Roles of CD4 and Coreceptors in Binding, Endocytosis, and Proteolysis of gp120 Envelope Glycoproteins Derived from Human Immunodeficiency Virus Type 1

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Infections by human immunodeficiency virus type 1 (HIV-1) involve interactions of the viral envelope glycoprotein gp120 with CD4 and then with a coreceptor. R5 isolates of HIV-1 use CCR5 as a coreceptor, whereas X4 isolates use CXCR4. It is not known whether coreceptors merely trigger fusion of the viral and cellular membranes or whether they also influence the energetics of virus adsorption, the placement of the membrane fusion reaction, and the metabolism of adsorbed gp120. Surprisingly, the pathway for metabolism of adsorbed gp120 has not been investigated thoroughly in any cells. To address these issues, we used purified 125I-gp120s derived from the R5 isolate BaL and from the X4 isolate IIIB as ligands for binding onto human cells that expressed CD4 alone or CD4 with a coreceptor. The gp120 preparations were active in forming ternary complexes with CD4 and the appropriate coreceptor. Moreover, the cellular quantities of CD4 and coreceptors were sufficient for efficient infections by the corresponding HIV-1 isolates. In these conditions, the kinetics and affinities of 125I-gp120 adsorptions and their subsequent metabolisms were strongly dependent on CD4 but were not significantly influenced by CCR5 or CXCR4. After binding to CD4, the 125I-gp120s slowly became resistant to extraction from the cell monolayers by pH 3.0 buffer, suggesting that they were endocytosed with half-times of 1–2 h. Within 20–30 min of endocytosis, the 125I-gp120s were proteolytically degraded to small products that were shed into the media. The weak base chloroquine strongly inhibited 125I-gp120 proteolysis and caused its intracellular accumulation, suggesting involvement of a low pH organelle. Results supporting these methods and conclusions were obtained by confocal immunofluorescence microscopy. We conclude that the energetics, kinetics, and pathways of 125I-gp120 binding, endocytosis, and proteolysis are determined principally by CD4 rather than by coreceptors in cells that contain sufficient coreceptors for efficient infections. Therefore, the role of coreceptors in HIV-1 infections probably does not include steerable or subcellular localization of adsorbed virus.

Infections by human immunodeficiency virus type 1 (HIV-1) involve binding of the oligomeric viral gp120-gp41 complexes onto cell surface CD4 followed by interactions with a coreceptor which result in fusion of the viral and cellular membranes (1, 2). The HIV-1 coreceptors are G protein-coupled receptors for chemokines (3–8). The major coreceptor for macrophage-tropic (R5) isolates of HIV-1 is CCR5, whereas that for T cell-tropic (X4) isolates is CXCR4 (9–11). Recent evidence suggests that purified monomeric gp120 forms a ternary complex with CD4 and an appropriate coreceptor and that the initial association with CD4 induces a conformational change in gp120 which enhances its affinity for the coreceptor (1, 2, 12–15). This secondary interaction competitively displaces 125I-chemokines and antagonizes chemokine-dependent signaling by CCR5 and CXCR4 (1, 2, 14, 16, 17). In the absence of chemokines, gp120s may also act as CCR5 and CXCR4 agonists in certain cells (18, 19). The observation that infections occur efficiently in cells that contain only a trace of coreceptor and a vast excess of CD4 has implied that membrane fluidity can contribute to formation of the ternary complexes that are necessary for infection (20).

The above results raise the possibility that coreceptors might substantially modify the kinetics and energetics of gp120 adsorption onto CD4-positive cells and that they might also control the route for subsequent virus or gp120 metabolism. To address these issues, we used 125I-gp120s derived from R5 and X4 isolates of HIV-1 for binding onto human cells that lack CD4 and coreceptors and onto derivative cells that contain CD4 alone or CD4 plus CCR5 or CXCR4. Although studies of CD4 endocytosis in clathrin-coated pits have been reported using 125I-monomoclonal antibodies (21–29), we are unaware of previous studies that used purified 125I-gp120s to investigate the metabolism of gp120-CD4 complexes. The rate of CD4 endocytosis is retarded by its association with the Lck tyrosine kinase (23, 24) and is enhanced by the HIV-1 protein Nef (25–29). Moreover, chemokines stimulate endocytosis of their receptors, and this inhibits infection by corresponding HIV-1 isolates (30–32). Because gp120s bind to both CD4 and coreceptors, either of these cell surface associations could possibly have a dominating influence on the pathways of viral entry into cells and on gp120 metabolism.

EXPERIMENTAL PROCEDURES

Cells and Viruses—HeLa cells and HeLa clonal derivatives expressing CD4 alone or CD4 plus CCR5 were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS) as described previously (20, 33). Human astrogliaoma U87MG cells were from the American Type Culture Collection (Manassas, VA). Clones of U87MG expressing CD4 described previously by Kozak et al. (37) were

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used to prepare derivatives that stably express CXCR4 or CCR5. The plasmid encoding CXCR4 (pDNA3-LESTR) was provided by Marcel Loetscher (Theodor Kocher Institute, University of Bern, Bern, Switzerland), whereas the CCR5 expression vector was constructed by cloning the CCR5 cDNA into the BamHI and Xhol sites of pCDN3 (Invitrogen, Carlsbad, CA). U87MG cells and its derivatives were maintained in minimum essential medium supplemented with 10% FBS, minimum essential medium nonessential amino acids (0.1 mM) (Life Technologies, Inc.), and minimum essential medium sodium pyruvate (1 mM) (Life Technologies, Inc.).

The M-tropic HIV-1 isolate BaL and the plasmids encoding the 5’-region (p83-2) and 3’-region (p83-10) of the genome of the T cell-tropic HIV-1 isolate NL4-3, contributed by Suzanne Gartner, Mikulas Popovic, and Robert Gallo, and by Ronald DeRosiers, respectively, were obtained from the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, National Institutes of Health. HIV-1 BaL was amplified by brief passage in fresh phytohemagglutinin-stimulated peripheral blood mononuclear cells. To propagate the T cell-tropic HIV-1 isolate BaL, NL4-3, HeLa cells were cotransfected with linearized p83-2 and p83-10 DNAs using the calcium phosphate precipitation method (34, 35). Culture medium was collected 72 h after transfection and passed through a filter (0.45-µm pore size). This initial viral stock was amplified in HeLa CD4 clone HI-J cells until high viral titers were achieved. HIV-1 isolates BaL and NL4-3 were used to infect U87MG CD4 cells expressing CCR5 and CXCR4, respectively, and the cells were assayed for infection using the foci infectivity method as described previously (36, 37).

gp120 Preparation and Labeling—Monomeric T cell-tropic gp120 IIIB was a gift from Shiu-Lok Hu (Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle). Monomeric M-tropic gp120 BaL was purified as described previously from the culture medium of Schneider 2 Drosophila cells that were generously donated by Dr. Raymond Sweet (Smith-Kline Beecham Pharmaceuticals, King of Prussia, PA) (38). Samples (10–25 µg) of the gp120 preparations were labeled either on amine groups with 125I-Bolton-Hunter reagent (ICN Pharmaceuticals, Inc., Costa Mesa, CA) according to the manufacturer’s directions, or on tyrosine residues with 125I (NEN Life Science Products) using the lactoperoxidase method as described previously (39). Unlabeled gp120 and 125I-gp120 were analyzed for purity by electrophoresis in 10% polyacrylamide gels containing 0.1% SDS followed by Coomassie Blue dye staining or autoradiography, respectively. Densitometric scanning suggested that these gp120 preparations were 92–95% pure. As expected, the major components in these gp120 preparations specifically bound antibodies to gp120.

Binding Assays—Cells (2.5 × 10^5) were plated in 2-cm² wells of a 24-well tissue culture plate the day before the assay. The cells were incubated with 1–3 µM 125I-gp120 in 0.2 ml of medium with 10% FBS at 37 °C. At various times, the cells were washed twice with medium with 10% FBS and once with phosphate-buffered saline (Life Technologies, Inc.). The cells were then solubilized in 0.1 N NaOH for measurement of radioactivity in a gamma counter. An aliquot of each solubilized sample was used for protein determination using the Coomassie Blue method (Bio-Rad). Before being solubilized, some wells were treated with cold pH 3.0 acid buffer to remove the surface 125I-gp120 as described previously (21–23). To study the metabolism of 125I-gp120, cells were incubated with 125I-gp120 for 60 min at 37 °C, washed, replenished with fresh medium, and subsequently incubated at 37 °C for various periods of time. The media were collected, precipitated by adjusting to 10% trichloroacetic acid, and centrifuged; and the radioactivity in the tri-chloroacetic acid-soluble -insoluble fractions was determined (21). The cells were then solubilized and processed as described above. In some experiments, chloroquine (20 µM) (Sigma) was present in the chase medium.

To determine the Kd for binding, the cells were incubated with 3 µM 125I-gp120 in the presence of increasing amounts of competing unlabeled gp120 at 37 °C for 1 h. The cells were washed, solubilized and counted as above.

To study the competitive displacement of the natural CCR5 ligand by gp120 BaL, the cells were initially incubated with various amounts of unlabeled gp120 in 0.1 ml/well at 37 °C for 30 min. Then 0.5 µM 125I-MIP1b (2.200 Ci/mmol; NEN Life Sciences Products) was added and incubated for another 30 min, after which the cells were processed and counted as described above.

Confocal Immunofluorescence Microscopy—HeLa CD4/CCR5 cells were seeded on eight-chambered coverglasses (Nunc, Inc., Naperville, IL) at 2 × 10^5 cells/chamber. All incubations were in DMEM and 10% FBS and at 37 °C unless otherwise noted. After 24 h, the cells were incubated with or without BaL gp120 at 10 µg/ml for 90 min. They were then incubated for 90 min either in the presence or in the absence of 20 µM chloroquine as indicated. After this chase period, surface gp120 was removed from the indicated wells by treatment with cold pH 3.0 acid buffer for 2 min with subsequent recovery in DMEM, 10% FBS, 20 µM Hepes, pH 7.5, at 37 °C for 10 min. The cells were then stained for either surface-bound gp120 or intracellular gp120. To detect surface gp120, the viable cells were incubated sequentially with sheep anti-Drosophila bound antibodies to gp120.

**FIG. 1. Analysis of gp120 preparations.** Two preparations of gp120 were electrophoresed on a 10% polyacrylamide gel in the presence of 0.1% SDS. In panel A, a preparation of gp120 BaL that had been purified from *Drosophila* culture medium (see “Experimental Procedures”) was labeled with either 125I-Bolton-Hunter reagent (lane 1) or 125I (lane 2) before electrophoresis and visualization by autoradiography. In panel B, a preparation of gp120 IIIB was labeled with 125I-Bolton-Hunter reagent. Densitometry suggested that the gp120s were 92–95% pure.

**FIG. 2. Competitive displacement of 125I-MIP1b by unlabeled gp120 BaL.** HeLa-CD4/CCR5 (clone JC53) cells were incubated with various amounts of unlabeled gp120 BaL or gp120 IIIB at 37 °C for 30 min before the addition of 0.5 nM 125I-MIP1b for another 30 min. The cells were washed, solubilized in 0.1 N NaOH, and counted in a gamma counter. HeLa-CD4 cells that lack CCR5 had a low background level of binding which was subtracted from the HeLa-CD4/CCR5 binding data. Binding values are expressed as a percentage, with no competing gp120 as 100%. The R5 gp120 BaL, competitively displaced the 125I-MIP1b that had been bound to the HeLa-CD4/CCR5 cells, whereas the X4 gp120 IIIB was ineffective. The data points are the mean of duplicate assays, and the error bars represent the range.
gp120 serum (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: contributed by Dr. Michael Phelan) at 1:300 for 60 min and Alexa 594 donkey anti-sheep IgG conjugate (Molecular Probes, Inc., Eugene, OR) at 1:250 for 60 min. Finally, cells were fixed in ice-cold methanol for 10 min. Intracellular gp120 was detected in cells that were acid-treated after the chase period by fixing them in ice-cold 3.7% formaldehyde in phosphate-buffered saline for 20 min. They were then permeabilized with 0.2% Triton X-100 in phosphate-buffered saline for 10 min and quenched in 20 mM glycine, 20 mM Heps, pH 7.5, in phosphate-buffered saline for 10 min. The cells were then incubated sequentially with sheep anti-gp120 serum at 1:400 for 90 min and Alexa 594 donkey anti-sheep IgG conjugate at 1:250 for 60 min and fixed again with ice-cold methanol for 10 min. All stained cells were stored under FluoroGuard antifade mounting reagent (Bio-Rad) and viewed by fluorescence confocal microscopy with a Bio-Rad MRC 1024 ES laser confocal imaging system.

Relative Rates of Endocytosis and Degradation—Assuming that the cell surface (S) and endocytosed (E) pools of $^{125}$I-gp120 are homogeneous and that there is no ligand recycling to cell surfaces (see "Results"), then $d[S]/dt = -k_e[S]$, where $k_e$ is the rate constant for endocytosis, and

$^{125}$I-gp120 IIB is slowly endocytosed and proteolytically degraded in HeLa-CD4 cells. Panel A, after an initial 60-min adsorption of $^{125}$I-gp120 IIB onto the cells at 37 °C, they were washed and incubated for various times with fresh medium in the presence and absence of chloroquine (20 μM). Chloroquine had no effect on the metabolism of cell surface $^{125}$I-gp120 IIB (with and without chloroquine; closed circles and closed squares, respectively), whereas chloroquine treatment resulted in an increase of $^{125}$I-gp120 IIB in the intracellular fraction (with and without chloroquine; open circles and open squares, respectively). Panel B, the culture media (with and without chloroquine) were collected at different times and were precipitated with trichloroacetic acid (see "Experimental Procedures"). Chloroquine caused a decrease in release of trichloroacetic acid-soluble radioactivity into the medium (trichloroacetic acid-soluble counts with and without chloroquine; closed circles and closed squares, respectively) but had no significant effect on the trichloroacetic acid-insoluble products in the medium (trichloroacetic acid-insoluble counts with and without chloroquine; open circles and open squares, respectively). The latter may represent $^{125}$I-gp120 that dissociated from the cell surfaces.

FIG. 3. Kinetics of $^{125}$I-gp120 IIB binding and its subsequent metabolism in HeLa-CD4 cells. Panel A, HeLa-CD4 cells in a 24-well tissue culture plate were incubated with a trace amount of $^{125}$I-gp120 IIB at 37 °C for various amounts of time until a steady state in labeling was reached after 1–2 h (closed circles). In contrast, control HeLa cells showed insignificant binding (×). Some cells were incubated with $^{125}$I-gp120 IIB for only 60 min, at which time fresh medium was added, and the cells were returned to the incubator (the arrow indicates the beginning of this chase). At various times the cells were either washed and solubilized (total cell-associated counts, closed squares) or extracted with a cold pH 3.0 acidic buffer before solubilization. The radioactivity that was extracted with the acidic buffer is the $^{125}$I-gp120 IIB cell surface fraction (open circles). After this acid extraction, the cells were solubilized and counted, and these counts are the acid-resistant intracellular $^{125}$I-gp120 IIB fraction (open squares). Panel B, the chase medium was collected, precipitated by adjusting to 10% trichloroacetic acid, and centrifuged to separate the trichloroacetic acid-soluble fraction (closed circles) from the trichloroacetic acid-insoluble fraction (closed squares).

FIG. 4. $^{125}$I-gp120 IIB is slowly endocytosed and proteolytically degraded in HeLa-CD4 cells. Panel A, after an initial 60-min adsorption of $^{125}$I-gp120 IIB onto the cells at 37 °C, they were washed and incubated for various times with fresh medium in the presence and absence of chloroquine (20 μM). Chloroquine had no effect on the metabolism of cell surface $^{125}$I-gp120 IIB (with and without chloroquine; closed circles and closed squares, respectively), whereas chloroquine treatment resulted in an increase of $^{125}$I-gp120 IIB in the intracellular fraction (with and without chloroquine; open circles and open squares, respectively). Panel B, the culture media (with and without chloroquine) were collected at different times and were precipitated with trichloroacetic acid (see "Experimental Procedures"). Chloroquine caused a decrease in release of trichloroacetic acid-soluble radioactivity into the medium (trichloroacetic acid-soluble counts with and without chloroquine; closed circles and closed squares, respectively) but had no significant effect on the trichloroacetic acid-insoluble products in the medium (trichloroacetic acid-insoluble counts with and without chloroquine; open circles and open squares, respectively). The latter may represent $^{125}$I-gp120 that dissociated from the cell surfaces.
were resistant (BaL strain of HIV-1, whereas U87MG-CD4 control cells lacking CCR5 U87MG-CD4 cells lacking CXCR4 (ble to infection by the X4 NL4-3 strain of HIV-1, whereas control Similarly, U87MG-CD4/CCR5 cells 
 Cultures are shown at a magnification of 

RESULTS

Properties of gp120s—The gp120 preparations, which were highly purified as seen by polyacrylamide gel electrophoresis, were labeled either on amine groups with 125I-Bolton-Hunter reagent or on tyrosines with 125I (see Fig. 1). The gp120s labeled by these methods had very similar properties and adsorbed specifically onto CD4-positive cells. However, the 125I-labeled gp120s were less stable and lost much of their activity within several days. Typically, the nonspecific binding onto 

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**Fig. 5.** Immunoperoxidase-stained focal assays for infections by X4 and R5 strains of HIV-1 in derivatives of human U87MG astrogloma cells. U87MG-CD4/CXCR4 cells (panel A) were susceptible to infection by the X4 NL4-3 strain of HIV-1, whereas control U87MG-CD4 cells lacking CXCR4 (panel B) were completely resistant. Similarly, U87MG-CD4/CCR5 cells (panel C) were susceptible to the X5 BaL strain of HIV-1, whereas U87MG-CD4 control cells lacking CCR5 were resistant (panel D). The infected cells (panels A and C) are heavily stained because of viral antigens and show typical large syncytia. Cultures are shown at a magnification of × 285.

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HeLa cells was approximately 5% or less of the binding onto HeLa-CD4 (clone HI-J) cells (see below). As determined by competitive displacement of 125I-gp120 by unlabeled gp120 (20, 40, 41), the $K_d$ for binding of the X4 gp120 IIIB onto HeLa-CD4 cells was $51 \pm 7$ nM ($n = 2$), whereas the $K_d$ for the R5 gp120 BaL was approximately $144 \pm 20$ nM ($n = 2$) (see below).

We have shown previously that these gp120 preparations interact specifically with the appropriate coreceptors (16). Thus, the X4 gp120 IIIB specifically antagonized CXCR4 signaling in response to the chemokine SDF-1, whereas the R5 gp120 BaL specifically antagonized CCR5 signaling responses to MIP1α (16). As shown in Fig. 2, the gp120 BaL preparation also competitively displaced the CCR5-specific ligand 125I-MIP1α from surfaces of HeLa-CD4/CCR5 (clone JC 53) cells, whereas the X4 gp120 IIIB preparation was inactive. Control HeLa cells lack CCR5 and do not bind 125I-MIP1α (40). Although we attempted the converse experiment using 125I-SDF-1α and U87MG-CD4/CXCR4 cells, this was unsuccessful because of a very high background of binding onto control U87MG cells. Because U87MG cells lack CXCR4 (see below), we infer that the background may be caused by 125I-SDF-1α binding to proteoglycans (43).

Characterization of 125I-gp120 IIIB Binding and Metabolism Using HeLa-CD4 Cells—Initially, we analyzed the binding and metabolism of 125I-gp120 IIIB using HeLa-CD4 (clone HI-J) cells that contain a large amount of CD4 (about 4–4.5 × 10^5 CD4/cell) and CXCR4 and are highly susceptible to LAV/IIIB and other X4 isolates of HIV-1 (20). In agreement with our previous evidence (16), the trace concentration of 125I-gp120 adsorbed slowly onto the HeLa-CD4 cells for 1–2 h, after which a steady state of labeling was achieved (see Fig. 3A). In contrast, negligible binding occurred onto the control HeLa cells.

Presumably, negligible binding occurred onto the control HeLa cells.

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**Fig. 6.** Competitive binding and Scatchard analysis of 125I-gp120 IIIB adsorption onto cells that contain or lack the CXCR4 coreceptor. Competitive binding assays were done on U87MG-CD4/CXCR4 cells (panel A) and U87MG-CD4 cells lacking CXCR4 (panel B) using a fixed concentration of 125I-gp120 IIIB and increasing concentrations of unlabeled gp120 IIIB. Background binding of 125I-gp120 IIIB, measured on control U87MG cells, was approximately 30 cpm. Scatchard analysis of the binding data (insets) performed to determine the binding affinities of 125I-gp120 IIIB, shows that the $K_a$ values and numbers of binding sites were not significantly influenced by the presence of the CXCR4 coreceptor.

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$d[E]/dt = k_a[S] - k_d[E]$, where $k_d$ is the rate constant for degradation. After removal of 125I-gp120 from the medium, the ratio of $(d[S]/dt)/ (d[E]/dt)$ was observed to reach a steady-state value that is identical to the ratio of $[S]/[E]$ (see “Results”). These ratios differ somewhat for different cells but range between approximately 1.3 and 2.0. By algebra, these considerations imply that $k_d$ is 2.3–3.0 times greater than $k_a$. 

extracted with acidic pH 3.0 buffer at 0 °C, suggesting that it was on cell surfaces, but this percentage declined somewhat during further incubation of the cells. Consistent with previous studies using other ligands (e.g. 21–23, 29), these brief rinses at pH 3.0 did not reduce cellular viability or adherence to the dishes, and additional rinses did not extract more 125I-gp120 from the cells. Because 125I-gp120 did not accumulate in the acid-resistant presumptive intracellular fraction during incubation of the cultures at 37 °C, we presumed that it might be degraded and released from the cells with a half-time that was considerably shorter than the half-time of endocytosis (see “Discussion”). Consistent with this interpretation, small radioactive products that were soluble in 10% trichloroacetic acid were released into the culture medium (see Fig. 3B).

The latter results implied that 125I-gp120 IIIB might be endocytosed slowly and then degraded proteolytically in HeLa-CD4 cells. To address this further, we incubated 125I-gp120 IIIB with the cells for 60 min at 37 °C and then rinsed the cells and compared the metabolism in the presence and absence of the weak base chloroquine. Weak bases including chloroquine accumulate in lysosomes, raise their pH, and inhibit their degradative functions (44). As shown in Fig. 4A, chloroquine had no effect on removal of 125I-gp120 IIIB from the acid-extractable cell surface fraction, suggesting that it did not perturb endocytosis. However, chloroquine caused a substantial increase of 125I-gp120 IIIB in the intracellular fraction and a corresponding decrease in release of trichloroacetic acid-soluble radioactive products into the culture medium (Fig. 4B). These results strongly suggest that 125I-gp120 IIIB is endocytosed slowly into HeLa-CD4 cells (half-time of approximately 100 min) and that it is then proteolytically degraded relatively rapidly. Furthermore, these data substantiate our methods for distinguishing cell surface and endocytosed pools of 125I-gp120.

Previous evidence suggested that endocytosed 125I-monoclonal antibody-CD4 complexes are partially recycled to cell surfaces (21, 29, 45). This evidence relied on the observation that the endocytosed complexes were resistant to pH 3.0 extraction at 0 °C and that this treatment did not reduce cell viability. After adding fresh culture medium at 37 °C, however, some of the labeled complexes slowly returned to the cell surface fraction. We performed similar analyses using 125I-gp120 IIIB but did not detect any recycling of this ligand to cell surfaces (results not shown).

**Interactions of 125I-gp120 IIIB with U87MG-CD4 and U87MG-CD4/CXCR4 Astroglioma Cells**—Because HeLa-CD4 cells contain CXCR4, the previous results could conceivably have been influenced by gp120 IIIB interactions with both CD4 and CXCR4. In contrast, U87MG-CD4 cells are completely resistant to HIV-1 infections, suggesting that they lack coreceptors (46). Consequently, we made stable clones of U87MG-
Binding and Metabolism of gp120s

CD4, U87MG-CD4/CXCR4, and U87MG-CD4/CCR5 cells. As expected, the U87MG-CD4/CXCR4 cells were highly and specifically susceptible to X4 isolates of HIV-1, whereas U87MG-CD4/CCR5 cells were specifically susceptible to R5 isolates (see Fig. 5). Infections were detected by immunoperoxidase staining for viral proteins and by syncytia formation.

We anticipated that CXCR4 might influence the energetics of gp120 IIIB binding onto U87MG-CD4 and that ternary gp120-CD4-CXCR4 complexes might be stronger than binary gp120-CD4 complexes. However, the effect of CXCR4 is somewhat difficult to predict, in part because the ternary complexes presumably form and disassociate in an ordered manner (1, 2, 13, 14) and because the affinity of CXCR4 for the gp120-CD4 complexes is unknown. In addition, cells with more than a trace quantity of any cell surface receptor generally behave as multivalent absorbers, and this results in a diffusion-limited rate of ligand binding and in an extremely low rate of dissociation that differs from the rates in free solution (47). To address this, we used a standard competition method (20, 40, 41) in which increasing amounts of unlabeled gp120 IIIB were added to a constant trace amount of $^{125}$I-gp120 IIIB before absorption onto the cells. As expected, binding equilibrium was achieved relatively quickly at higher concentrations of gp120 IIIB, but the equilibrium levels of cellular labeling were decreased because of the competition. The results are plotted in Fig. 6, with the insets showing Scatchard analyses of the data. The $K_d$ estimates of 85 ± 6 nM for U87MG-CD4/CXCR4 cells and 58 ± 10 nM for U87MG-CD4 cells were not significantly different.

We then compared the binding and subsequent metabolism of $^{125}$I-gp120 IIIB using U87MG-CD4 and U87MG-CD4/CXCR4 cells (see Fig. 7). The results were similar to those obtained with HeLa-CD4 cells (see Fig. 3), with $^{125}$I-gp120 IIIB initially binding to cell surfaces as indicated by its susceptibility to acid extraction and then being slowly endocytosed with a half-time of 60–120 min and degraded to form products soluble in 10% trichloroacetic acid. Interestingly, the data were unaffected by the presence of CXCR4 in the cells. This experiment was repeated four times with the same basic results.

**Adsorption and Metabolism of $^{125}$I-gp120 BaL in HeLa-CD4**

**Compared with HeLa-CD4/CXCR5 Cells**—The HeLa-CD4 and HeLa-CD4/CCR5 cell clones used for this investigation express similar quantities of CD4 and CCR5 and were described previously (20). Infections of these cells by R5 HIV-1 isolates absolutely require CCR5 (20, 37). Fig. 8 shows analysis of the binding affinities of $^{125}$I-gp120 BaL onto the cells in the presence of different concentrations of unlabeled gp120 BaL. In agreement with the results described above using gp120 IIIB, we did not observe a significant effect of coreceptor expression on the binding affinity of $^{125}$I-gp120 BaL.

We also analyzed the adsorption and metabolism of the $^{125}$I-gp120 BaL (see Fig. 9). Similar to the above results, this gp120 was also endocytosed and degraded proteolytically to form products that were predominantly soluble in 10% trichloroacetic acid. The rates and extents of cell surface adsorption, endocytosis, and proteolysis were not significantly influenced by the presence of CXCR5. We should mention in this context that our clones of HeLa-CD4/CXCR5 cells differ somewhat in their levels of CD4 and in the corresponding steady-state levels of $^{125}$I-gp120 BaL which they adsorb. These clonal variations also influenced the binding of the X4 $^{125}$I-gp120 IIIB, indicating that they were not caused by CCR5 (results not shown). We conclude that CCR5 did not significantly affect the rate or extent of $^{125}$I-gp120 BaL adsorption, endocytosis, or proteolysis.

**Confocal Immunocytochemistry**—By using double labeling with confocal immunofluorescence microscopy, we were able to localize adsorbed gp120s and CCR5 in the cells. During incubations at 37 °C, the adsorbed gp120s became localized both at cell surfaces and in intracellular organelles (see Fig. 10), consistent with the above evidence for endocytosis. Extraction with the pH 3.0 buffer removed the cell surface but not the intracellular gp120. Moreover, treatment of the cells with 20 μM chloroquine resulted in a relative accumulation of gp120 intracellularly (Fig. 10). In addition, the double labeling analyses the CCR5 coreceptor appeared to be situated principally at sites that were distinct from the gp120. Although some overlap in these localizations was evident, we believe that this overlap was caused in part by random factors rather than by

![Fig. 8: Competitive binding and Scatchard analysis of $^{125}$I-gp120 BaL adsorption onto HeLa-CD4 cells in the presence and absence of the CCR5 coreceptor.](image-url)
specific complexation because it was not noticeably different for the X4 gp120 IIIB and the R5 gp120 BaL (results not shown).

**DISCUSSION**

We have used highly purified gp120 preparations derived from the X4 HIV-1 isolate IIIB and from the R5 isolate BaL to investigate the effects of CD4 and coreceptors on 125I-gp120 binding to cells and on its subsequent metabolism. These gp120s interacted with CD4 (Figs. 3 and 6–9) and with coreceptors in a specific manner. For example, 125I-MIP1β binds specifically to CCR5 (2, 42) and was displaced from HeLa-CD4/CCR5 cells by gp120 BaL (Fig. 2), consistent with the coreceptor specificity of the corresponding virus. In contrast, gp120 IIIB had a considerably higher affinity for the cells but did not displace 125I-MIP1β. Furthermore, we found previously that the same gp120 IIIB preparation specifically antagonized SDF-1α-induced activation of CXCR4 signal transduction, whereas the gp120 BaL preparation specifically antagonized MIP1α-induced signaling by CCR5 (16). These antagonist effects required coexpression of CD4 with the appropriate coreceptors on the cell membranes (16). In the present study we have in addition used cell clones constructed for specific expression of CD4 and coreceptors and have demonstrated that infections by HIV-1 isolates were highly efficient and absolutely dependent on presence of both CD4 and the appropriate coreceptor (see Fig. 5). For example, the HeLa-CD4 and HeLa-CD4/CCR5 cell clones used in this investigation expressed large amounts of both CD4 (4 × 10^4 CD4/cell) and CCR5 (1.5 × 10^4-1.3 × 10^5 CCR5/cell), and we showed previously that the cells with CCR5 are infected by R5 HIV-1 isolates as efficiently as normal human peripheral blood mononuclear cells (20). In contrast, HeLa-CD4 cells that lack CCR5 are completely resistant to R5 HIV-1 infections (20). Similarly, infections of human U87MG/CD4/CXCR4 astrogloma cells by X4 HIV-1 isolates were efficient and completely dependent on CD4 and CXCR4 (see Fig. 5). Conversely, R5 HIV-1 isolates could only infect the U87MG-CD4/CCR5 cells.

An important result of this investigation is that the 125I-gp120s were bound to cells and metabolized by a process that was highly dependent on CD4 but not significantly influenced by coreceptors (see Figs. 7 and 9). After removal of unabsorbed ligand, the 125I-gp120s were not substantially shed back into the medium; rather, they were removed slowly from the cell surfaces (half-times approximately 1 h) as indicated by their resistance to extraction from the cell monolayers by a pH 3.0 buffer that has been used widely to study ligand endocytosis (e.g. 21–23). This removal was indeed caused by endocytosis as indicated by confocal immunofluorescence microscopy (Fig. 10). In agreement with this interpretation, the 125I-gp120 that was removed from cell surfaces was subsequently released into the culture medium as small degradation products that were soluble in 10% trichloroacetic acid (Figs. 3, 4, 7, and 9), and this degradation was blocked by chloroquine (Fig. 4), a weak base that raises the pH of acidic organelles and inhibits lysosomal proteolysis (44). In the absence of chloroquine, the endocytosed 125I-gp120s did not substantially accumulate intracellularly, suggesting that the rate of their degradation was rapid compared with the rate of their endocytosis. Indeed, based on mathematical analysis (see “Experimental Procedures”) and the observation that the ratio of label on cell surfaces relative to intracellular sites reaches a steady-state value of approximately 1.3–2.0 after removal of 125I-gp120 from the culture medium (Figs. 3, 4, 7, and 9), we conclude that the rate of degradation is at least 2.3–3.0 times faster than the rate of endocytosis in the conditions of our experiments. This implies that the half-time for degradation of endocytosed 125I-gp120 is approximately 20–25 min. This is a minimum estimate be-

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**FIG. 9.** Kinetics of 125I-gp120 BaL binding and its subsequent metabolism in HeLa-CD4 cells in the presence or absence of CCR5. Experiments similar to those described in Figs. 3 and 7 were performed with both HeLa-CD4/CCR5 cells (panels A and B) and HeLa-CD4 cells (panels C and D). Total 125I-gp120 BaL binding onto control HeLa cells at 60 min was approximately 0.6 cpm/µg of protein.
cause some of the intracellular label may have been partially or fully degraded but not yet released from the cells.

Although other investigators have used immunocytochemical methods to study interactions of HIV-1 virions or gp120s with cells (e.g., 32, 48) and 125I-monomoclonal antibodies to study the endocytosis of CD4 (21–29), we are unaware of previous studies that used 125I-gp120 s to investigate these issues. The endocytosis of CD4 detected in these previous analyses occurred with half-times of approximately 40 min to 1.5 h depending on the cells employed; and a fraction of the endocytosed CD4 was apparently recycled to cell surfaces (21, 29, 45). Moreover, anchoring of CD4 to the cytosolic protein tyrosine kinase Lck retards its rate of endocytosis (23, 24). In contrast, the HIV-1-encoded Nef protein increases formation of clathrin-coated pits and enhances endocytosis of CD4 and of HLA proteins (25–29), thereby facilitating virus release and protecting the infected cell from cytotoxic T lymphocytes. Thus, CD4 endocytosis can be slowed or increased by other proteins. Although we have not analyzed effects of Lck or Nef on endocytosis and proteolysis of adsorbed 125I-gp120s, our results are compatible with these earlier studies and additionally suggest that CD4 has a predominant effect on the adsorption, endocytosis, and proteolysis of these HIV-1 glycoproteins, whereas coreceptors have much weaker influences in the conditions of our analysis.

Although gp120s from X4 strains of HIV-1 have been reported to induce endocytosis of CXCR4 in HeLa cells, it was surprising that this occurred independently of the presence of CD4 in the cells (32). Furthermore, these X4 gp120s can cause CXCR4 capping on surfaces of some other cells (48). In contrast, we did not observe any stimulation of CCR5 endocytosis by gp120s from R5 or X4 strains of HIV-1 (results not shown). Conversely, we did not detect any significant effect of coreceptors on adsorption or metabolism of 125I-gp120s. This is probably not a consequence of the gp120 labeling modifications because we obtained the same results with protocols that label different amino acids (see “Experimental Procedures”). Moreover, compatible results were obtained by confocal immunofluorescence microscopy using unlabeled gp120s (see Fig. 10). The lack of effect of coreceptors on apparent affinities of 125I-gp120s for surfaces of CD4-positive cells could be a consequence of a relatively low affinity of coreceptors for the membrane-associated 125I-gp120-CXCR4 complexes. However, it could alternatively be a consequence of the substantial concentrations of CD4 on the cells (20, 37). Use of these cells was required to obtain adequate amounts of 125I-gp120 adsorption. Because gp120s bind first to CD4 and secondarily to coreceptors (1, 2, 12–15), the latter would be expected to have little effect on the initial rates of gp120 association with the cells. Moreover, at high CD4 concentrations the rates of gp120 dissociation from the cells would become negligible (47) even in the absence of coreceptors. In this circumstance, an effect of coreceptors on gp120 affinity might be difficult to discern. Although significant effects of coreceptors might conceivably be observed using other gp120s or cells that contain less CD4 and/or more coreceptors, the gp120s we used were derived from highly infectious laboratory-adapted strains of HIV-1, and the cells we used were efficiently infected by these viruses. Additional studies will be required to determine whether similar results would occur with other cells or by using whole virions or oligomers of gp120s.

Previous evidence has indicated that gp120 adsorption onto uninfected cells may sensitize them to immune destruction by antibody and complement-dependent cytoxicity or to apoptosis (49–51). The presentation of endocytosed and partially degraded gp120 peptides by HLA class II proteins could also result in T cell killing (50, 52). Moreover, gp120s adsorbed onto CD4 can activate the associated Lck tyrosine kinase with resultant activation of the mitogen-activated protein kinase cascade (53); and gp120s may also act as agonists (18, 19) and antagonists (16) of their coreceptors. These and other pathogenic effects of gp120s such as neural degeneration (54, 55) would presumably be substantially attenuated by the process of CD4-mediated endocytosis and lysosomal degradation which we have demonstrated.

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