# The extracellular processing of HIV-1 envelope glycoprotein gp160 by human plasmin

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**Abstract Cleavage of the envelope glycoprotein precursor gp160 of HIV-1 is a prerequisite for the infectivity of HIV-1, and occurs at least in part before gp160 reaches the cell surface. Kexin/subtilisin-related endopeptidases are proposed enzyme candidates for this intracellular processing. In this study, we reveal the possibility that plasminogen binds to the cell surface and part of gp160 escaping intracellular processing is cleaved by plasmin extracellularly. Plasmin cleaves gp160 precisely at the C-terminal arginine residue of gp120, and the processing is effectively inhibited by an analogue peptide of the cleavage motif (RXK/RR) and by plasmin inhibitors.**



*Key words:* Plasmin; gp160; Processing; Human immunodeficiency virus

## 1. Introduction

The processing of the HIV-1 envelope glycoprotein gp160 has been proposed to be an essential step in the production of infectious viral particles [1,2]. As in the case of other retroviruses, the envelope glycoprotein of HIV-1 is synthesized as a precursor polypeptide (gp160) that is cleaved by a protease at a site in the multi-basic motif (RXK/RR) between gp120 and gp41. To date, kexin/subtilisin-related endopeptidases and viral envelope glycoprotein maturase (VEM), which are proprotein convertases in the Golgi apparatus, have been shown to be processing proteases for HIV-1 gp160 [3–10], and inhibitors of these enzymes, candidates for the treatment of AIDS, suppress multiple cycles of HIV-1 replication, thus inhibiting the spread of HIV. However, part of gp160 escapes intracellular processing and is transported to the cell surface and/or secreted into the extracellular milieu [11]. In this regard, we hypothesized a cell-surface proteolytic activation mechanism for gp160, and searched for an extracellular processing protease that converts gp160 to gp120 and gp41. The results show that only plasmin among the extracellular trypsin-like proteases tested is a processing enzyme for gp160. Plasminogen in serum binds to the surface of human T cells and is converted to plasmin. This processing of gp160 is inhibited by the plasmin inhibitors aprotinin and HI-30, and by a substrate analogue, decanoyl (dec)-RVKR-chloromethyl ketone (cmk). Judging from these results, unprocessed gp160 on the cell surface, which has escaped intracellular processing and/or has resulted from the inhibition of intracellular processing by inhibitors of kexin/subtilisin-related endopeptidases and VEM, is converted to gp120 and gp41 on the cell surface by human plasmin.

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*Abbreviations:* HIV-1, human immunodeficiency virus type 1; VEM, viral envelope glycoprotein maturase; AIDS, acquired immune deficiency syndrome; dec, decanoyl; cmk, chloromethyl ketone; mAb, monoclonal antibody; Boc, *N-tert*-butyloxycarbonyl; MCA, 4-methylcoumaryl-7-amide; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; uPA, urokinase-type plasminogen activator; PBS, phosphate-buffered saline; BSA, bovine serum albumin; FCS, fetal calf serum; FITC, fluorescein isothiocyanate

## 2. Materials and methods

### 2.1. Materials

Glycosylated recombinant HIV-1(IIIB) gp160, which is produced in insect cells using the baculovirus expression system, was purchased from MicroGeneSys, Inc. (West Haven, CT, USA). Anti-gp41 monoclonal antibody (mAb) was from Daiichi Pure Chemicals (Tokyo, Japan). Anti-bovine plasminogen antibody, which reacts with both plasminogen and plasmin, was from American Diagnostica (Greenwich, CT, USA). Human plasmin was purchased from Boehringer Mannheim Biochemicals (Mannheim, Germany). Urokinase and protease inhibitors, aprotinin, $\alpha_1$-antitrypsin, and chymostatin were from Sigma Chemical Co. (St. Louis, MO, USA). Leupeptin, *N-tert*-butyloxycarbonyl (Boc)-LKR-4-methylcoumaryl-7-amide (MCA), and Boc-RVRR-MCA were from the Peptide Institute (Osaka, Japan). Inhibitors of furin, dec-RVKR-cmk and dec-FAKR-cmk, which are analogue peptides of the multi-basic cleavage motif RXK/RR found in various prohormones and retrovirus envelope glycoprotein precursors, were a gift from Dr. H.D. Klenk, University of Marburg (Germany). HI-30 was a gift from Prof. H. Fritz, University of Munich (Germany). Tryptase from human lung was purified to homogeneity by the method of Smith et al. [12]. Thrombin and factor Xa from bovine plasma were purified by the method of Hashimoto et al. [13].

### 2.2. Cell culture

Molt-4, clone 8 cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) or in serum-free GIT medium (Wako, Osaka, Japan) at 37°C with 5% $CO_2$. The cells in the early stationary phase after five passages were collected by centrifugation, washed twice with saline and used for subcellular fractionation.

### 2.3. Preparation of membrane fraction of human T cells

Molt-4, clone 8 cells ($1\times10^8$) cultured in RPMI 1640 medium supplemented with 10% FCS or in serum-free medium were homogenized with 10 volumes of 10 mM Tris-HCl buffer, pH 7, containing 135 mM NaCl without $MgCl_2$ in a Potter-Elvehjem homogenizer at 0°C. The homogenate was centrifuged at $12\,000\times g$ for 60 min to remove nucleus, mitochondria, lysosomes and a large portion of microsomes, and the supernatant was further centrifuged at $105\,000\times g$ for 1 h. Under the conditions used, the precipitate is mainly composed of plasma membrane. The precipitate was then suspended in 2 ml of 0.1 M Tris-HCl buffer, pH 8.5, homogenized and centrifuged at $105\,000\times g$ for 30 min. The resulting supernatant was used for the analysis of gp160 processing.

### 2.4. Conversion of gp160 to gp120 and gp41

The gp160 (1.0 μg) was incubated with various proteases (1.35pmol)


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for 7.5–30 min at 37°C under optimal reaction conditions for each enzyme in 20 μl of buffer (plasmin and urokinase, 0.1 M Tris-HCl buffer, pH 7.0; human lung tryptase, 0.1 M Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 2.5 μg/ml heparin; thrombin, 50 mM Tris-HCl buffer, pH 7.8, containing 0.1 M NaCl and 20 mM EDTA; and factor Xa, 50 mM Tris-HCl buffer, pH 7.5, containing 0.15 M NaCl and 10 mM $CaCl_2$). The enzyme/substrate ratios were 1:10. For assays of gp160 processing by membrane fraction, aliquots of the membrane fraction of human T cells (80 μg) were incubated with 0.1 μg of $^{125}$I-labeled gp160 prepared with Iodo-Beads [10] in 20 μl of 100 mM Tris-HCl, pH 7, and then incubated at 37°C for 5–10 h. The reactions were terminated by cooling on ice and adding 6×SDS-PAGE sample loading buffer [14]. The reaction mixtures were then subjected to SDS-PAGE under reducing conditions and analyzed by Western immunoblotting.

*2.5. Electrophoresis and Western immunoblotting*

SDS-PAGE (4–20% gradient gel; Daiichi Pure Chemicals) was performed at room temperature by the method of Laemmli [14]. The SDS-PAGE high range standards (Bio-Rad) used as molecular weight markers were myosin (213 kDa), β-galactosidase (119 kDa), bovine serum albumin (BSA) (83 kDa), and ovalbumin (47 kDa). For Western blot analysis, the reaction samples were subjected to SDS-PAGE, and then transferred electrophoretically to an Immobilon transfer membrane (Millipore); excess sites were blocked with 3.5% skim milk in PBS. The membrane was then probed with a mAb against gp41, 1:200, in 1% BSA in Tris-buffered saline (TBS: 20 mM Tris-HCl buffer, pH 7.5/0.5 M NaCl) overnight at 4°C. After extensive washing with TBS, the membrane was reproved with anti-mouse IgG conjugated with horseradish peroxidase (Amersham, Amersham, UK), 1:1000, in 3.5% skim milk in PBS for 1 h at room temperature. The bound antibodies were detected with ECL Western blotting detection reagents (Amersham, Amersham, UK) according to the manufacturer's instructions.

*2.6. Amino acid sequences*

The N-terminal amino acid sequences of the proteolytic products were determined by the microsequencing method with an Applied Biosystems 492 model gas-phase sequencer/140C high performance liquid chromatography system after the reaction samples had been electrophoretically transferred to a ProBlot membrane (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

*2.7. Inhibition of gp160 processing*

The gp160 (1.0 μg) was incubated with plasmin (1.35 pmol) in reaction buffer in the presence or absence of protease inhibitors (10 μM) for 30 min at 37°C, and the reaction was terminated by cooling on ice and adding 6×SDS-PAGE sample loading buffer [14]. The samples were then subjected to SDS-PAGE under reducing conditions and analyzed by Western immunoblotting.

*2.8. Flow cytometry*

Molt-4, clone 8 cells ($1\times10^6$) cultured in RPMI 1640 medium supplemented with 10% FCS or in serum-free GIT medium were incubated with or without anti-bovine plasminogen antibody, 1:10, in 3.5% skim milk in PBS for 1 h at 4°C followed by anti-goat IgG conjugated with FITC, for 30 min as described [15]. They were then washed three times with PBS by centrifugation ($300\times g$ for 1 min), resuspended in 0.6 ml of 1% paraformaldehyde in saline, and analyzed with a EPICS Elite ESP model cell sorter (Coulter).

## 3. Results and discussion

### *3.1. Selective conversion of gp160 to gp120 and gp41 by human plasmin*

We examined the effects of various extracellular trypsin-type proteases on the processing of gp160 (Fig. 1A). Among the extracellular processing proteases tested, human plasmin (lane 2) specifically converted gp160 to gp120 and gp41. The other extracellular proteases and secretory proteases tested, including human lung tryptase, thrombin, factor Xa, and urokinase, did not give gp41. We then examined the time course

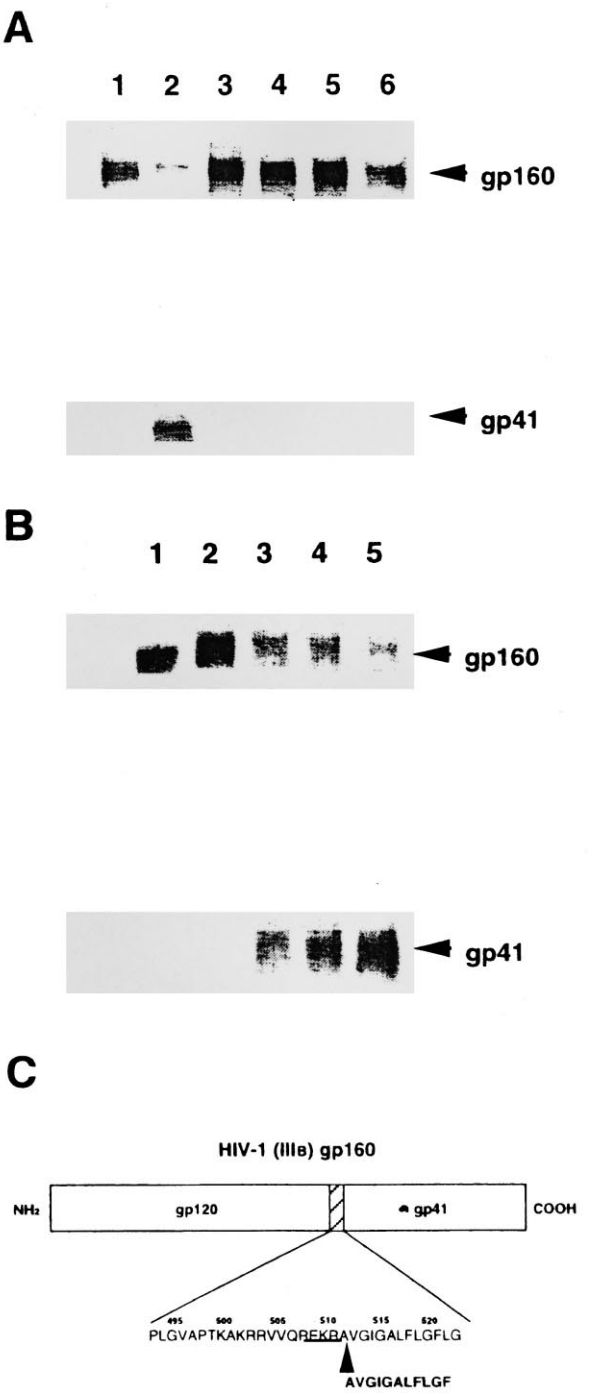


Fig. 1. Processing of gp160 by extracellular trypsin-type proteases. A: Effect of various extracellular proteases on gp160. gp160 (1.0 μg) (lane 1) was incubated for 30 min with various extracellular and/or secretory proteases (1.35 pmol) including plasmin (lane 2), human lung tryptase (lane 3), thrombin (lane 4), factor Xa (lane 5), and urokinase (lane 6). The reaction products were separated by SDS-PAGE followed by detection by Western immunoblotting. B: Time course of gp160 processing by human plasmin. gp160 (1.0 μg) (lane 1) was incubated with plasmin (1.35 pmol) for 0 (lane 2), 7.5 (lane 3), 15 (lane 4), or 30 min (lane 5), and then subjected to SDS-PAGE and Western immunoblotting. C: The N-terminal sequence of the gp41 generated by plasmin was analyzed as described in Section 2, and is indicated in bold letters. The aligned amino acid sequence is the sequence of HIV-1(IIIB) gp160 [22]. The multi-basic cleavage motif of gp160 is underlined.

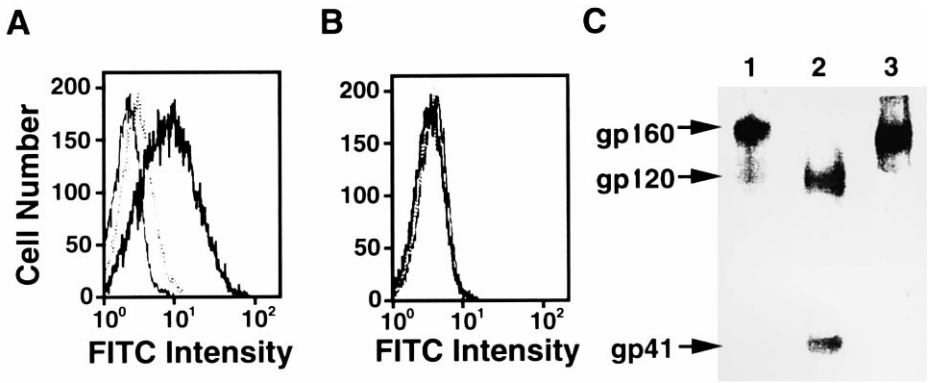


Fig. 2. Binding of plasminogen to the cell surface (A and B) and processing of gp160 by the membrane fraction (C). A and B: Molt-4, clone 8 cells were cultured in RPMI 1640 medium supplemented with 10% FCS (A) or in serum-free GIT medium (B) and bound plasminogen and/or plasmin on these cells after five passages was detected by anti-bovine plasminogen antibody and FITC-labeled anti-goat antibody under the conditions given in Section 2. Cells were then analyzed by flow cytometry. The thin line indicates control cells treated with buffer alone, while the thick line refers to cells treated with anti-plasminogen antibody and FITC-labeled second antibody. The dotted line indicates cells treated with FITC-labeled second antibody alone. C: Membrane fraction (80 μg) of cells cultured in RPMI 1640 medium supplemented with 10% FCS (lane 2) and that of the cells in serum-free GIT medium (lane 3) were incubated with 0.1 μg of $^{125}$I-labeled gp160 (lane 1) at 37°C for 10 h under the conditions given in Section 2. The reactions were then subjected to SDS-PAGE (4–20% gradient gel).

of gp160 processing by plasmin, and the products were analyzed by Western immunoblotting with a mAb against gp41 (Fig. 1B). As expected, the amount of gp160 decreased in a time-dependent manner along with an increase in gp41.

To determine the cleavage site of gp160 by human plasmin, the N-terminal amino acid sequence of the protein with a molecular mass of 41 kDa, which corresponds to gp41, was analyzed (Fig. 1C). Although the protein band was diffuse because it is a glycoprotein, a single amino acid sequence was detected without contamination. The sequence of the 41 kDa fragment revealed A(4.6)-V(4.5)-G(4.2)-I(3.8)-G(4.1)-A(3.9)-L(3.6)-F(3.2)-L(3.5)-G(3.3)-F(3.1) (the values in parentheses are the yields in pmol). This indicates that the $R^{511}$–$A^{512}$ bond on the C-terminal side of the multi-basic cleavage motif $R^{508}EKR^{511}$ between gp120 and gp41 is selectively cleaved by human plasmin. Another sequence of clustered basic amino acids, $K^{502}RRVV^{506}$, was found not to be susceptible to this enzyme. This site cleaved by human plasmin is the same site as that cleaved by kexin/subtilisin-related endopeptidases and VEM [4–6,10]. This finding is supported by our previous data that plasmin exhibits similar $K_m$ values for a synthetic plasmin substrate (Boc-LKR-MCA) ($K_m = 4.2 \times 10^{-4}$ M) and a synthetic kexin/furin substrate (Boc-RVRR-MCA) ($K_m = 4.8 \times 10^{-4}$ M) [16]. Taken together, the results indicate that the consensus cleavage motif of gp160, $R^{508}EKR^{511}$, is recognized by human plasmin, and that the $R^{511}$–$A^{512}$ bond is cleaved by the enzyme.

To determine the biological activity of gp120 produced after the processing, we analyzed the affinity constant for the interaction between gp120 and soluble CD4 as described [17]. Scatchard analyses revealed a single class of binding with an apparent dissociation constant ($K_d$) of 8.3 nM for soluble CD4 and this value was comparable to that reported [18].

### 3.2. *Binding of plasminogen to the cell surface and conversion of gp160 to gp120 and gp41 by the membrane fraction*

We next attempted to directly detect binding of plasminogen to the membrane of Molt-4, clone 8 cells cultured in RPMI 1640 medium supplemented with 10% FCS. By fluorescence-activated cell-sorting analyses, plasminogen and/or plasmin was detected on the membrane but not on that of the cells cultured in serum-free GIT medium (Fig. 2A,B, respectively). So far there was no antibody that can distinguish between plasminogen and plasmin, these immunoreactive proteins in the membrane fraction were subjected to SDS-PAGE followed by detection by Western immunoblotting. The membrane fractions of Molt-4, clone 8 cells cultured in RPMI 1640 medium supplemented with 10% FCS revealed a main immunoreactive protein band with a molecular mass of plasminogen and minor immunoreactive protein bands with molecular masses of two subunits of plasmin (data not shown). However, plasminogen and plasmin in the membrane fractions of the cells cultured in serum-free medium were not detected at all.

The membrane fractions of the cells cultured in the medium supplemented with 10% FCS but not those of the cells cultured in serum-free medium converted gp160 to gp120 and gp41 (Fig. 2C) and this conversion was completely inhibited by plasmin inhibitors aprotinin and HI-30, an active fragment

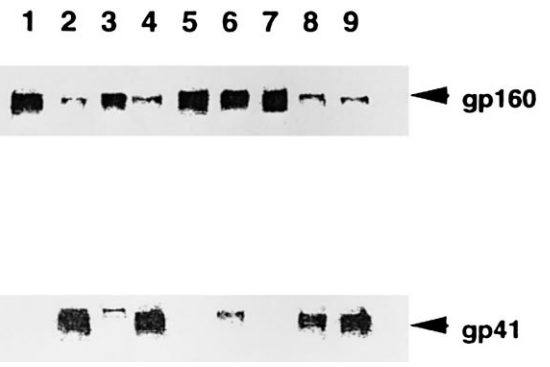


Fig. 3. Inhibition of gp160 processing by various protease inhibitors and substrate analogues. gp160 (1.0 μg) (lane 1) was incubated for 30 min with plasmin (1.35 pmol) in the absence (lane 2) or presence of various inhibitors and substrate analogues (10 μM) including dec-RVKR-cmk (lane 3), dec-FAKR-cmk (lane 4), aprotinin (lane 5), leupeptin (lane 6), HI-30 (lane 7), $\alpha_1$-antitrypsin (lane 8), and chymostatin (lane 9). The reactions were then subjected to SDS-PAGE and Western immunoblotting.

of human inter-α-trypsin inhibitor (data not shown). The results were consistent with those in Fig. 3.

### 3.3. Inhibition of gp160 processing by natural inhibitors and synthetic peptides

We further analyzed the effect of various natural and fungal serine protease inhibitors and substrate analogue peptides on gp160 processing by human plasmin. As shown in Fig. 3, this processing is markedly inhibited by the natural plasmin inhibitors aprotinin and HI-30, as well as by a substrate analogue, dec-RVKR-cmk, having basic amino acids at the P1, P2, and P4 positions. However, another substrate analogue tested, dec-FAKR-cmk, which has phenylalanine at the P4 position instead of a basic amino acid, and the other serine protease inhibitors tested, leupeptin, chymostatin, and $\alpha_1$-antitrypsin, caused little inhibition of the processing. In addition to the importance of a basic amino acid at the P1 position of the cleavage site [1,2], the data imply that a basic amino acid at the P4 position of the motif may also be important for processing by plasmin. The $K_i$ values of aprotinin and dec-RVKR-cmk against plasmin are $5.3\times10^{-9}$ M and $1.3\times10^{-6}$ M, respectively [16].

These data suggest that plasmin plays a role in the processing of gp160 on the cell surface, in addition to kexin/subtilisin-related endopeptidases and VEM, which convert gp160 to gp120 and gp41 intracellularly. The cell surface plasmin formation is controlled by urokinase-type plasminogen activator (uPA) [19]. Indeed, it has recently been reported that freshly isolated T lymphocytes and monocytic cells from HIV-positive donors show an increase in the expression of uPA receptor [20,21], and, hence, this may increase in the expression of cell surface-bound uPA and thus plasmin formation, which in turn facilitates conversion of gp160 to the mature forms on the plasma membrane. To evaluate the role of the extracellular gp160 processing by human plasmin in production of infectious viral particles, further studies on determination of the amount of gp160 on the cell surface, that has escaped from intracellular processing in HIV-1-infected cells and those cells treated with inhibitors of kexin/subtilisin-related endopeptidase and VEM, are now in progress. Besides proteolysis on the cell surface being involved in processes like adhesion, chemotaxis and migration that are important for viral spreading, cell surface processing of gp160 by plasmin may also provide a specific advantage to HIV.

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