JOURNAL OF VIROLOGY, May 1993, p. 2566–2574
0022-538X/93/052566-09$02.00/0

Vol. 67, No. 5

Human Immunodeficiency Virus Type 1 Envelope gp120 Is Cleaved after Incubation with Recombinant Soluble CD4

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Received 14 October 1992/Accepted 1 February 1993

Human immunodeficiency virus type 1 (HIV-1) infects human CD4$^+$ cells by a high-affinity interaction between its envelope glycoprotein gp120 and the CD4 molecule on the cell surface. Subsequent virus entry into the cells involves other steps, one of which could be cleavage of the gp120 followed by virus-cell fusion. The envelope gp120 is highly variable among different HIV-1 isolates, but conserved amino acid sequence motifs that contain potential proteolytic cleavage sites can be found. Following incubation with a soluble form of CD4, we demonstrate that gp120 of highly purified HIV-1 preparations is, without addition of exogenous proteinase, cleaved most likely in the V3 loop, yielding two proteins of 50 and 70 kDa. The extent of gp120 proteolysis is HIV-1 strain dependent and correlates with the recombinant soluble CD4 sensitivity to neutralization of the particular strain. The origin of the proteolytic activity in the virus preparations remains unclear. The results support the hypothesis that cleavage of gp120 is required for HIV infection of cells.

Human immunodeficiency virus type 1 (HIV-1), the cause of AIDS, initiates infection of human CD4$^+$ cells by a high-affinity interaction between its envelope glycoprotein gp120 and the CD4 molecule on the cell surface (13, 28, 33, 35, 36). Subsequently, the fusion domain of the viral transmembrane protein gp41 is presumed to be exposed, and a pH-independent fusion of the viral and cellular membranes takes place (17, 35, 51). Binding of the HIV-1 gp120-gp41 envelope structure of HIV-1 to CD4 alone is not sufficient to support entry into cells, since certain HIV-1 strains are unable to infect human CD4-expressing cell lines (5, 7–9, 11, 14, 27, 44, 50). Moreover, HIV-1 binds to human CD4-expressing mouse cells but fails to enter them. These results suggest that, in addition to CD4 attachment, other processes are involved in HIV infection (for a review, see reference 42).

Viral determinants for cellular tropism map to a domain of gp120 which includes the third variable (V3) region of gp120 (10, 22, 50). This V3 region contains a disulfide-linked loop of about 35 amino acids that has a major HIV-1 neutralizing epitope (23, 47). Although highly variable among different HIV-1 isolates (30, 38), conserved amino acid motifs which resemble a proteolytic cleavage site (12, 20, 29) can be identified in the V3 loop (30). Moreover, antibodies against rat tryptase M inhibit HIV-1 syncytium formation (20). In addition, the V3 loop interacts with proteinase-like molecules at the surface of the human T-cell line Molt4 clone 8 (Molt4/8) (43). A membrane-bound serine protease, TL2, which binds HIV-1 gp120 and is inhibited by V3 loop peptides (20, 25, 26) has been purified from the Molt4/8 cells (27). Furthermore, the V3 loop of gp120 can be cleaved in vitro by addition of serine proteases (e.g., thrombin) to virus preparations, resulting in breakdown products of approximately 70 and 50 kDa (49). All of these observations suggest that cleavage of gp120 could be important for HIV infection.

To study the potential role of proteolytic cleavage of HIV-1 gp120 in virus infection, we analyzed by immunoblot procedures highly purified, concentrated viable virus preparations after incubation with recombinant soluble CD4 (rsCD4). Without addition of an exogenous protease, the outer envelope protein gp120 of all tested HIV-1 strains was cleaved, most likely at the V3 loop, relative to the rsCD4 concentration and the incubation time. The extent of gp120 cleavage was dependent on the HIV-1 strain, not the cell type used for its propagation, and the process correlated with the rsCD4 sensitivity to neutralization of the particular strain. The origin of this proteolytic activity in the virus preparations remains unclear. Copurification or incorporation of a cellular protease in the viral membrane during budding or self-cleavage of gp120 upon binding to rsCD4 could be involved.

MATERIALS AND METHODS

Cells and cell lines. Ficoll-Hypaque gradient-purified peripheral blood mononuclear cells (PBMC) were prepared and pretreated with 3 μg of phytohemagglutinin per ml of culture fluid 3 days prior to use as described elsewhere (4). PBMC from HIV-seronegative individuals were provided by Irwin Memorial Blood Bank (San Francisco, Calif.). The PBMC were grown in RPMI 1640 medium supplemented with 10% heat-inactivated (30 min, 56°C) fetal bovine serum (Irvine Scientific, Irvine, Calif.), 2 mM L-glutamine, 100 IU of penicillin per ml, 100 μg of streptomycin per ml, and 5% interleukin-2 (Electronucleonics, Silver Spring, Md.). The origin and maintenance of the established T-cell lines SupT1, Jurkat, HuT 78, and Molt4/8 used in these experiments have been described previously (2, 3). Human glioma cells (SK-N-MC) were obtained from the American Type Culture Collection. The cell lines were grown in supplemented RPMI 1640 medium without interleukin-2.

Viruses. Virus isolation procedures, biological characterization, molecular cloning, and DNA sequencing of the HIV-1 wild-type strains (HIV-1_{SF2}, HIV-1_{SF13}, HIV-1_{SF33}, and HIV-1_{SF162}) and the biologically active molecular clones (HIV-1_{SF2mc}, HIV-1_{SF33mc}, and HIV-$1_{SF162mc}$) have been described elsewhere (5, 32, 34, 48).

Virus stocks and titration. High-titer virus stocks were prepared from bulk infections of phytohemagglutinin-stimu-

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lated normal human PBMC as previously described (2) and diluted to obtain the 50% tissue culture infectious dose ($TCID_{50}$). In brief, serially diluted virus stocks were inoculated at threefold dilutions in quadruplicate wells onto PBMC in round-bottom 96-well plates. Cell culture supernatants were scored for positivity by an HIV-1 enzyme-linked immunosorbent assay (ELISA) every second day, beginning at day 6 after infection. By this procedure, 100-μl fluid aliquots from each well were taken, added to ELISA plates, treated with 0.05% Triton X-100, and incubated at room temperature overnight to allow viral antigens to adhere. Plates were absorbed with gelatin, washed, incubated with a 1:500 dilution of anti-HIV-1-specific immunoglobulin G (IgG) for 1 h, and then washed four times and incubated with anti-human IgG conjugated with horseradish peroxidase (Sigma, St. Louis, Mo.) for 1 h. The optical density was measured at 592 nm. HIV-1-specific IgG was prepared as previously published (46). $TCID_{50}$s were determined by standard analysis (24). Virus replication was also measured in some assays by a standard reverse transcriptase (RT) procedure (21).

HIV-1 neutralization with rsCD4. rsCD4 was a generous gift from R. W. Sweet (SmithKline and French Laboratories, King of Prussia, Pa.). HIV-1 neutralization by rsCD4 was evaluated in rsCD4 concentration-dependent assays in 96-well plates in quadruplicate by incubation of 100 $TCID_{50}$s of each virus with various dilutions of rsCD4 (20, 10, 5, 2.5, 1.25, 0.6, and 0.3 μg/ml) for 1 h at 37°C prior to infection of PBMC. Virus replication was measured at days 8, 12, and 14 after infection by the ELISA procedure described above and RT activity assays of the cell culture supernatants. Results were evaluable when either the optical density at 592 nm was ≥1.5 or the RT activity of the positive control (without rsCD4) was $\geq 5 \times 10^5$ cpm/200 μl of cell culture supernatant.

Virus purification. Infected cell culture supernatants were assayed for RT activity three times a week, and supernatants with high RT activity (>800 cpm/ml of cell culture supernatant) were cleared of cells and cell debris by centrifugation for 20 min at 1,500 rpm in a Beckman TJ6 followed by centrifugation for 30 min at 10,000 rpm in a Sorvall SS34 at 4°C. After filtration through 0.45-μm-pore-size filters (Nalgene, Rochester, N.Y.), supernatants were ultracentrifuged through a 32% sucrose cushion for 3.5 h at 28,000 rpm at 4°C (SW28 rotor; Beckman Instruments, Palo Alto, Calif.). Pellets were suspended in phosphate-buffered saline (PBS), placed on top of a preformed linear sucrose gradient (16 to 60%), and then ultracentrifuged for 12 h at 36,000 rpm at 4°C (SW41 rotor; Beckman Instruments). Twenty-four 0.5-ml fractions were collected and tested for density and virus content by RT activity and immunoblot procedures. Virus-positive fractions were pooled and further purified by gel exclusion chromatography (37, 41) on 5-ml Sephacryl S-1000 columns (Pharmacia Fine Chemicals, Uppsala, Sweden). Proteins were eluted with PBS, and at least 18 fractions of 4 drops each (approximately 150 μl) were collected and analyzed for virus content by RT and immunoblot procedures.

Immunoblot analysis. Immunoblotting was performed by following the procedure previously published (2, 45). Briefly, virus proteins were separated on sodium dodecyl sulfate (SDS)–10% polyacrylamide gels and transferred to Immobilon-P filters (Millipore Corp., Bedford, Mass.). After incubation in PBS–0.6% polyethylenesorbitan monolaurate (Tween 20; Sigma) for 1 h, the filters were incubated with goat anti-gp120 antibody (donated by Chiron Corp., Emeryville, Calif.) overnight at 4°C. After two washes, the filters were incubated with horseradish-labeled protein G (Bio-Rad Laboratories, Richmond, Calif.) for 2 h at 21°C. After three subsequent washes, filters were developed with H_2O_2–3′,3′-diaminobenzidine (Sigma) in PBS. Some of the filters were cut in half; one half was used to assess the molecular range from 300 to 30 kDa, and the other half denoted the range from 30 to 15 kDa. They were incubated with anti-gp120 antiserum (upper half) or anti-HIV-1 patient serum (lower half) for separate detection of the envelope gp120 and core p25 proteins.

HIV-1 gp120 cleavage assay. Purified HIV-1 virus preparations (15 μl) in PBS were incubated with 1 μg of rsCD4 at 37°C for 0.5 to 8 h. Alternatively, 15-μl virus preparations were incubated with various amounts of rsCD4 (0.01 to 2 μg) at 37°C for 8 h. Blocking of gp120 cleavage with monoclonal antibodies (MAb) was assessed by incubating the virus preparations with MAb and rsCD4 at 37°C for 8 h. After incubation, virus preparations were assayed for gp120 as well as cleavage by immunoblot procedures. The protease inhibitors aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), soybean trypsin inhibitor, and EDTA (Sigma) were used in effective concentrations as described previously (18).

MAb. Human anti-V3 loop MAb 257-2D and 268-11D were gifts from Susan Zolla-Pazner (16). Antibody 257-2D recognizes KRIHI, which is to the left of the conserved tip of the V3 loop, and antibody 268-11D is reactive with HIGPGR, which is at the tip of the V3 loop. Both antibodies are able to neutralize HIV-1_{SF2} (16, 28a). MAb L77 anti-CD4 (Leu-3a epitope) was provided by Becton Dickinson (San Jose, Calif.). MAb OKT4 (Ortho Diagnostics Inc., Heidelberg, Germany), which is unable to prevent HIV-1 infection in vitro (53), was used as a control antibody.

RESULTS

Virus purification. HIV-1 virions were obtained from HIV-1_{SF13}-infected cell culture supernatants by sucrose gradient banding and gel exclusion chromatography on Sephacryl S-1000 columns (Fig. 1). The resulting virus preparations were found to be highly pure as analyzed by SDS-polyacrylamide gel electrophoresis and silver staining of the gels and contained infectious virus as tested by infection of PBMC with aliquots of the fractions (data not shown). In some highly purified virus preparations small amounts of two breakdown products of gp120 with molecular sizes of approximately 70 and 50 kDa could be detected (see lanes 1, Fig. 4 and 5). Even with further purification, e.g., a second run on a Sephacryl S-1000 column or a second sucrose gradient purification, it was not possible to remove those breakdown products from the virus preparation, suggesting that they are associated with the virion.

The content of gp120 in comparison with that of the core p25 protein (gp120/p25 ratio), estimated by immunoblot procedures, was low in SF2, SF13, and SF33 preparations. In contrast, the SF162 and SF162mc viruses yielded large amounts of gp120, suggesting that spontaneous shedding of gp120 from the virions is reduced in the last two virus preparations (see Fig. 3C, 4, and 5).

Neutralization of HIV-1 infection by rsCD4. Infection of PBMC with 100 $TCID_{50}$s of the HIV-1 strains SF2, SF2mc, SF33, SF33mc, and SF13, preincubated with rsCD4 at 37°C, resulted in inhibition of infection, measured by ELISA and RT activity assay of the cell culture supernatant. Most HIV-1 isolates used in this study were neutralized by rsCD4 concentrations between 0.15 and 0.3 μg/ml. The HIV-1_{SF162} and HIV-$1_{SF162mc}$ strains, which readily infect primary pe-

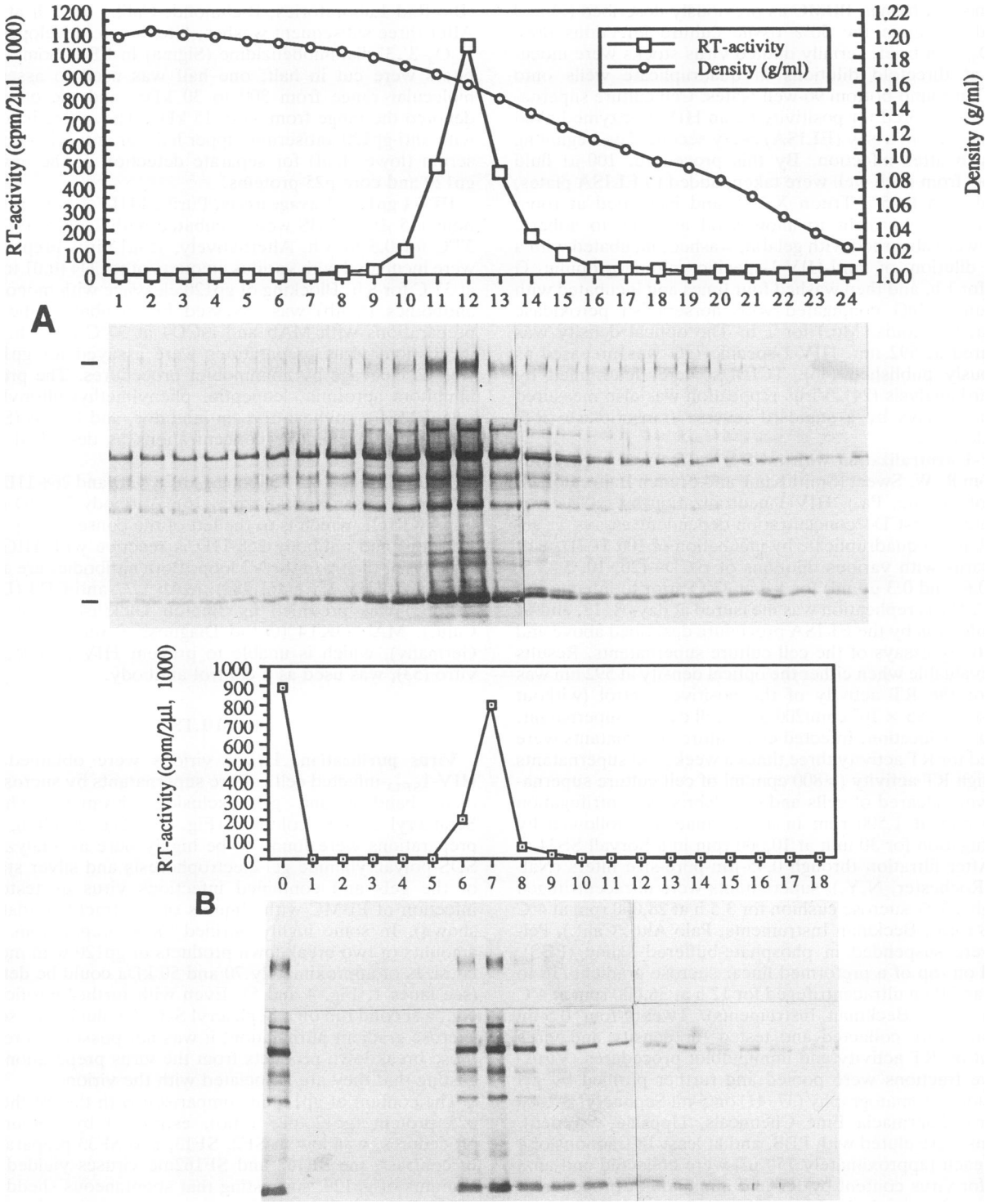


FIG. 1. Purification of HIV-1_{SF13} by sucrose gradient banding and gel exclusion chromatography on Sephacryl S-1000 columns. (A) Culture supernatants from cells infected with HIV-1_{SF13} were ultracentrifuged through a sucrose cushion, and resuspended pellets were subjected to a preformed linear sucrose gradient (16 to 60%) followed by ultracentrifugation. Fractions were collected, and aliquots were analyzed for density and RT activity and by immunoblot procedures. Each lane of the immunoblot analysis represents an aliquot of one fraction. Immunoblots were stained with goat anti-gp120 antibodies (upper half) and with pooled anti-HIV-1 IgG (lower half) purified from HIV-1-positive serum. Lines on the left indicate gp120 and p25. (B) The HIV-1-positive fractions were further purified by gel exclusion chromatography on Sephacryl S-1000 columns. Aliquots of collected fractions from the above-described experiment were analyzed for RT activity and by immunoblot procedures. Immunoblots were stained with goat anti-gp120 antibodies (upper half) and with anti-HIV-1 IgG (lower half) as described above. Lane 0, aliquot of virus preparation before Sephacryl S-1000 chromatography purification; lanes 1 to 18, aliquots of fractions after Sephacryl S-1000 chromatography.

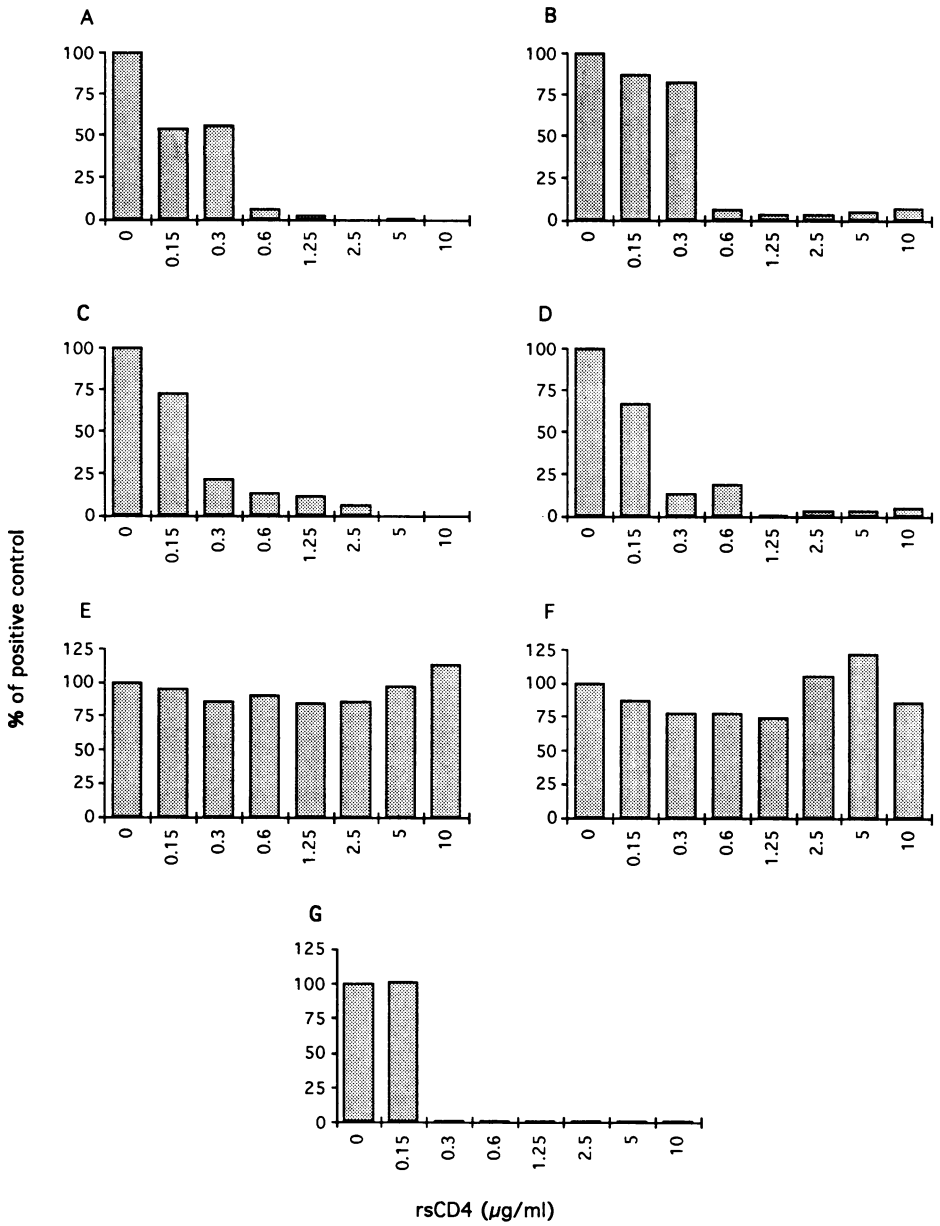
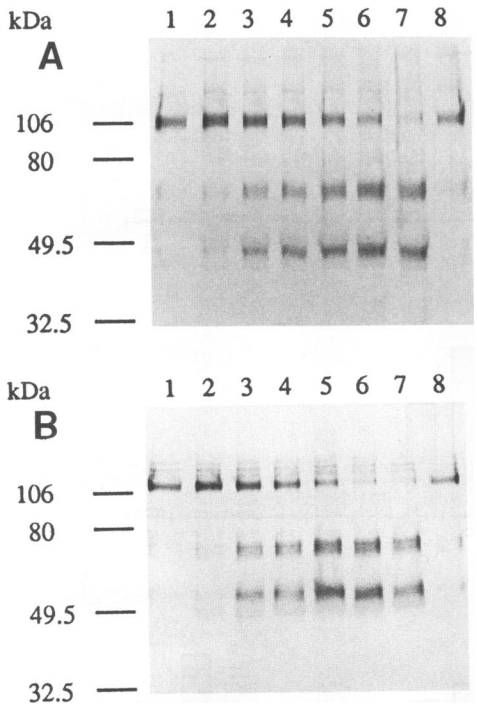


FIG. 2. Blocking of PBMC infection with HIV-1 by preincubation with rsCD4. SF2 (A), SF2mc (B), SF33 (C), SF33mc (D), SF162 (E), SF162mc (F), and SF13 (G) (100 $TCID_{50}$s) were incubated with the indicated dilutions of rsCD4 in quadruplicate for 1 h at 37°C prior to infection of PBMC. Virus replication was measured at days 8, 12, and 14 after infection by ELISA and RT activity assay in the cell culture supernatants. Assays were evaluated when either the RT activity of the positive control (without rsCD4) was $\geq 2.5 \times 10^5$ cpm/200 μl of cell culture supernatant or the optical density at 592 nm was ≥1.5. Values represent the averages of quadruplicate measurements in two independent experiments.

ripheral blood macrophages and do not replicate efficiently in T-cell lines (5), were relatively resistant to neutralization by rsCD4. They were not inhibited by 10 μg of rsCD4 per ml. Molecularly cloned and biologically active HIV-1 strains SF2 and SF33 were inhibited by the same concentration of rsCD4 as the corresponding HIV-1 wild-type strain (Fig. 2). Thus, their reactivity resembled that of the uncloned parental HIV-1 strain.

Incubation of virus preparations with rsCD4 and analysis by immunoblot procedures. In the study of the effects of the CD4-HIV envelope interaction, aliquots of the purified virus preparations were incubated with rsCD4 for 0.5 to 8 h. In other experiments, various rsCD4 concentrations were used for 8 h at 37°C. Representative results, analyzed by immunoblot procedures, were obtained by time-dependent assays performed with SF33mc, SF2mc, and SF162mc and one rsCD4 concentration-dependent assay performed with SF2 (Fig. 3 and 4). Incubation of purified virus preparations with rsCD4 leads, without addition of an exogenous protease, to cleavage of full-length gp120 into two protein species of approximately 70 and 50 kDa. The cleavage reaction is highly specific, since there is no additional cleavage of the



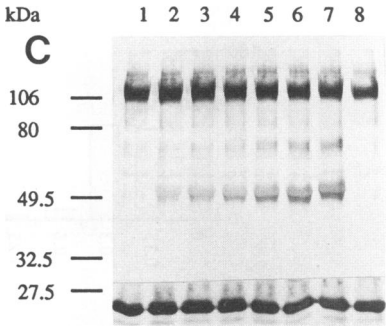


FIG. 3. Examples of purified virus preparations incubated with rsCD4 over time and analyzed by immunoblot procedures. (A) HIV-1_{SF33mc}; (B) HIV-1_{SF2mc}; (C) HIV-1_{SF162}. Lanes 1, virus preparations without rsCD4, incubation for 0 h; lanes 2, virus preparations with 1 μg of rsCD4, incubation for 0 h; lanes 3 to 7, virus preparations with 1 μg of rsCD4, incubation for 0.5, 1, 2, 4, and 8 h; lanes 8, virus preparations without rsCD4, incubation for 8 h.

breakdown products, even with incubation for 8 h. Cleavage occurs only in the presence of at least 0.06 μg of rsCD4 and is not induced by incubation of the virus preparations at 37°C for 8 h alone (Fig. 3, lanes 8; Fig. 4, lane 12). These two cleavage-derived proteins were the same size as those found in small amounts with some purified HIV-1 strains (Fig. 4, 5). To rule out the possibility that the rsCD4 preparations used in these experiments were contaminated with a protease, purified SF2 recombinant gp120 (V3 loop sequence: IYIGPGRAFHTTGRIIGDIRKA) served as a control and was not cleaved after incubation with rsCD4 for over 8 h. Moreover, rsCD4 from Pharmacia Inc. at the same concentration as the rsCD4 from SmithKline and French Laboratories also induced cleavage of virion-associated gp120 but not of purified SF2 recombinant gp120 (data not shown).

The extent of gp120 cleavage is dependent on the HIV-1 strain used: the gp120 of SF162 is poorly cleaved, whereas the gp120's of the SF2, SF13, and SF33 strains are almost completely digested, although a refractory unclipped portion of gp120 was always detectable after an incubation period of 8 h (Table 1 and Fig. 3). This phenomenon was reproducible with at least four independently purified virus preparations of each HIV-1 strain used in this study.

To test whether the cleavage phenomenon is dependent on the cell type in which the viruses were propagated, HIV-1 strains were purified from cell culture supernatants of infected HuT 78, Jurkat, SupT1, MT-4, and Molt4/8 T-cell lines as well as, in the case of SF33, from SK-N-MC, a CD4-negative glioma cell line (6). As summarized in Table 1, there was no substantial difference in the extent of gp120 cleavage of SF2, SF2mc, SF33, SF33mc, and SF13, regardless of the cell line in which the virus was propagated, including the glioma cell line SK-N-MC (for SF33). SF162 and SF162mc cannot replicate efficiently in T-cell lines (5); therefore, virus purification was possible only from infected PBMC culture supernatants.

Blocking of cleavage by MAb. Cleavage of virion-associated gp120 can be blocked by MAb L 77 (rsCD4/MAb ratio: 0.1 μg/0.05 μg) (Fig. 5, lane 6), which binds to the HIV-1 gp120-binding site of the CD4 molecule (1) and blocks HIV-1 infection in vitro (data not shown). The MAb OKT4, used in the same concentration as L 77, served as a control and was unable to prevent proteolysis. These data further suggest that binding of rsCD4 to gp120 is necessary to induce proteolysis of virion-associated gp120.

To determine the cleavage site in the virion-associated gp120, a purified SF2mc preparation and an SF33mc virus preparation were incubated with rsCD4, MAb 257-2D, which recognizes KRIHI on the left of the conserved tip of the V3 loop, and MAb 268-11D, which reacts with HIGPGR at the tip of the V3 loop. Figure 6A shows that MAb 268-11D is able to block cleavage of virion-associated SF2 gp120 with 0.05 μg/15 μl of virus preparation, whereas MAb 257-2D (Fig. 6B) cannot prevent proteolysis in a concentration of 0.1 μg/15 μl of virus preparation. Virion-associated SF33 gp120 (V3 loop sequence at the tip: TSGPGK) served as a control, and proteolysis was not inhibited with MAb 257-2D and 268-11D (data not shown). These data indicate that MAb 268-11D protected the putative proteolytic cleavage site at the tip of the V3 loop from cleavage (12, 20, 29).

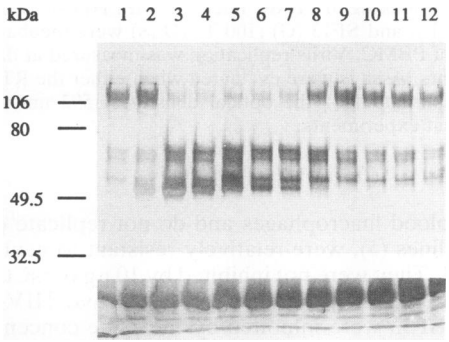


FIG. 4. Immunoblot analysis of a purified HIV-1_{SF2} preparation incubated with decreasing rsCD4 concentrations. Lane 1, SF2 without rsCD4, incubation for 0 h; lane 2, SF2 with 1 μg of rsCD4, incubation for 0 h; lanes 3 to 11, SF2 with 1, 0.5, 0.25, 0.125, 0.06, 0.03, 0.015, 0.006, and 0.003 μg of rsCD4, incubation for 8 h at 37°C; lane 12, SF2 without rsCD4, incubation for 8 h at 37°C.

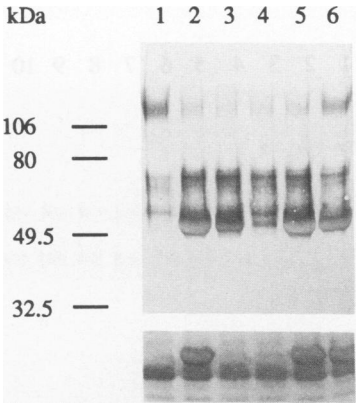


FIG. 5. Immunoblot analysis of purified HIV-1_{SF2mc} preparations incubated with rsCD4, OKT4, and anti-CD4 MAb. Lane 1, SF2mc, incubation for 0 h; lane 2, SF2mc with 0.1 μg of rsCD4 and 0.05 μg of OKT4, incubation for 8 h at 37°C; lane 3, SF2mc with 1 μg of rsCD4, incubation for 8 h at 37°C; lane 4, SF2mc with 0.1 μg of rsCD4, incubation for 8 h at 37°C; lane 5, SF2mc with 1 μg of rsCD4 and 0.05 μg of L 77 (Leu-3a epitope of CD4), incubation for 8 h at 37°C; lane 6, SF2mc with 0.1 μg of rsCD4 and 0.05 μg of L 77, incubation for 8 h at 37°C. Additional bands in lanes 2, 5, and 6 are heavy and light chains of MAb detected by horseradish peroxidase-conjugated protein G, which was used as the second antibody.

Inhibition of proteolysis with protease inhibitors. HIV-1-induced syncytium formation can be inhibited by several serine protease inhibitors (29). Consequently, we tried to inhibit rsCD4-induced proteolysis of SF2mc and SF33mc gp120s with protease inhibitors. The proteolytic activity in the virus preparations could not be blocked completely, but partial elimination of proteolysis was observed with aprotinin, leupeptin, PMSF, and EDTA at concentrations of 2 μg/ml, 2 μg/ml, 50 μg/ml, and 1 mM, respectively. Soybean trypsin inhibitor had no measurable effect on gp120 cleavage (Table 2).

DISCUSSION

These studies were conducted to assess the effects of HIV-1 interaction with the CD4 molecule. They indicated that proteolysis of gp120 can be demonstrated with all of the HIV-1 strains tested, regardless of the cell type that propagated the virus. The extent of proteolytic cleavage was substantially lower with the monocytotropic HIV-1 strain SF162 than with the T-cell line-tropic strains (Fig. 3; Table 1). In support of these biological differences was the relative sensitivity of these HIV-1 strains to rsCD4 neutralization. The T-cell line-tropic strains were neutralized by low concentrations of rsCD4, whereas the monocytotropic HIV-1 strains, both the wild-type SF162 and SF162mc, were relatively resistant to rsCD4 neutralization.

The mechanism of rsCD4 neutralization of HIV-1 strains is presumably a function of rsCD4-induced gp120 shedding (19, 31, 40, 41). This induction of gp120 loss is reduced with many primary HIV-1 isolates associated with a decreased binding affinity of gp120 to the CD4 molecule (39). Our results suggest that rsCD4 resistance might also reflect less rsCD4-induced proteolysis of virion-associated gp120 in comparison with that in the readily neutralizable HIV strains. In this regard, the density of virion-associated gp120 is definitely greater on the HIV_{SF162} strains than on the SF2 and SF33 strains, as estimated from the gp120-p25 content by immunoblot analysis (Fig. 3C, 4, and 5).

These results suggest that a gp120-CD4 interaction is necessary to bring about a conformational change in the gp120, thereby exposing a previously protected domain of gp120 to proteolytic cleavage. Thus, the susceptibility of cells to HIV-1 infection could be determined by expression of an appropriate protease. This concept is in accordance with the observation that addition of thrombin to virion-associated gp120 results in cleavage at the V3 loop when rsCD4 is added (12, 49, 52). Moreover, conserved amino acid sequence motifs in the V3 loop of HIV-1 strains resemble a proteolytic cleavage site (12, 20, 29), and the V3 loop interacts with proteinase-like molecules at the surface of the human T-cell line Molt4/8 cell (43). In addition, a membrane-bound serine protease, TL2, which binds HIV-1-gp120 and is inhibited by V3 loop peptides (20, 25, 26) has been purified from Molt4/8 cells (27).

Our results were supported by results of studies with an anti-V3 loop human MAb (268-11D) that is directed against a predicted cleavage site in the V3 loop and that can neutralize HIV-1_{SF2mc} in vitro (16). The finding that it prevents rsCD4-mediated cleavage of gp120 is in agreement with the hypothesis that antibodies to the V3 loop could mediate neutralization by preventing the gp120 proteolysis needed for virus

TABLE 1. HIV-1 strains tested for cleavage of virion-associated gp120

HIV-1 strain	rsCD4 sensitivity (μg/ml)	Cleavage[a] of gp120 associated with virus propagated on:						
		PBMC	T-cell line:					$CD4^-$ glioma cell line SK-N-MC
			Hut 78	Jurkat	Molt4/8	SupT1	MT-4	
SF33	0.15	+++[b]	+++	+++	+++	+++	+++	+++
SF33mc	0.15	+++	+++	ND	+++	+++	+++	NR
SF2	0.3	+++	+++	ND	+++	+++	ND	NR
SF2mc	0.3	+++	ND	ND	+++	ND	ND	NR
SF13	0.15	+++	ND	ND	+++	+++	ND	NR
SF162	>10	+[b]	—	—	—	—	—	—
SF162mc	>10	+	—	—	—	—	—	—

[a] Proteolytic cleavage of gp120 was induced by incubation of purified virus preparations with rsCD4 for 8 h at 37°C. ND, not done; NR, no replication; +++, proteolysis of full-length gp120 is almost complete after 8 h of assay, and very little full-length gp120 can be detected (Fig. 2A and B); +, proteolysis of gp120 is clearly detectable, but the decrease of full-length gp120 is low (Fig. 2C); —, does not replicate in these established cell lines.

[b] The extent of proteolysis was estimated by immunoblot procedures from the quantity of full-length gp120 and the quantity of breakdown products (70 and 50 kDa) after incubation of virus preparations with 1 μg of rsCD4 for 8 h at 37°C. The rating is based on at least four independently purified and analyzed virus preparations.

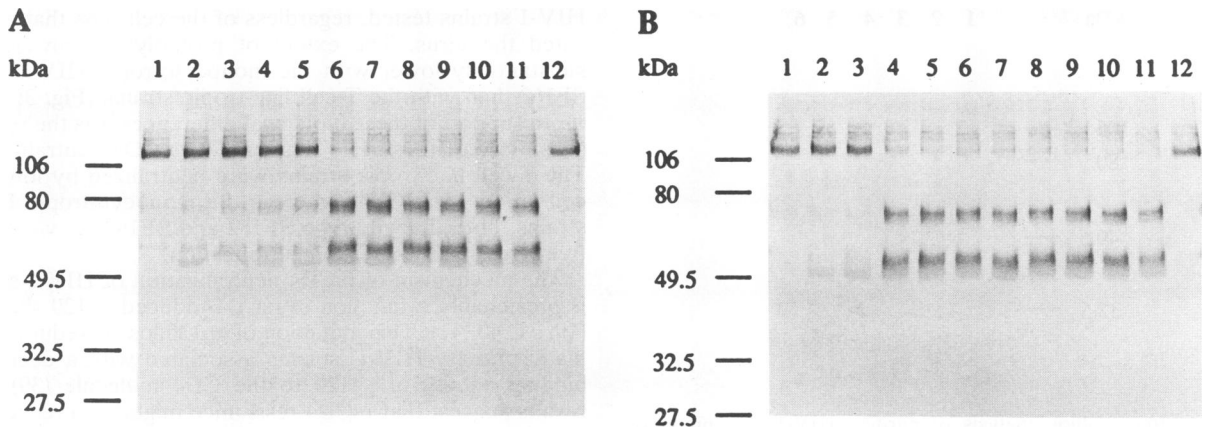


FIG. 6. Immunoblot analysis of purified HIV-1_{SF2mc} incubated with rsCD4 and anti-V3 loop MAb. SF2mc preparation was incubated for 8 h at 37°C with rsCD4 and MAb 268-11D (A) and MAb 257-2D (B). Lanes 1, SF2mc without rsCD4 and without MAb, incubation for 0 h; lanes 2, SF2mc with 1 μg of rsCD4, incubation for 0 h; lanes 3, SF2mc with 1 μg of rsCD4 and 0.1 μg of MAb, incubation for 0 h; lanes 4 to 11, SF2mc with 1 μg of rsCD4 and 0.1, 0.05, 0.025, 0.0125, 0.006, 0.003, 0.0015, and 0.0007 μg of MAb, incubation for 8 h at 37°C; lanes 12, SF2mc with 0.1 μg of MAb, incubation for 8 h at 37°C.

infection (52). The inability to inhibit completely this proteolytic cleavage of gp120 with several protease inhibitors could reflect not knowing the specific requirements for complete inhibition of an unknown and uncharacterized protease.

The importance of CD4 in the process was demonstrated by the lack of a protease associated with this molecule and the blocking of cleavage of virion-associated gp120 by MAb L 77 (Fig. 5), which binds to the HIV-1 gp120 binding site for the CD4 molecule (1).

The origin and nature of the proteolytic activity found in the virus preparations remain unclear. Although the virus was extensively purified by sucrose gradient banding and gel exclusion chromatography (37, 41) on Sephacryl S-1000 columns (Fig. 1), we cannot exclude the possibility that a cellular, membrane-bound protease was copurified. Since it has been shown that cellular membrane proteins can be included in the viral membrane (15), the proteolytic activity could be associated with the virions but difficult to eliminate without destroying the integrity of the HIV-1 particles. Alternatively, the results could reflect an autoproteolytic activity of gp120. These possibilities are under study.

Taken together, these studies demonstrate for the first time that proteolysis of HIV-1 virion-associated gp120 is induced, without addition of an exogenous protease, by incubation with rsCD4. The process is both dose and time dependent. Since it is very likely that proteolysis of HIV-1 gp120 also occurs upon binding to the CD4 on the cell surface, the biological importance of these findings needs to be evaluated.

TABLE 2. Inhibition of proteolytic cleavage of gp120 with protease inhibitors[a]

Inhibitor[b]	Concn (μg/ml or mM)	Mean inhibition ± SD (%)
Aprotinin	2	53.7 ± 4.5
	0.2	19.4 ± 3.3
	0.02	0
Leupeptin	2	22.7 ± 4.6
	0.2	0
	0.02	0
PMSF	10	4.5 ± 2.1
	1	7.9 ± 4.1
	0.1	0
EDTA	2	29.4 ± 4.9
	0.2	27.2 ± 3.2
	0.02	0

[a] Induced proteolytic cleavage of gp120, without addition of inhibitor, resulted in 79.5% (±3.2%) cleavage of input gp120. The extent of inhibition of cleavage was estimated by measuring the density of each gp120 band on the immunoblot with a densitometer. The averages from two independent experiments are given. The integral of the peak of each band was calculated, and the degree of inhibition was determined as follows: inhibition (%) = (inhibited reaction − residual gp120)/(input virus − residual gp120) × 100, where inhibited reaction is the area of the peak of gp120 band from the inhibited reaction, residual gp120 is the area of the peak of gp120 band from the residual gp120 after incubation with rsCD4 for 8 h, and input virus is the area of the peak of gp120 band from input virus (uncleaved because it was not incubated with rsCD4).

[b] Aprotinin and leupeptin concentrations are in micrograms per milliliter; PMSF and EDTA concentrations are in millimolars. Soybean trypsin inhibitor caused no inhibition at 0.5, 5, or 50 μg/ml.

ACKNOWLEDGMENTS

This research was supported by grants from the National Institutes of Health (UOI A1 26471 and ROI AI 29394). A.W. is a fellow of the AIDS-Stipendienprogramm of the Deutsches Krebsforschungszentrum, Heidelberg, Germany.

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