferred to clean wells of a 96-well tissue-culture plate. When cells were infected, washed free of virus, and cultured in the same wells of a tissue-culture plate, peak RT activity was found to increase in direct relation to the culture concentration of α1PI (Figure 3A-C). When cells were infected in microfuge tubes, washed free of virus, and transferred to wells, there was little detectable RT activity after 12 days of culture in serum-free medium containing any concentration of α1PI (Figure 3A). The HIV-1 nonpermissive cells remained nonpermissive in all conditions tested. The loss of HIV-1 infectivity at the lowest concentration of α1PI even in the presence of the highest MOI demonstrates α1PI dose-dependence and suggests that cells infected in microplates were not prejudiced by residual inoculum. Further, that α1PI dose-dependence is identically replicated at all 3 MOIs and that decreasing MOI produce the expected decrease in infectivity suggests that α1PI is necessary for successful HIV-1 infectivity in serum-free medium and may stimulate adherence and selection of significant cell populations and influence subsequent detection of infectivity outcome using traditional infectivity assays. Further, these results suggest the hypothesis that α1PI might influence HIV-1 infectivity by its influence on receptor colocalization.

Since HLE is not constitutively expressed on the cell surface of clone 17, it was considered that LPS-induced cell-surface expression of HLE might confer permissivity. Clone 17 was cultured in serum-free medium and stimulated with LPS in the presence or absence of LBP prior to addition of HIV. Cells were subsequently infected in the presence or absence of physiologic concentrations of α1PI in serum-free medium. In the presence of LPS and LBP, the kinetics of appearance and disappearance of RT activity in nonpermissive cells were similar to those of permissive cells (Figure 3D). The delayed appearance of RT activity when cells were stimulated with LPS in the absence of LBP is consistent with previous evidence of the diminished level of LPS monomer formation and receptor recognition of LPS in the absence of LBP.29 These results suggest membrane expression of an additional HIV-1 cofactor in response to LPS. In the absence of α1PI, detectable RT activity occurred neither in permissive nor nonpermissive cells under any condition examined. These results suggest that α1PI and a cell-surface receptor are HIV-1 cofactors in these promonocytic cells.

To examine the possibility that patching might occur during HIV-1 entry, clone 10 was incubated at 37°C with infectious HIV-1NL4-3 and cells were fixed and examined at various time points by CLSM for colocalization of HIV-1 and coreceptors. HIV-1 was detected using a monoclonal antibody with specificity for epitopes proximate to the V3 loop (Research Diagnostics). This antibody has recently been shown not to recognize α1PI in contrast to a different monoclonal antibody (1C1; Repligen, Cambridge, MA) with specificity for epitopes proximate to the HIV-1 fusion domain.12 After incubation of cells with virus for 15 minutes, HIV-1 was detected in several patches on the cell surface colocalized with CD4, CXCR4, and HLE (Figure 3E-G). HIV-1 appeared to patch (Figure 3F) and coalesce with these receptors on extensions of the plasma membrane (Figure 3E).

Figure 2. Binding and patching of HLE in response to the fusion domain of HIV. (A) Wells in a microtiter plate were uncoated (■) or were coated (●) with 10 mM HIV-1 fusion peptide (FLGFL) and incubated with 125I-U937 clone 10 solubilized cytoplasmic membrane proteins. Hyperionic saline (0.5 M NaCl) was used to limit nonspecific ionic interactions between solubilized membranes and the hydrophobic FLGFL. Maximum bound cpm/well at saturation was 1.3 × 10^6 (r^2 = 0.94). Binding to uncoated wells was 9.0 ± 0.3 × 10^5 cpm. Specific binding to fusion peptide is represented as the difference between coated and uncoated wells, and binding to uncoated wells is depicted for comparison. (B) Scatchard analysis revealed a linear character (r^2 = 0.81) to the interactions and association constant 1 × 10^3 M^−1 in hypertonic saline. Interaction of cell-surface HLE with FLGFL (C) or α1PI (D) stimulated patching. Bound monoclonal antibody specific for HLE was detected using biotinylated anti-IgG and FITC-conjugated streptavidin. Significantly, HIV-1 (green) was found to copatch with CXCR4 (red) in response to crosslinked polyclonal (E) or monoclonal (F) anti-HLE. Cells were stimulated, fixed, stained, and examined by suspension under coverslip. Negative-control cells stained for HLE in the absence of HIV-1 fusion peptide, α1PI, or other HLE ligands depicted no detectable staining (not shown). (G) Colocalization of α1PI (green) and SDF-1α (red) on the attached surface of clone 10 was detected when cells were stimulated by α1PI and postincubated with SDF-1α. (H) In contrast, when cells were first stimulated with SDF-1α and postincubated with α1PI, SDF-1α was detected in an internal section, but α1PI was not detected in any section. In the cell represented, SDF-1α was detected 2.4 μm from the attached surface of clone 10. Cells were stimulated, slides were prepared by cytopsin, fixed, and stained. Colocalization by CLSM was analyzed using 8-step 0.6-μm sectional scanning from the attached surface toward the unattached surface of the cells. Patching and colocalization were performed at least 3 times and examined by 2 investigators independently. A representative cell is depicted. All photomicrographs were magnified to the same scale; bar represents 25 μm. Homogeneous receptor density at equilibrium was achieved by exposing cells to α1PI by culturing in medium containing 10% FBS prior to staining.
Requirement for cell-surface HLE during HIV-1 infection

The data presented thus far suggest the participation of HLE as a receptor during HIV-1 capture and infection. Linking HIV-1 entry with the novel expression of CD4 and chemokine receptor cDNA in cells lacking their expression has established this method as the gold standard for identifying HIV-1 receptors. On the other hand, silencing gene expression has proven to be an equally powerful tool for demonstrating dependence of HIV-1 entry on CD4 expression. Since novel expression of HLE cDNA is technically not possible at this time, the importance of HLE during HIV-1 entry and infectivity was examined by inhibiting HLE expression. Morpholino antisense oligonucleotides complementary to HLE mRNA and pre-mRNA were introduced into HIV-1 permissive clone 10 and cultured for 48 hours. Antisense oligonucleotides complementary to β-globin were used as a negative control. Analysis by flow cytometry showed that cells transfected with antisense oligonucleotides specific for the start codon or for the 3’ splice site of HLE exon 4 diminished surface expression of HLE to approximately 30% relative to the control β-globin oligonucleotide (Table 1 and Figure 4A). Neither antisense oligonucleotide influenced cell-surface expression of CXCR4 or CD4.

Oligonucleotide-treated cells were incubated with HIV-1NL4-3, washed free of virus, and cultured for 48 hours. The presence of minus-strand, strong-stop DNA, the earliest RT product following HIV-1 entry, was determined by PCR amplification of cell lysates using R/U5 primer pairs. As was found with HLE expression, minus-strand, strong-stop DNA was diminished in cells transfected with antisense oligonucleotides complementary to the HLE start codon or the 3’ splice site of exon 4 (Figure 4B). In contrast, this DNA was easily detected in cells transfected with antisense oligonucleotide complementary to β-globin. We conclude that in addition to CD4 and CXCR4, cell-surface HLE is necessary for HIV-1 binding and infection.

Discussion

Cell-surface HLE ligation by αvPI, anti-HLE, and HIV-1 fusion peptide was shown here to induce copatching of HLE, CD4, and chemokine receptors. A model is proposed in which HLE ligation increases the odds of the dense focus of HIV-1 envelope glycoprotein, induces clustering of HIV-1 receptors, and that this dense focus of receptors increases the odds of the dense focus of HIV-1 envelope glycoprotein.
proteins to interact with remaining free receptors. Ligand-occupied receptors thereby produce a tangible increase in proximal receptors rather than producing steric interference and decreased accessibility of HIV-1 receptors. Consistent with this model, SDF-1α was found to prevent receptor clustering, and this suggests that chemokines may suppress HIV-1 infectivity by decreasing HIV-1 receptor clusters rather than by directly blocking receptors as previously believed. Increased circulating αvβ6 is correlated with increased viral load in the HIV-1 population, suggesting the hypothesis that αvβ6 might facilitate HIV-1 binding and infectivity. Here we have shown that RT activity produced by HIV-1 infectivity is dependent on αvβ6 in a dose-dependent manner, that αvβ6 facilitates copatching of HLE with the canonical HIV-1 receptors, that live HIV-1 preferentially binds copatched receptors, and that binding of virus to copatched receptors does not occur in the absence of αvβ6. We have further demonstrated that translocation of HLE from granules to the cell surface conveys HIV-1 permissivity to nonpermissive cells and that inhibiting HLE mRNA proportionally diminishes detection of the earliest RT product, minus-strand, strong stop DNA. In support of these findings, previous studies have demonstrated that in contrast to permissive clones, nonpermissive clones are unable to efficiently form complexes between gp120, CD4, and CXCR4.

The critical parameters determining the kinetics of HIV-1 infectivity have been defined as virus concentration, number of virions produced by one cell, and the time for one complete cycle of infection. In a homogeneous cell population, the time to peak RT activity has been shown to be directly related to virus concentration. We found that time to peak RT activity produced by clone 10 was diminished by 2 days for each logarithmic dilution of HIV-1NL4-3, and these results are consistent with previous kinetic studies. However, clone 10 produced negligible RT activity when cells were infected in serum-free medium lacking αvβ6, and RT activity increased in a dose-dependent manner as αvβ6 increased suggesting αvβ6 is necessary for infectivity. That time to peak RT activity was not increased by αvβ6 suggests that the influence of αvβ6 on peak RT levels was not related to viral inoculum or time of infection. Rather, these results suggest that increased peak RT levels resulted either from an increased number of virions produced by each cell or from an increased number of infected cells.

The dose-dependent manner in which αvβ6 influenced HIV-1 infectivity suggested a receptor-dependent mechanism. HIV-1 permissive and nonpermissive U937 clones differ with respect to cell-surface localization of HLE. We found that HIV-1 infectivity was permitted when granule-released HLE was bound to the cell surface of HIV-1 nonpermissive clone 17. Significantly, HIV-1 infectivity of clone 17 also required the participation of αvβ6, and this supports the identity of the responsible granule-released component as HLE. During the acute phase of inflammation coincident with LPS-stimulated HLE release, the concentration of αvβ6 increases as much as 4-fold. In this scenario, the acute phase of opportunistic infection would produce the phenomenon of HIV-1 receptor copatching and thereby facilitate increased viral load.

Evidence that HIV-1 gp120 binds CD4 includes the demonstration that binding is reversible and saturable, introducing CD4 cDNA expression in CD4 null cells results in a corresponding increase in HIV-1 infectivity, and that silencing CD4 mRNA expression blocks HIV-1 infectivity. Since HIV may reside either on the cell surface or in granules, and since HIV localization is dependent neither on DNA, mRNA, nor protein structure, but on whether the cell is producing granules, an as-yet-undefined process, introduction of HLE mRNA into HLE-negative cells is not a good test of its receptor function. The true test is to identify cells lacking HLE on the cell surface; test whether these cells are not susceptible to HIV-1 entry in the presence of CD4, CCR5, and CXCR4; and determine whether coexpression specifically of HLE on the cell surface now rescues HIV-1 entry into these cells. Reversible, saturable binding of the HIV-1 fusion domain to HLE has been shown here and elsewhere. We have demonstrated here that inhibiting HLE mRNA expression blocks HIV-1 infectivity and that favoring HLE association with the plasma membrane favors HIV-1 infectivity. Clark’s receptor occupancy model states that in addition to reversible, saturable binding of the ligand to the receptor, binding must produce a biologic response proportional to the number of receptors bound. Evidence presented suggests that suppression of HLE expression proportionally suppresses the earliest HIV-1 RT product, and this supports a requirement for HLE during HIV-1 binding and the earliest steps of infection consistent with a model of HIV-1 entry that requires HLE as a fusion receptor. We have previously found that in vitro viral infectivity outcome correlates with HLE, but not CD4, CXCR4, or CCR5, and this suggests HLE is a rate-limiting receptor for viral entry potentially by a mechanism involving the phenomenon of receptor copatching.

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References


HIV-1 preferentially binds receptors copatched with cell-surface elastase

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