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A Role for Urokinase-Type Plasminogen Activator in Human Immunodeficiency Virus Type 1 Infection of Macrophages

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Urokinase-type plasminogen activator (uPA), a proteinase which activates plasminogen by cleaving at -CPGR ↓ V-, was shown to cleave the V3 loop in recombinant gp120 of human immunodeficiency virus type 1 (HIV-1) IIIB and MN strains, as well as a synthetic, cyclized peptide representing the clade B consensus sequence of V3. Proteolysis occurred at the homologous -GPGR ↓ A-, an important neutralizing determinant of HIV-1. It required soluble CD4 and was prevented by inhibitors of uPA but not by inhibitors of likely contaminating plasma proteinases. It was accelerated by heparin, a known cofactor for plasminogen activation. In immune capture experiments, tight binding of uPA to viral particles, which did not depend on CD4, was also demonstrated. Active site-directed inhibitors of uPA diminished this binding, as did a neutralizing antibody to V3. Addition of exogenous uPA to the laboratory-adapted IIIB strain of HIV-1, the macrophage-tropic field strains JR-CSF and SF-162, or a fresh patient isolate of indeterminate tropism, followed by infection of macrophages with the various treated viruses, resulted in severalfold increases in subsequent viral replication, as judged by yields of reverse transcriptase activity and p24 antigen, as well as incorporation, as judged by PCR in situ. These responses were reversible by inhibitors or antibodies targeting the proteinase active site or the V3 loop. We propose that uPA, a transcriptionally regulated proteinase which is upregulated when macrophages are HIV infected, can be bound and utilized by the virus to aid in fusion and may be an endogenous component that is critical to the infection of macrophages by HIV-1.

Entry of human immunodeficiency virus type 1 (HIV-1) into primate cells occurs through interactions at several levels between viral envelope proteins gp120 and gp41 and the host cell membrane. After binding of gp120 to the receptor CD4 (32), structural changes that permit an N-terminal hydrophobic peptide of gp41 to penetrate the lipid bilayer, mediating fusion, are believed to occur (11, 36). The site of origin of these changes is obscure, but strongly implicated is the crown of the third variable (V3) loop region of gp120, which is distinct and remote from the domains involved in CD4 binding and contains the amino acid sequence -GPGRA- or -GPGRV- (12, 28). This sequence is highly conserved among (and, indeed, defines) clade B strains (35) of HIV-1, despite the mutability of the surrounding amino acids which earns the region its name. An important neutralizing determinant for monoclonal or patient-derived antibodies to gp120 also resides in -GPGR-, underscoring its importance in viral behavior. -GPGRA- and its counterparts in other clades, frequently -GPGQA- and -GPGQV-, adopt a type II β-turn structure (13) that remains accessible to antibodies in the assembled viral envelope (33). Variants of HIV-1 altered in -GPGRA/V- can escape antibody neutralization (23): some are not replication competent and, specifically, not fusogenic (39), despite the fact that binding to CD4 persists unimpaired (4, 11, 43).

As predicted from its homology to -GPCRA-, a sequence found at the binding sites of proteinase inhibitors such as trypstatin and inter-α-trypsin inhibitor (18, 24, 34), gp120 at the V3 loop is also a potential substrate or binding site for certain secreted or cellular proteinases (3, 7, 17, 47). Given the strong homology of -GPGRA/V- to the activation sequence -CPGRV- in human plasminogen, we investigated whether gp120 is recognized by urokinase-type plasminogen activator (uPA), a receptor-bound proteinase produced by macrophages as part of their tissue-invasive machinery and inflammatory response. We have found that not only does uPA bind and cleave gp120 at V3, but it is also responsible for significant increases in the infectivity of HIV-1 toward these cells.

(This work was presented in preliminary form [16] at the May 1994 meeting of the American Federation for Clinical Research/American Society for Clinical Investigation/Association of American Physicians in Baltimore, Md.)

MATERIALS AND METHODS

Cleavage of gp120. Recombinant full-length, fully glycosylated gp120 of HIV-1 IIIB strain (Intracel Corp.) or HIV-1 MN strain (Agmed) was incubated at 20 μg/ml with two-chain recombinant uPA (ruPA; 600 IU or ~6 μg/ml; Abbott Laboratories), recombinant soluble CD4 (rsCD4; 5 μg/ml; DuPont-NEN), and heparin (25 U/ml), which increases uPA proteolytic activity (45). When necessary, sCD4 was pretreated with DEGR-CMK (1,5-dansyl-Glu-Gly-Arg-chloromethyl ketone dihydrochloride; 5 μM for 30 min) to inactivate a contaminating proteinase derived from the CHO cell of origin (7). Reagents tested for the ability to inhibit cleavage of gp120 included (i) murine immunoglobulin G1 (IgG1) antibody to the uPA catalytic site (antibody 394; American Diagnostica); (ii) IgG1 antibody to the uPA receptor-binding domain (antibody 3921; American Diagnostica); (iii) endothelial cell PAI-1 (American Diagnostica); (iv) α_2-plasmin inhibitor (American Diagnostica); (v) a neutralizing monoclonal antibody (antibody 9205; DuPont-NEN) to an epitope in V3 of HIV-1 IIIB spanning the cleavage site, GPGR ↓ A (9); and (vi) a nonneutralizing antibody (antibody IIIB-V3-01) to the sequence IGKIGNMRQ four residues C terminal to the putative cleavage site (27). Samples of gp120 denatured and reduced in 5 mM EDTA–40 mM imidazole–1 M glycerol–0.05% bromophenol blue–1% sodium dodecyl sulfate (SDS)–5% 2-mercaptoethanol (pH 6.4) were electrophoresed in 10% polyacrylamide (26). After the gels were washed in 20% methanol–25 mM Tris–0.2 M glycine, proteins were transferred (Pharmacia Novablot) to Immobilon P (Millipore). Blots were blocked overnight in 5% nonfat milk–0.1 M NaCl–20 mM Tris (pH 7.5), then incubated with sheep antibody to gp120 C5 region (BioDesign International)–2 μg of IgG per ml for 4 h, followed by alkaline phosphatase-conjugated donkey anti-sheep IgG (1/30,000) absorbed with rodent

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and other sera (Jackson Immunoresearch), and developed with 5-bromo-4-chloro-3-indolylphosphate toluidinium (BCIP)–nitroblue tetrazolium (NBT) premixed substrate (Kirkegaard & Perry Laboratories).

High-pressure liquid chromatography (HPLC). The 35-residue, disulfide-bonded consensus sequence for V3 loop peptide of HIV-1 clade B (Intracel) was incubated at 1.0 mg/ml with ruPA (600 U/ml) or human thrombin (10 μg/ml) in phosphate-buffered saline (PBS) for up to 6 h. No sCD4 or heparin was present. Samples of 5 μg, reduced with 0.25 M dithiothreitol, were injected via a cartridge precolumn (Brownlee) into a 5-cm-long, 4.6-mm-diameter, 300-Å (30-nm)-pore-size LC-18 reverse-phase column (Supelcosil). Elution was with a 0 to 60% CH_3CN gradient in 0.1% phosphoric acid–0.01 mM dithiothreitol–water. Effluent absorbance was monitored at 210 nm. The marker for proteolysis was the 18-residue peptide that would derive C terminally from cleavage at -GPGR ↓ A- and subsequent reduction. This peptide was synthesized by standard resin-based methodology and verified by sequencing.

Cell culture. Mononuclear cells of healthy uninfected donors were obtained, with informed consent, from 200 ml of heparinized blood by centrifugation through Ficoll-Hypaque (Pharmacia) (5). After collection from the interface, the cells were washed three times in RPMI 1640 medium (Whittaker), transferred to flasks, and incubated under humidified 5% CO_2. Nonadherent cells were removed after 20 h by replacing with RPMI 1640–20% fetal bovine serum with endotoxin <30 pg/ml (Sigma)–5% autologous heat-inactivated human serum, glutamine, and antibiotics. On day 4, adherent macrophages were lifted by scraping, washed, resuspended, and replated in 12- or 24-well plates at 10^6 per well. They were >90% viable by trypan blue exclusion and >98% positive for monocyte nonspecific esterase. Analysis by flow cytometry (FACStar Plus; Becton Dickinson) typically indicated 99% positivity for LeuM5 plus HLA-DR; >96% positivity for CD14 (monocytes); and ≤1% positivity for CD3 (pan-T), CD4 (T4), CD19 (pan-B), CD15 (neutrophils), or CD56 (natural killer cells). At 2 h, aliquots of HIV-1 IIIB from H9 lymphoblastoma cells (40) were added at 5,000 to 20,000 cpm of reverse transcriptase (RT) activity (19) after treatment of some aliquots with ruPA (600 U/ml) for 4 h at 37°C, with or without antibody 394 (50 μg/ml) or PAI-1 (800 U/ml). The virus was washed free of unbound uPA, etc., before infection. Control cultures were infected with untreated HIV, up to 100,000 cpm of RT (50% tissue culture infective dose, $\sim 8 \times 10^4$), or sham infected with medium alone. During culture, the medium was replaced every 4 days. Samples from alternate days were assayed for p24 antigen by capture enzyme-linked immunosorbent assay (ELISA; DuPont-NEN) and for RT by a colorimetric method (46).

Other strains. In experiments investigating additional viral strains, HIV-$1_{JR\text{-}CSF}$ and HIV-$1_{SF\text{-}162}$ were cultured for 5 days in phytohemagglutinin-stimulated peripheral blood lymphocytes in the presence of interleukin-2 (Boehringer Mannheim) (20) and quantified by RT activity (19). Patient isolate 5056 was recovered from culture supernatants of the patient's peripheral blood lymphocytes cocultured with normal donor lymphocytes at a ratio of 1:5 (20). Aliquots ranging between 160 and 5,000 cpm of RT activity were treated at a fixed uPA concentration (600 U/ml) and used to infect 10^6 macrophages per aliquot as described above.

PCR in situ. The procedure was performed as described previously (37). Macrophages lifted by scraping 72 h after infection with HIV-1 IIIB were fixed in 10% formaldehyde–PBS for 5 min. Aliquots of 10^5 cells were spun onto siliconized slides and ethanol fixed, treated for 5 min with proteinase K (20 μg/ml), and then subjected to 24 cycles of PCR (2 min, 55°C; 1 min, 94°C; 1 min, cooling; 5 min, 61 to 57°C) with *Taq* polymerase (0.18 U/ml; Cetus) in 10 mM Tris–50 mM KCl–1.5 mM $MgCl_2$ containing 0.2 mM digoxigenin-labeled deoxynucleoside triphosphates and primer SK19 of *gag* (38). Amplified HIV-1 DNA was detected with the Genius system (Boehringer Mannheim) as instructed by the manufacturer. Cells were counterstained with nuclear fast red. Nuclei stained blue-black with NBT were counted by two observers unaware of the applicable conditions, and the values were averaged.

Association of uPA with HIV-1 virions. ruPA (500 U/ml) was added to HIV-1 IIIB (7.5×10^6 cpm of RT per ml) in the presence or absence of PAI-1 (500 U/ml), neutralizing monoclonal antibody 9205 to the V3 loop, or antibody 394 to the uPA catalytic site (50 μg/ml). After incubation for 1 h, the viral pellet was recovered, washed twice by resuspension, dissociated in SDS, electrophoresed, and immunoblotted as described above. The blot was probed with goat anti-uPA and alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma) and developed with BCIP-NBT.

Immunocapture of HIV-1 by antibody to uPA. HIV-1 IIIB (60 cpm of RT activity) was incubated with 50 U of ruPA in 100 μl of PBS for 4 h at 37°C. Goat anti-human uPA (antibody 398; 25 ng; American Diagnostica) was added, the mixture was incubated for 1 h at ambient temperature, and then 10 ng of rabbit anti-goat IgG heavy and light chains (Sigma) was added for a further 1 h. Complexes containing rabbit IgG were captured on 400 μl of staphylococcal protein A-agarose, washed with 40 volumes of PBS, and solubilized in 5% Triton X-100–PBS before assay for HIV-1 p24 antigen by ELISA. Data were corrected for nonspecific binding of rabbit antibody in the absence of goat IgG. Binding was also measured after treatment of uPA with DEGR-CMK (up to 1.0 μM) or PAI-1 (200 IU/ml).

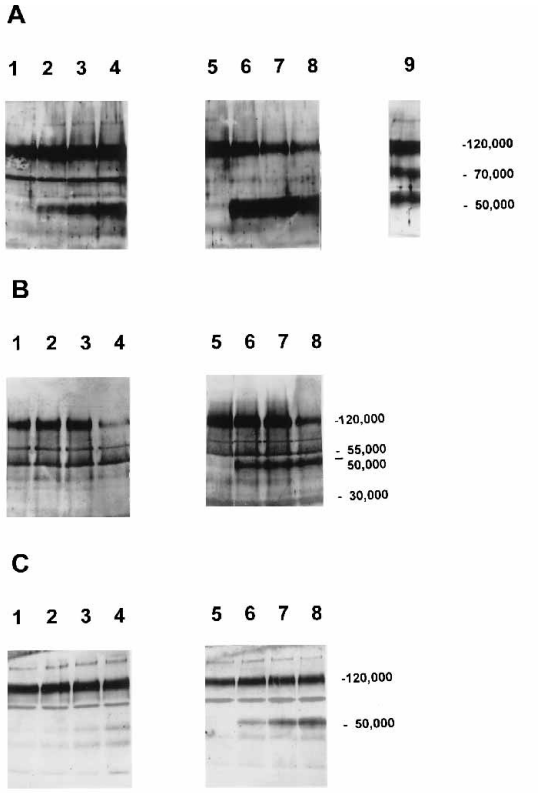

FIG. 1. Cleavage of gp120 by uPA and prevention by specific uPA inhibitors. (A) Cleavage of gp120 by uPA (600 U/ml; lanes 1 to 4), or thrombin, (2.5 μg/ml; lanes 5 to 8) at 0, 2, 4, and 6 h. The 70-kDa cleavage product could be visualized by immunoblotting with human rather than sheep antibody (lane 9). Approximate molecular weights are relative to prestained standards. (B) Cleavage by uPA at 0, 2, 4, and 6 h in the presence of antibody to the uPA catalytic site (lanes 1 to 4) or antibody to the receptor-binding domain (lanes 5 to 8). (C) Cleavage by uPA at 0, 2, 4, and 6 h in the presence of PAI-1 (lanes 1 to 4) or human α_2-plasmin inhibitor (lanes 5 to 8). Residual cross-reactivity of the detecting antibodies with albumin in ruPA, and with heavy and light chains of murine antibodies, accounts for immunopositive bands at ~70, ~55, and 30 kDa.

RESULTS

Specific cleavage of HIV-1 gp120. When recombinant glycosylated gp120 of HIV-1 IIIB was incubated with uPA, a 50-kDa fragment was detectable on Western blots (immunoblots) with sheep antibody to the C terminus of gp120, which increased in intensity over time (Fig. 1A, lanes 1 to 4) and resembled a fragment obtained by cleavage with thrombin (7) (lanes 5 to 8). The N-terminal, 70-kDa fragment of gp120 was visible when similar blots were probed with IgG from a donor with high-titer antibody to gp120 (lane 9). Similar 50- and 70-kDa fragments were generated when rgp120 of HIV-1 MN was incubated with uPA or thrombin (not shown).

A murine IgG1 monoclonal antibody to the catalytic site of uPA blocked cleavage over time (Fig. 1B, lanes 1 to 4), whereas IgG1 antibody to the receptor-binding domain of uPA, with no effect on catalytic activity in the absence of the uPA receptor, did not (Fig. 1B, lanes 5 to 8). Similarly, the endothelial plasminogen activator inhibitor PAI-1 inhibited proteolysis (Fig. 1C, lanes 1 to 4), but α_2-plasmin inhibitor had no effect (Fig. 1C, lanes 5 to 8). Antithrombin, an inhibitor of thrombin and related proteinases, also had no effect on cleavage, nor did Ca^{2+} or EDTA (data not shown). Therefore, proteolysis apparently occurred without the intervention of

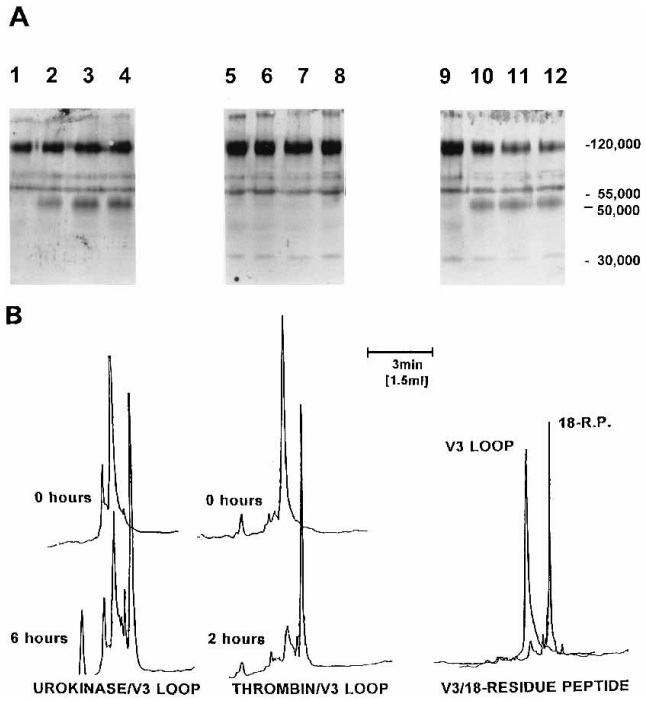

FIG. 2. Identification of the site of cleavage as the V3 loop. Lanes 1 to 4, control without antibody; lanes 5 to 8, samples over time with neutralizing antibody spanning the cleavage site; lanes 9 to 12, samples with nonneutralizing antibody to V3. Residual cross-reactivity of the detecting antibodies with albumin in ruPA, and with heavy and light chains of murine antibodies, accounts for immunopositive bands at ~70, ~55, and 30 kDa. (B) Separation of peptides generated during cleavage of V3 loop by uPA or thrombin. Effluent absorbance at 210 nm from a C_{18} reverse-phase HPLC column is shown. 18-R.P., the marker for proteolysis, is the C-terminal peptide that would derive from cleavage and reduction of V3. This peptide lagged 0.75 ml behind intact V3 loop when a mixture of the two was chromatographed (far right). Incubation of V3 with thrombin (center) or uPA (left) generated cleavage product over time.

plasmin or other plasma-derived proteinases; it resulted from uPA as such.

Site of cleavage in gp120. To verify the site of proteolysis, the effects of monoclonal antibodies to epitopes in V3 were compared. Relative to nonimmune murine IgG1 (Fig. 2A, lanes 1 to 4), a neutralizing monoclonal IgG1 antibody (9) to an epitope spanning -GPGR- prevented cleavage (Fig. 2A, lanes 5 to 8), whereas a nonneutralizing IgG1 antibody to a flanking sequence close by (27) did not (Fig. 2A, lanes 9 to 12). This result shows that neutralization occurs specifically at the potential cleavage site. Furthermore, when the synthetic, 35-residue cyclized V3 peptide was incubated as such with uPA or thrombin, cleavage products generated by either proteinase had the same chromatographic characteristics as the 18-residue C-terminal product of cleavage at -GPGR ↓ A- (Fig. 2B). This reaction occurred in the absence of CHO cell-derived recombinant reagents, eliminating the CHO cell protease known to cleave gp120 (7) as responsible for V3 cleavage in our experiments.

Effects of uPA on viral infectivity and replication. Mature monocyte-derived macrophages readily increase expression of uPA in response to hematopoietic or inflammatory stimuli (14, 29). Therefore, the possibility that uPA proteolytic activity and infection of macrophages by HIV-1 are related was investigated. Blood monocytes from healthy donors were isolated and allowed to adhere and differentiate in culture. After cellular homogeneity was confirmed by flow cytometry, the macrophages were infected with HIV-1 IIIB which had been previously cultured in H9 lymphoblastoma cells (40) and preincubated (without addition of sCD4) in aliquots of 5,000 cpm of RT with pyrogen-free ruPA (600 U/ml) immediately prior to infection. This plan was designed to avoid any effects of uPA on macrophages that might alter viral behavior, as well as interference by proteinase inhibitors in serum-supplemented media. The infectivity of the proteinase-treated virus was determined in three ways: (i) RT assay of culture supernatants (46) and (ii) p24 antigen assay, both of which assessed production of new virions 7 to 9 days after infection; and (iii) PCR in situ (37), which indicated the proportion of cells containing proviral DNA 3 days after infection. Infection was at a low 50% tissue culture infectious dose of 2.2×10^4, at which, without prior uPA treatment, the yield of RT was at or near the background for uninfected media (Fig. 3A). In contrast, after exposure of HIV-1 to uPA, the RT yield increased to >0.6 absorbance units; this uPA-dependent increase was attenuated 100% on day 7 and ~70% on day 9 when antibody to the uPA active site was present during the preincubation step (Fig. 3A).

In this and a second identical experiment, undetectable replication was increased to these highly significant levels by pretreatment of the HIV-1 inoculum with uPA, as shown (Fig. 3A). In six more experiments, in which there was some baseline

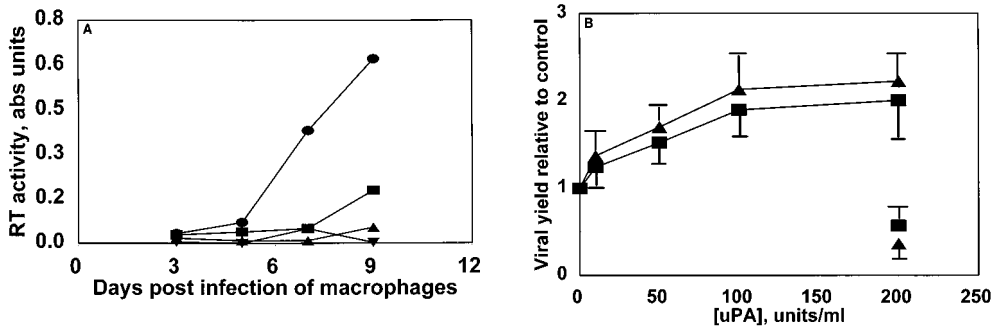

FIG. 3. Increased yield of HIV-1 from macrophages after treatment of the inoculum with uPA. (A) Yield of RT activity over time from a culture infected with HIV-1 that was (●) or was not (▲) pretreated with uPA. ■, inoculum treated in the presence of antibody to uPA; ▼, baseline activity from uninfected cells. This experiment is typical of eight representing different donors, with each analysis performed in duplicate or triplicate. Statistical analysis is given in the text. abs, absorbance. (B) Dose response to uPA concentrations of between 0 and 200 U/ml. The input level of HIV-1 was 10,000 to 20,000 cpm of RT activity per aliquot. Data shown are means from six experiments, each analysis performed in duplicate. ■, RT activity; ▲, p24 antigen; solitary points, virus treated with uPA (200 U/ml) in the presence of PAI-1 (800 U/ml).

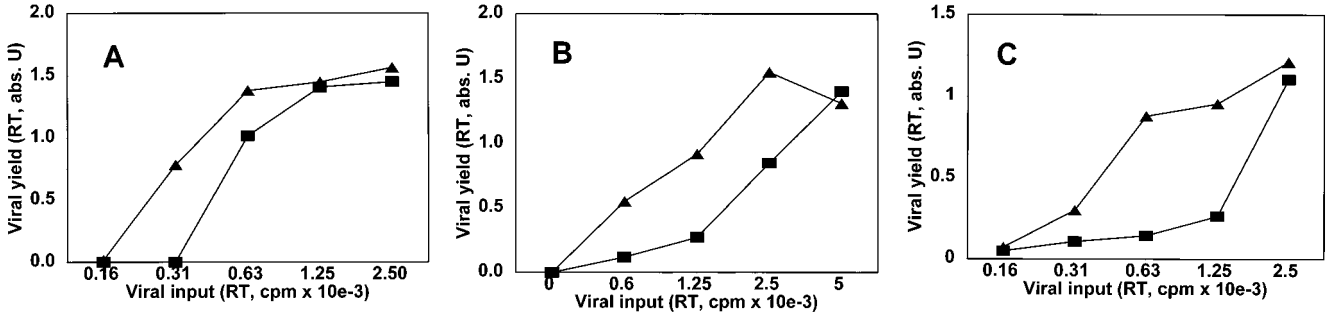

FIG. 4. Responses of field strains of HIV-1 to pretreatment with uPA at a fixed concentration over a range of HIV-1 inoculum size (■) relative to untreated HIV-1 (▲). Each experiment was performed twice, and one example of each is shown. (A) HIV-$1_{JR\text{-}CSF}$; (B) HIV-1_{SF162}; (C) a fresh isolate, 5056. abs., absorbance.

infection without exposure of virus to proteinase, RT activity after uPA treatment was increased from a minimum of 5-fold to a maximum of 100-fold ($P < 0.0001$ by pairwise t test).

By PCR in situ analysis (six experiments), the number of nuclei staining for incorporated viral DNA with the *gag* SK19 probe (38) on day 3 postinfection, before replication was detectable, increased from 26.1% ± 4.6% to 78.1% ± 1.4% (means ± standard errors of the means; $p = 0.0001$ by pairwise t test). This was equal to the percentage of cells (75.6% ± 3.2%) containing proviral DNA in control cultures that were infected maximally with 20 times more HIV-1 IIIB. Theoretically these results could be affected by the presence of positively stained but unintegrated, circular forms of HIV-1 DNA. However, it seems unlikely that a change in the number of these forms could account solely for the effects of uPA, acting extracellularly on the virion or macrophages, particularly as upregulation is most pronounced at low viral input levels (see below).

In additional experiments, it was confirmed that the mean increase in viral yield was dose dependent with respect to uPA (Fig. 3B). In six cultures, mean yields of RT activity rose as uPA concentrations ranged between 0 and 200 U/ml, while p24 antigen increased concomitantly. Both p24 and RT were decreased to levels at or below baseline in the presence of PAI-1 (500 U/ml), confirming that the uPA was responsible for the enhancement of infectivity. Moreover, this upregulation, like cleavage, occurs independently of plasminogen activation, as levels of plasmin(ogen) in the ruPA are very low indeed, >12 to 36 pg per mg of uPA. Also, α_2-plasmin inhibitor did not reverse the increase in infectivity caused by uPA (data not shown). However, it cannot be entirely ruled out that the viral particle carries with it traces of plasminogen from prior exposure to serum-supplemented culture medium.

The HIV-1 used in the preceding experiment was the laboratory-adapted IIIB strain, with its possible idiosyncrasies caused by multiple passaging and the insertion of two amino acids into V3 adjacent to the cleavage site (35). Therefore, we sought to determine whether uPA would influence infectivity of HIV-1 of strains deriving from two field isolates, SF162 (6) and JR-CSF (25), from clade B and minimally passaged in a primary lymphocyte culture, as well as a fresh patient isolate treated in a similar manner. It was found that these strains displayed different intrinsic infectivities toward macrophages when the inoculum size was based on RT activity. Therefore, the inoculum of HIV-1 used was titrated from 0 to 5,000 cpm of RT activity, with or without prior treatment at a fixed concentration of uPA, 600 U/ml. The results are shown in Fig. 4 as a function of viral input. One of two experiments performed with each of three isolates in different donor cells is shown (Fig. 4A, B, and C). The data are corrected for the low background activity of uninfected cells (<0.1 absorbance units). As for IIIB, levels of viral input had to be decreased well below maximum infection for enhancement by uPA to be the most striking. Under these conditions, the yield of RT was increased severalfold in response to uPA in all three strains. This increase was greatest at viral inputs of between 300 and 1,250 cpm of RT, depending on the strain. Taken together, these results indicate that upregulation of infectivity by uPA is by no means exclusive to the laboratory-adapted IIIB strain but extends to three others that had been minimally passaged.

Binding of uPA to HIV-1 particles. The rates of cleavage of gp120 versus the kinetics of upregulation of infectivity are widely different, and a possible problem with proposing uPA as an agent for obligatory cleavage is that, under the conditions described here, the rate is quite slow. One explanation may be that the in vitro system lacks physiologic components that would considerably enhance the kinetics of plasminogen activation, importantly the uPA receptor (10, 30). Another possibility is that the uPA active site serves a binding rather than catalytic function, with cleavage occurring only incidentally and at a slower pace. Therefore, the ability of HIV-1 gp120 to bind uPA was investigated.

In experiments in which uPA was added to a high-titer suspension of HIV-1 IIIB (7.5×10^6 cpm of RT activity per ml), virus recovered from the suspension by centrifugation carried virtually all of the uPA detectable by immunoblotting. This apparent association of uPA with viral particles was prevented equally well by antibody to the uPA catalytic site, PAI-1, or neutralizing antibody to the V3 loop (Fig. 5A).

Mindful of the possibility that cellular debris contributed to this apparent binding, we investigated the phenomenon further in immune capture experiments not dependent on centrifugation. When uPA, goat antibody to uPA, a rabbit anti-goat IgG, and staphylococcal protein A-agarose were added sequentially to suspensions of HIV-1, the complexes recovered by elution from protein A at low pH contained 79.6 ± 7.3 pg (mean ± standard deviation) of p24 antigen per ml (Fig. 5B). This was significantly more than the mean amount of p24 recovered from complexes containing no exogenous uPA (seven experiments; $P < 0.0001$ by pairwise t test). However, the latter did contain 16.5 ± 3.9 pg of p24 antigen per ml compared with controls containing no goat anti-uPA antibody in which p24 antigen was undetectable (Fig. 5B), suggesting a possible association of endogenous uPA with viral particles. Moreover, the association between uPA and p24 was decreased, in a dose-dependent fashion, by the uPA active site inhibitor DEGR-CMK at concentrations of up to 1.0 μM ($n = 6$; $P = 0.003$) or by PAI-1 at 200 U/ml ($n = 4$; $P = 0.048$) (Fig. 5B).

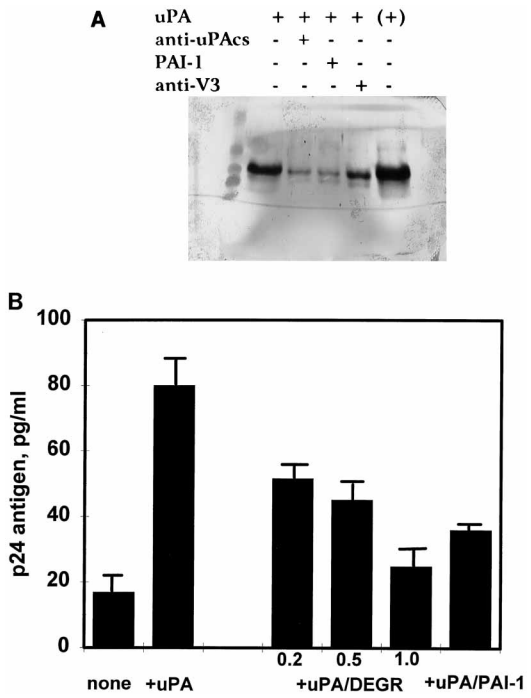

FIG. 5. Association of uPA with viral particles and prevention of association by active site-directed inhibitors. (A) HIV at high titer was mixed with uPA with or without various inhibitors as indicated above the blot and recovered by centrifugation. uPA with the same mobility as the parent ruPA (far right) was visualized by immunoblotting (see Materials and Methods). Anti-uPA cs, antibody to the uPA catalytic site. (B) HIV at low titer was mixed with uPA with or without DEGR-CMK (at the concentrations [micromolar] indicated at the bottom) or PAI-1, recovered by immunocapture with anti-uPA antibody, and quantified in an ELISA from the content of p24 antigen. Data are shown as means ± standard deviations (bars) and are corrected for assay background, determined from *p*-nitrophenol generation in the absence of primary antibody to uPA.

Therefore, by several criteria, the binding of uPA to HIV-1 particles appears to be specific and mediated through interaction between the exposed V3 loop of the virus and the proteinase active site.

DISCUSSION

We have shown that uPA binds tightly to HIV-1 particles via gp120 at V3 and can also cleave gp120 of IIIB and MN strains within the V3 loop in the presence of sCD4. These reactions are prevented with a variety of inhibitors targeted to either side of the interaction (uPA or viral envelope), whereas inhibitors of potential contaminating blood proteinases are without effect. uPA also causes a large, dose-dependent increase in the infectivity of both laboratory-adapted and field strains of HIV-1. Neutralizing antibody targeting V3 of IIIB prevents this effect also, as well as preventing the proteolysis of V3 and infection. As uPA is a critical enzyme in the inflammatory response and in the invasive properties of macrophages, we believe that its interaction with the viral envelope is an important element in the infection of activated macrophages by HIV-1.

Although the sites of interaction between virus and uPA have been identified, the extent and type of the interaction are still somewhat in doubt, as it is not known whether cleavage as such is obligatory for fusion. Given the disparity in rate between binding and proteolysis of gp120 by uPA, we propose as a working hypothesis that the binding of uPA to virus particles, which probably occurs independently of CD4, may be the decisive interaction. This would be consistent with observations that other cellular proteinases, such as cathepsin G and surface proteinases of MOLT-4 cells, recognize and bind to gp120 or peptides derived from the -GPGRA/V- sequence (3, 17, 42). Given the binding interaction, it is conceivable that uPA acquired from the vicinity of the macrophage is carried with viral particles into other surroundings and to other cells. The roles of this mode of transport in infection and in cellular tropism are currently being investigated.

Although the susceptibility of gp120 to proteases is not a new observation, it has not previously been linked to altered viral behavior in HIV-1 or to a pathway that would particularly assist in fusion of HIV-1 with macrophages. The phenomenon of increased infectivity after treatment with proteinases was seen with another retrovirus, however (1), while intracellular cleavage of gp160, the gp120 precursor, by eukaryotic proteinases such as furin or its homologs (8) is known to be required for competence in subsequent fusion (2). Proteolytic cleavage of spike proteins by cellular proteinases, and associated increases in infectivity, are widespread among the ortho- and paramyxoviruses. Such processing can influence both virulence and host cell range (22). Bacterial plasminogen activators, in particular, influence the replicative cycle of influenza virus, with consequences for pathogenesis (41), and the virulence of *Yersinia pestis* (44).

It is particularly interesting that in previous experiments, the secretion of uPA by macrophages was found to be increased in the presence of HIV-1 virions that had been derived from prior culture in macrophages exactly as described here (15). In those experiments, uninfected macrophages exposed (under lipopolysaccharide-free conditions) to HIV-1 IIIB particles for just 1 h expressed uPA at levels that were severalfold higher than those in resting macrophages. Given that the association of gp120 with uPA or analogous proteinases may be critical to fusion of HIV-1 with these cells, our data suggest the existence of an infective cycle in which HIV-1, encountering its target cell, induces expression of uPA that is then used for fusion with the membrane. It follows that fusion is a target at which a proteinase inhibitor with appropriate specificity could be expected to act (31).

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