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Blocking of Human Immunodeficiency Virus Type-1 Virion Autolysis by Autologous p2^{gag} Peptide

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Our previous study suggested that the p2^{gag} peptide, AEAMSQVTNTATIM, inhibits human immunodeficiency virus type 1 (HIV-1) protease (PR) activity *in vitro*. In this study, Ala substitutions (Met4Ala and Thr8Ala) and deletion of amino acid Asn9 within the nona p2^{gag} peptide (AEAMSQVTN) were found to decrease the inhibitory effect on HIV-1 PR activity. Furthermore, treatment of PMA-activated latently infected T lymphocytes, ACH-2 cells, with the p2^{gag} peptide (100 and 250 μM) resulted in a decrease in the amount of p24^{gag} in the resultant viral lysates derived from the cell-free supernatant. In addition, the HIV-1-Tat-p2^{gag} fusion peptide was synthesized to effectively deliver the p2^{gag} peptide into the cells. The fusion peptide was incorporated into chronically infected T lymphocytes, CEM/LAV-1 cells, as detected on indirect immunofluorescence analysis using anti-p2^{gag} peptide monoclonal antibodies, which recognize the nona peptide (AEAMSQVTN) derived from the N-terminus of the p2^{gag} peptide, and cleaved by HIV-1 PR *in vitro*. Treatment of CEM/LAV-1 cells with the fusion peptide also resulted in a decrease in the amount of p24^{gag} in the resultant viral lysate derived from the cell-free supernatant. Taken together, these data suggest that the p2^{gag} peptide consequently blocks the autolysis of HIV-1 virions for the conservation of viral species.

Key words: HIV-1, HIV-1 protease, p2^{gag} peptide, processing, suicidal inhibition.

Abbreviations: HIV-1, human immunodeficiency virus type 1; PR, protease; MAP, multiple antigen peptide.

During HIV-1 particle assembly, HIV-1 PR cleaves Gag and Gag-Pol precursors into structural proteins (p17^{gag}, p24^{gag}, p2^{gag}, p7^{gag}, p1^{gag}, and p6^{gag}) and viral enzymes (PR, reverse transcriptase/RNase H, and integrase) required for viral replication (*1*, *2*). Although HIV-1 PR is activated in the membrane compartment of an HIV-1-infected cell, it has been controversial as to what inhibites the proteolytic activity of HIV-1 PR after the enzyme has completed precursor processing during the maturation phase of the viral life cycle. Our previous study suggested that the p2^{gag} peptide is an inherent suicidal inhibitor of HIV-1 PR because it (400 μM) strongly inhibited the proteolytic cleavage of the recombinant Gag precursor protein into functional structural units (p17^{gag} and p24^{gag}) *in vitro* (*3*). The p2^{gag} peptide concentration in the virion may be sufficient to completely inhibit HIV-1 PR activity because the Gag precursor protein exists in the viral particle at a concentration of 4 mM (*4*, *5*). Furthermore, Pettit *et al.* demonstrated that the p2^{gag} peptide is finally cleaved from the *C*-terminus of the p24^{gag} protein to produce fully infectious virions (*6*). As soon as viral maturation is completed, the p2^{gag} peptide may inhibit HIV-1 PR activity in released viral particles in order to block the autolysis of HIV-1 virions for the conservation of viral species.

In this study, we mapped the amino acids in the p2^{gag} peptide responsible for the functional inhibition of HIV-1 PR activity, and examined whether or not the p2^{gag} peptide actually inhibited the proteolytic cleavage of the Gag precursor protein when the p2^{gag} peptide was in the viral particles during the early maturation phase.

MATERIALS AND METHODS

Materials—Reagents were obtained from the following sources: sodium dodecyl sulfate, sodium deoxycholate, PMA, and polyethylene glycol (PEG; average molecular weight, 8,000) from Sigma-Aldrich Japan K. K. (Tokyo, Japan); a Wakosil-PTC™ HPLC column (4.0 × 150 mm) and Triton X-100 from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); a chemiluminescence kit from Perkin-Elmer Life Sciences, Inc. (Boston, MA); RPMI-1640 medium from Nissui Seiyaku Co. (Tokyo, Japan); HIV-1-positive plasma from Scripps Laboratories (San Diego, CA); and polyvinylidene difluoride from Nihon Millipore Ltd. (Tokyo, Japan). The purity of synthetic oligopeptides was confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Cell Lines—Latently infected T lymphocytes, ACH-2, and chronically HIV-1-infected T lymphocytes, CEM/LAV-1, were maintained at 37°C in RPMI-1640 medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

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Protease Inhibition Assay—The HIV-1 PR inhibition assay was performed according to Ref. *3*. Determination of the HIV-1 PR activity was carried out routinely with 1 mM succinyl (Suc)-SQNYPIVQ as a substrate in 50 mM MES buffer (pH 5.5) containing 100 mM NaCl, 0.5 mM DTT, and 0.5 mM EDTA with or without the peptide inhibitor. The peptide inhibitors were separately dissolved in 50 mM MES buffer (pH 5.5). A mixture of 30 μl of an inhibitor solution and 50 μl of the enzyme solution (0.134 μg/μl, in the same buffer containing 200 mM NaCl, 1 mM DTT, and 1 mM EDTA) was preincubated at 37°C for 30 min, and then the substrate solution (20 μl) was added. The mixture was maintained at 37°C for 120 min with various concentrations of the peptide inhibitors (0–250 μM). The enzymatic reaction was terminated by adding acetonitrile (100%, 11.1 μl) containing 1% trifluoroacetic acid (TFA). The amount of Suc-SQNY liberated was estimated by HPLC on Wakosil-PTC (4.0 × 150 mm), and determined by amino acid analysis of its acid hydrolysates (131°C, 2 h). The concentration of the inhibitor needed to reduce the activity by 50% (IC_{50}) was determined graphically from the curve of percentage inhibition versus inhibitor concentration.

Analysis of the Virion Protein Profile—ACH-2 cells (8 × 106) were labeled for 3 h in RPMI-1640 medium containing 1% dimethyl sulfoxide with the $p2^{gag}$ peptide (0, 100, or 250 μM), and cultured for an additional 24 h after phorbol 12-myristate 13-acetate (PMA, 0.5 ng/ml) stimulation. For the preparation of a viral extract, the cell-free supernatant was filtered through a 0.45-μm low-protein-binding filter. Virions in the cell-free supernatant were pelleted by adding 1/2 volume of 30% PEG precipitation buffer (30% PEG 8000 and 1.2 M NaCl). After overnight incubation, the virus-PEG mix was centrifuged at 950 ×*g* for 45 min. The resulting pellet was boiled for 1 min and lysed in 200 μl of lysis buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% sodium deoxycholate, 0.1% sodium dodecyl sufate, and 1% Triton X-100). Ethanol was added to the lysate. An equal amount of viral protein was normalized according to the procedure described previously (*7*) and analyzed by Western blot analysis.

CEM/LAV-1 cells (2 × 106) were labeled for 20 min in serum-free RPMI-1640 medium (9 ml) with the HIV-1 Tat-$p2^{gag}$ fusion peptide (Fusion-$p2^{gag}$ peptide) (0, 5.6, 11.1, 27.8, or 55.6 μM), and cultured for an additional 96 h at 37°C in RPMI-1640 medium (10 ml) supplemented with 10% fetal calf serum with the Fusion-$p2^{gag}$ peptide (final concentration: 0, 5, 10, 25, or 50 μM). Then, the cells were serially cultured up to at least passage 4 with the Fusion-$p2^{gag}$ peptide. Virions in the cell-free supernatant were subjected to Western blot analysis in the same way as in the case of ACH-2 cells. The cells were also lysed and the lysates were subjected to SDS-PAGE (4–20%) followed by Western blot analysis.

Western Blot Analysis—Proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane, and the Gag precursor, Gag intermediates, and $p24^{gag}$ were detected with HIV-1-positive plasma. Actin was detected with an anti-actin monoclonal antibody (Oncogene Research Products, Boston, MA, USA). Densitometric analysis of the Western blots was performed with a LAS-1000plus from Fuji Film.

Furthermore, heat-inactivated HIV-1-PR- or HIV-1-PR-digests of $Pr55^{gag}$ were also subjected to Western blot analysis with HIV-1 positive plasma and anti-$p2^{gag}$ peptide monoclonal antibody.

Antibody Preparation for the $p2^{gag}$-Multiple Antigen Peptide (MAP)—An antibody was raised against $p2^{gag}$-MAP as described previously (*8*). Female BALB/c mice were immunized intraperitoneally with $p2^{gag}$-MAP in Freund's adjuvant at 1-week intervals and administered an intravenous booster of $p2^{gag}$-MAP 3 days prior to splenectomy. Hybridomas were generated by the standard method, splenocytes being fused with P3U1 cells, and selected in hypoxanthine-, aminopterin-, and thymidine-supplemented media. During the screening, supernatants were tested for reactivity to $p2^{gag}$-MAP. Hybridomas that produced the most potent supernatants were then cloned by limiting dilution.

Antibody Specificity—The antibody specificity was determined by ELISA, which was performed according to the procedure described previously (*9*). The anti-$p2^{gag}$ peptide monoclonal antibody was incubated with $p2^{gag}$-MAP in the presence or absence of competitors in 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 0.1% polyoxyethylene (*20*) sorbitan monooleate (37°C, 60 min), and the reaction mixtures were subjected to ELISA.

Proteolytic Processing of the HIV-1-Tat-$p2^{gag}$ Peptide by HIV-1 PR—The HIV-1-Tat-$p2^{gag}$ peptide was dissolved in 50 mM MES buffer (pH 5.5). A mixture of 50 μl of the HIV-1-Tat-$p2^{gag}$ peptide and 50 μl of the enzyme solution (0.134 μg/μl) was incubated at 37°C for 2 h. The enzymatic reaction was terminated byadding acetonitrile (100%, 11.1 μl) containing 1% trifluoroacetic acid (TFA). The amount of $p2^{gag}$ peptide liberated was estimated by HPLC on Wakosil-PTC (4.0 × 150 mm).

Indirect Immunofluorescence Analysis—CEM and CEM/LAV-1 cells (5 × 10^5 cells/ml) were incubated in the presence or absence of the HIV-1-Tat-$p2^{gag}$ fusion peptide for 20 min and then harvested by centrifugation (260 ×*g*, 5 min). The cell pellets were washed with PBS(–) and then subjected to indirect immunofluorescence analysis using anti-$p2^{gag}$ antibodies according to the procedure described previously (*10*).

RESULTS

Inhibitory Effects of the Amino-Acid-Substituted $p2^{gag}$-Like Peptide on HIV-1 PR Activity—Our previous study suggested that the synthetic $p2^{gag}$ peptide inhibited HIV-1 PR activity at an IC_{50} of 10 μM, and completely inhibited HIV-1 PR activity at 250 μM *in vitro* (*3*). The inhibition constant Ki was 30 μM (*3*). To determine the minimum length of the $p2^{gag}$ peptide that exhibits inhibitory activity, various lengths of the $p2^{gag}$-like peptide were prepared and analyzed. The results suggest that the nona $p2^{gag}$ peptide, AEAMSQVTN, has strong inhibitory activity (IC_{50} = 5 μM) (*3*). In this study, a number of Ala-substituted $p2^{gag}$ peptides were synthesized to elucidate the detailed structure-activity relationships in the $p2^{gag}$ peptide sequence. The data obtained with substitutions of Ala within the nona and full-length $p2^{gag}$ peptides are summarized in Table 1. These Ala-substituted $p2^{gag}$ peptides were generated as nine-amino- acid *N*-terminal fragments of the $p2^{gag}$ peptide, because the nona $p2^{gag}$

Table 1. **Effects of alanine-substituted p2 of p2gag peptide on inhibition of HIV-1 protease activity.**

Peptide sequence	IC_{50} (μM)
Ala-Glu-Ala-Met-Ser-Gln-Val-Thr-Asn	5
Ala-Ala-Ala-Met-Ser-Gln-Val-Thr-Asn	100
Ala-Glu-Ala-Ala-Ser-Gln-Val-Thr-Asn	–[a]
Ala-Glu-Ala-Met-Ala-Gln-Val-Thr-Asn	124
Ala-Glu-Ala-Met-Ser-Gln-Val-Ala-Asn	–[a]
Ala-Glu-Ala-Met-Ser-Gln-Val-Thr	–[a]
Ala-Glu-Ala-Met-Ser-Gln-Val	–[a]
Ala-Glu-Ala-Met-Ser-Gln	–[a]
Ala-Glu-Ala-Met	–[a]
Val-Thr-Asn	–[a]
Val-Thr-Asn-Thr-Ala-Thr-Ile-Met	–[a]
Ala-Glu-Ala-Met-Ser-Gln-Val-Thr-Asn-Thr-Ala-Thr-Ile-Met	10
Ala-Glu-Ala-Ala-Ser-Gln-Val-Thr-Asn-Thr-Ala-Thr-Ile-Met	142
Ala-Glu-Ala-Ser-Gln-Val-Thr-Asn-Thr-Ala-Thr-Ile-Met	126
Ala-Glu-Ala-Met-Ser-Gln-Val-Ala-Asn-Thr-Ala-Thr-Ile-Met	110

[a]Not inhibited. The HIV-1 PR inhibition assay was performed according to Ref. *3*. Determination of HIV-1 PR activity was carried out routinely with 1 mM succinyl (Suc)-SQNYPIVQ as the substrate with or without a peptide inhibitor. The peptide inhibitors were separately dissolved in 50 mM MES buffer (pH 5.5). A mixture of 30 μl of an inhibitor solution and 50 μl of the enzyme solution (0.134 μg/μl) was preincubated at 37°C for 30 min, and then the substrate solution (20 μl) was added. The mixture was maintained at 37°C for 120 min with various concentrations of the peptide inhibitors (0–250 μM). The enzymatic reaction was terminated by adding acetonitrile (100%, 11.1 μl) containing 1% trifluoroacetic acid (TFA). The amount of Suc-SQNY liberated was estimated by HPLC on Wakosil-PTC, and determined by amino acid analysis of its acid hydrolysates (131°C, 2 h). The concentration of the inhibitor needed to reduce activity by 50% (IC50) was determined graphically from the curve of percentage inhibition versus inhibitor concentration.

peptide exhibited almost the same inhibitory effect as the full-length p2gag peptide. Ala substitutions at E2 and S5 reduced the activity by 20- and 24.8-fold, respectively. Ala substitutions at M4 and T8 had no inhibitory effect (Table 1). Ala substitutions at M4 or T8, and deletion of M4 from the full-length p2gag peptide also led to lower inhibitory activitiy (IC_{50} = 142, 110, and 126 μM, respectively) (Table 1). Furthermore, our study suggested that neither the hexa p2gag peptide, AEAMSQ, nor the one- and two-amino-acid expanded peptides (AEAMSQV and AEAMSQVT) exhibited inhibitory activity, while the nona p2gag peptide AEAMSQVTN had strong inhibitory effect. These results suggest that the side chains of M4, T8, and N9 are required for the inhibitory effect of the p2gag peptide on HIV-1 PR activity.

p2gag Peptide Inhibits HIV-1 Gag Precursor Processing—To determine the inhibitory effect of the authentic p2gag peptide on the processing of the HIV-1 Gag precursor of viral particles in cell culture, ACH-2 cells were preincubated with the p2gag peptide (0, 100, or 250 μM) for 3 h, and then cultured for an additional 24 h after PMA stimulation (0.5 ng/ml) for virus production. The effect of the p2gag peptide on HIV-1 Gag precursor processing was determined by monitoring the cleavage products produced on proteolytic processing in the virion by Western blot analysis. An increase in the p2gag peptide concentration resulted in a decrease in the amount of p24gag. The band representing p24gag was hardly detected in the presence of the 250 μM p2gag peptide (Fig. 1, lane 4). The results suggest that the p2gag peptide inhibits the processing of the HIV-1 Gag precursor by HIV-1 PR in the virion. Unfortunately, it was difficult to determine whether or not the amount of Pr55 increased with the decrease in the amount of p24gag because the band of Pr55 was overlapped by a nonspecific band due to nonspecific binding of the secondary antibody alone, as shown in Fig. 1, lane 1.

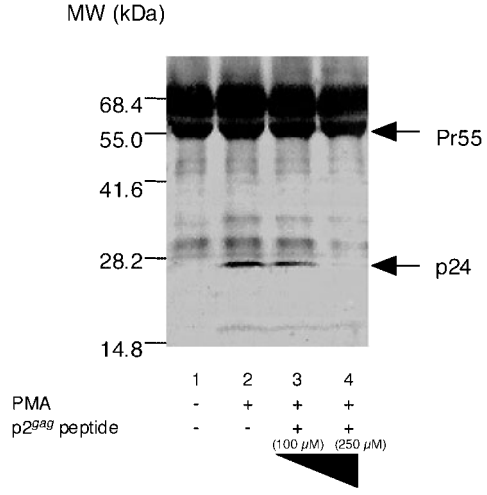

Fig. 1. **Effect of the p2gag peptide on proteolytic processing of Gag precursors in ACH-2 cells.** ACH-2 cells were preincubated in the presence of the p2gag peptide (0, 100, or 250 μM) for 3 h, and cultured for an additional 24 h after phorbol 12-myristate 13-acetate (PMA, 0.5 ng/ml) stimulation. Virions in the cell-free supernatant were subjected to Western blot analysis as described under "MATERIALS AND METHODS."

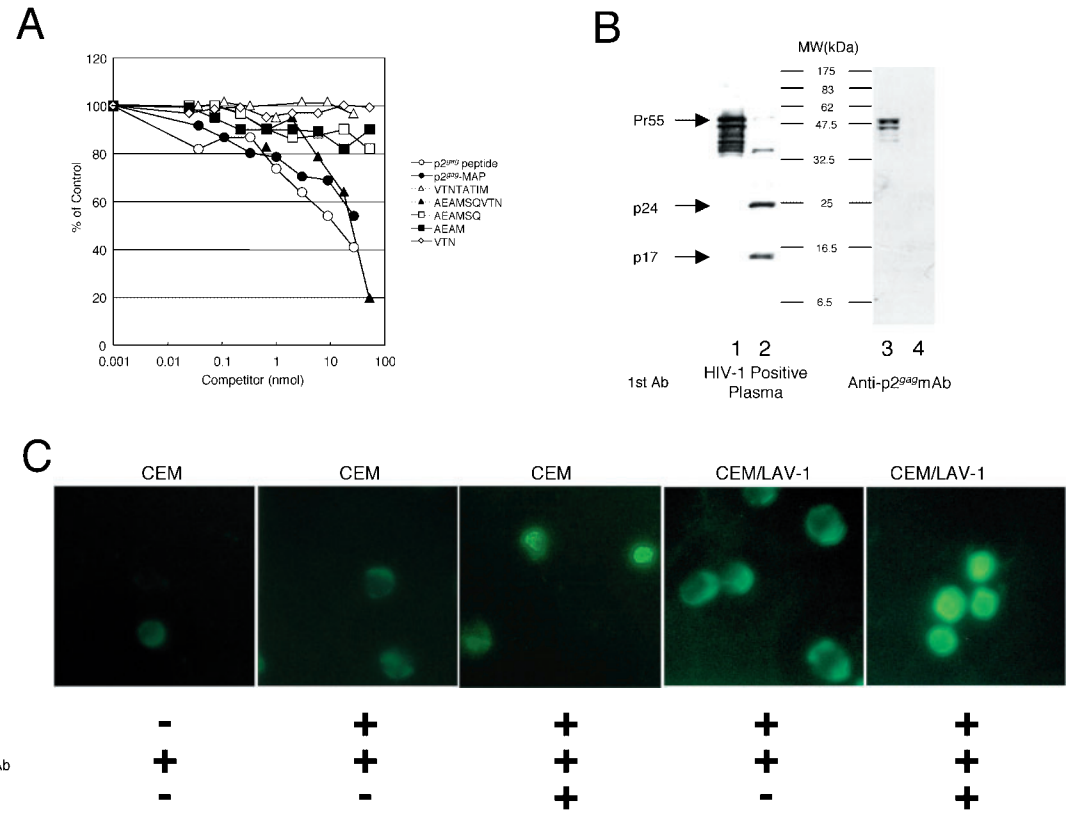

Fig. 2. **Specificity of the anti-p2^{gag} peptide monoclonal antibody and incorporation of the HIV-1-Tat-p2^{gag} fusion peptide into cells.** (A) Antibody specificity was determined by ELISA. The anti-p2^{gag} peptide monoclonal antibody was incubated with p2^{gag}-MAP in the presence or absence of competitors (open circles: p2^{gag} peptide; solid circles: p2^{gag}-MAP; open triangles: VTNTATIM; solid triangles: AEAMSQVTN; open squares: AEAMSQVTN; solid squares: AEAM; diamonds: VTN). (B) Heat-inactivated HIV-1-PR- (lanes 2 and 4) or HIV-1-PR-digests (lanes 1 and 3) of Pr55^{gag} were subjected to western blot analysis with HIV-1positive plasma (lanes 1 and 2) and anti-p2^{gag} peptide monoclonal antibodies (lanes 3 and 4). (C) CEM and CEM/LAV-1 cells (5×10^5 cells/ml) were incubated in the presence or absence of the HIV-1-Tat-p2^{gag} fusion peptide and then subjected to indirect immunofluorescence analysis as described under "MATERIALS AND METHODS."

Antibody Specifiicity—An anti-p2^{gag} peptide antibody was selected as a specific antibody against p2^{gag}-MAP. As shown in Fig. 2A, the immunochemical reaction was inhibited by p2^{gag} peptide, p2^{gag}-MAP, and nona p2^{gag} peptide (AEAMSQVTN), but not by VTNTATIM, AEAMSQ, AEAM, or VTN. This indicates that the monoclonal antibody recognizes the nonapeptide (AEAMSQVTN) derived from the N-terminus of the p2^{gag} peptide. Furthermore, to detemine whether or not the antibody could react with the recombinant Pr55^{gag} (*3*) containing the p2^{gag} domain, heat-inactivated HIV-1-PR- or HIV-1-PR-digest of Pr55^{gag} was subjected to Western blot analysis with the antibody. The Pr55^{gag} was processed by HIV-1 PR to p17^{gag}, p24^{gag}, and processing intermediates (Fig. 2B, lane 2). A major band of 55 kDa was specifically detected for the heat-inactivated HIV-1-PR-digest (Fig. 2B, lane 3) but not for the HIV-1-PR-digest (Fig. 2B, lane 4). These results indicate that the antibody can recognize not only the p2^{gag} peptide but also the protein containing the p2^{gag} domain.

Incorporation of the HIV-1-Tat-p2^{gag} Peptide into Cells—To effectively deliver the p2^{gag} peptide into cells across the lipid bilayer, a synthetic HIV-1 Tat-p2^{gag} fusion peptide (Fusion-p2^{gag} peptide: GRKKRRQRRRARVLAE-AMSQVTNTATIM) that includes residues 359–376 of the HIV-1 Gag precursor fused with the arginine-rich domain (G_{48}–R_{57}) of HIV-1 Tat was generated. Internalization of the Fusion-p2^{gag} peptide was monitored by indirect immunofluorescence microscopy using a mouse anti-p2^{gag} peptide monoclonal antibody and fluorescein isothiocyanate-conjugated anti-mouse IgM after 20-min incubation of the Fusion-p2^{gag} peptide with CEM/LAV-1. The Fusion-p2^{gag} peptide was obviously internalized into the cells (Fig. 2C).

Proteolytic Processing of the HIV-1-Tat-p2^{gag} Peptide by HIV-1 PR—To determine whether or not the synthetic HIV-1 Tat-p2^{gag} fusion peptide is cleaved by HIV-1 PR, the fusion peptide was incubated with HIV-1 PR for 2 h. As shown in Fig. 3A, the fusion peptide was cleaved by recombinant HIV-1 PR at an internal Leu-Ala site in vitro (Fig. 3A), and GRKKRRQRRRARVL and AEAM-SQVTNTATIM were eluted at the retention times of 14.4 and 17.0 min, respectively. These results indicated that the p2^{gag} peptide was released from the Fusion-p2^{gag} peptide.

Fusion-p2^{gag} Peptide Inhibits HIV-1 Gag Precursor Processing—In this study, CEM/LAV-1 cells were serially cultured up to at least passage 4 in the presence of the Fusion-p2^{gag} peptide. Each resultant viral lysate derived from cell-free supernatants at passages 2, 3, and 4 was subjected to Western blot analysis. An increase in the

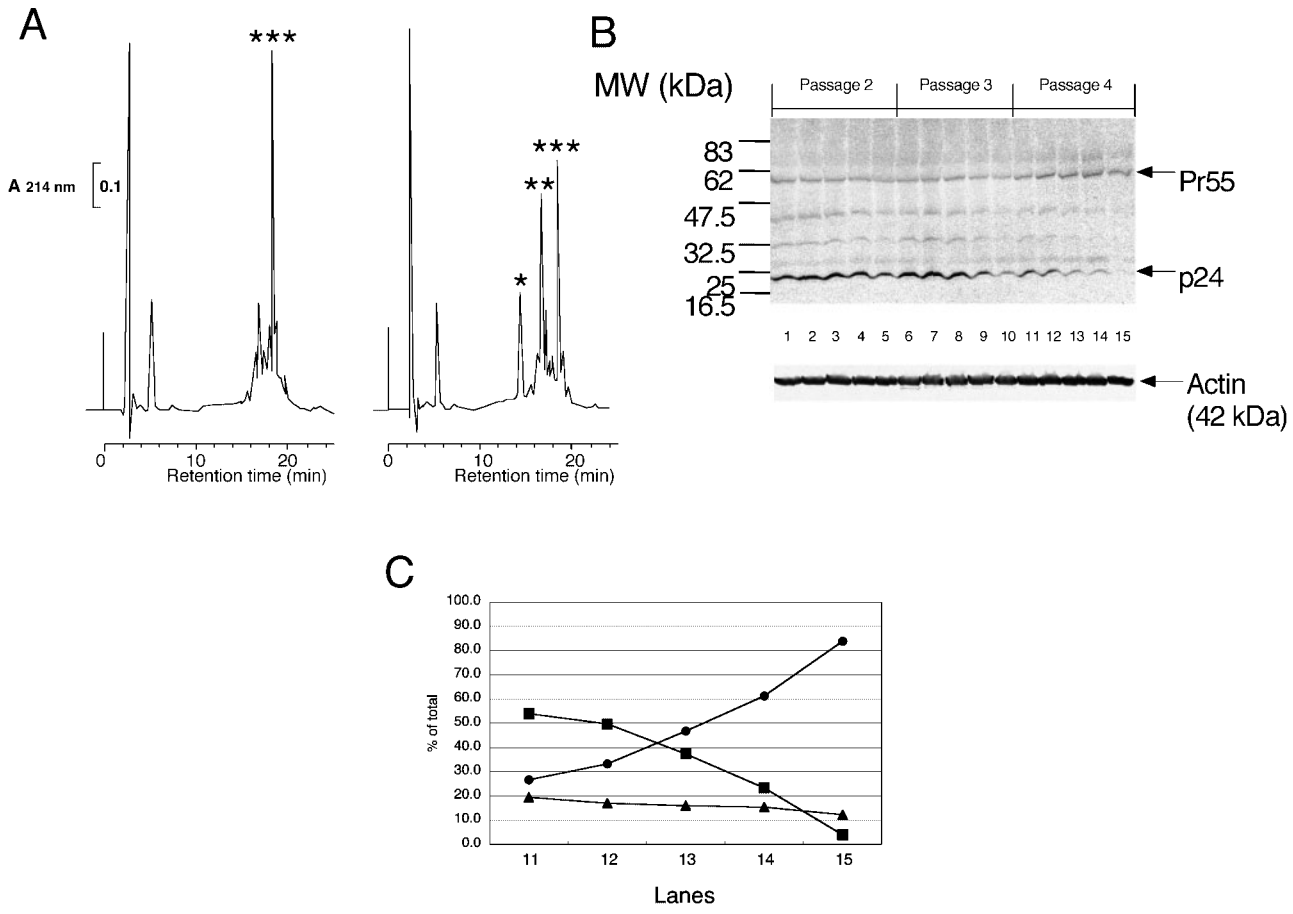

Fig. 3. **Proteolytic cleavage of the HIV-1-Tat-p2^{gag} fusion peptide and its effect on proteolytic processing of Gag precursors.** (A) HPLC of the HIV-1-Tat-p2^{gag} fusion peptide and its HIV-1-PR-digests. An aliquot of the HIV-1-Tat-p2^{gag} fusion peptide or one of its HIV-1-PR-digests was subjected to HPLC. Column: Wakosil-PTC (4.0 × 150 mm). Eluents: A, 10% CH_3CN containing 0.1% TFA; B, 100% CH_3CN containing 0.1% TFA. Gradient: 10–100% B in 20 min, linear, and then 100% B for 10 min. Detector: UV (214 nm) at 1.0 a.u.f.s. Flow rate: 0.8 ml/min. GRKKRRQRRRARVL, AEAMSQVTNTATIM, and GRKKRRQRRRARVLAEAMSQVYNTATIM are denoted by *, **, and ***, respectively. (B) After 2, 3, and 4 passages of CEM/LAV-1 with the HIV-1-Tat-p2^{gag} fusion peptide (lanes 1, 6, and 11, 0 μM; lanes 2, 7, and 12, 5 μM; lanes 3, 8, and 13, 10 μM; lanes 4, 9, and 14, 25 μM; lanes 5, 10, and 15, 50 μM), virions in the cell-free supernatant were subjected to Western blot analysis as described under "MATERIALS AND METHODS." (C) The intensities of the bands shown in Fig. 3B, lanes 11–15, in the densitometric scan. The values shown (circles: Pr55, triangles: p17^{gag}–p24^{gag}, squares: p24^{gag}) are percentages of the total Gag proteins detected in each lane.

Fusion-p2^{gag} peptide concentration resulted in a dose-dependent decrease in the amount of HIV-1 p24^{gag} at passage 4 (Fig. 3B, lanes 11–15), although the HIV-1 p24^{gag} amount in the virion at passage 2 was not significantly different (Fig. 3A, lanes 1–5). Furthermore, a densitometric scan suggested that the amount of Pr55 in the virion at passage 4 increased with the concentration of the HIV-1-Tat-p2^{gag} fusion peptide as compared to that of Pr55 in the absence of the HIV-1-Tat-p2^{gag} fusion peptide (Fig. 3C). Fortunately, the secondary antibody did not nonspecifically bind to viral lysates of CEM/LAV-1 cells. The actin level in the cell lysates remained unchanged (Fig. 3B), indicating that the Fusion-p2^{gag} peptide is not toxic to cells. These results suggest that the Fusion-p2^{gag} peptide also inhibits sequential processing of the HIV-1 Gag precursor in the virion.

DISCUSSION

The main structural proteins of HIV-1 are synthesized as a Gag precursor. Viral enzymes such as PR, reverse transcriptase, and integrase are synthesized as a Gag-Pol precursor, which is derived through ribosomal frameshifting at the rate of 5% (*11*). The cleavage of Gag and Gag-Pol precursors is essential for maturation and HIV-1 infectivity because mutation of HIV-1 PR or inhibition of its activity abolishes the production of infectious viruses (*6*, *12*). Studies for elucidation of the pathway leading to the formation of mature HIV-1 PR from the Gag-Pol precursor have been carried out (*13*, *14*). However, little is known about what eventually inhibits the proteolytic activity in the maturation phase of the viral life cycle. To elucidate the mechanism that blocks the autolysis of HIV-1 virions, we previously attempted to determine an inherent viral HIV-1 PR inhibitor derived from the HIV-1 PR substrate because the HIV-1 PR proteolytic activity should be inhibited after maturation has

Table 2. **Sequence variability within p2^{gag} peptide derived fromclade A to K**

Clade B HXB2															
A	E	A	M	S	Q	V	T	N	S	A	T	.	.	I	M
A	E	A	M	S	Q	V	T	N	T	A	.	.	.	I	M
(100)	(100)	(98.9)	(100)	(98.9)	(92.9)	(51.0)	(57.2)	(59.2)	(33.7)	(53.1)	(49.0)	(85.7)	(96.9)	(84.8)	(87.8)
		T		G	H	A	N	S	S	N	T	A	A	V	L
		(1.1)		(1.1)	(3.1)	(38.8)	(23.5)	(24.5)	(30.7)	(29.6)	(35.7)	(12.2)	(2.0)	(12.2)	(12.2)
					K	T	Q	Q	A	T	A	T	N	T	
					(3.1)	(8.2)	(11.2)	(6.1)	(20.4)	(7.1)	(12.2)	(2.1)	(1.1)	(1.0)	
					R	I	.	G	P	S	N			A	
					(0.9)	(1.0)	(3.1)	(6.1)	(9.2)	(6.1)	(3.1)			(1.1)	
						S	G	H	N	V				M	
						(1.0)	(2.0)	(3.1)	(2.0)	(2.0)				(1.0)	
							S	T	V	G					
							(1.0)	(1.0)	(2.0)	(2.0)					
							P		H						
							(1.0)		(1.0)						
							H		M						
							(1.0)		(1.0)						

The sequences of p2^{gag} peptide available at the Los Alamos HIV sequence database (http://hiv-web.lanl.gov/content/index) werealigned against that fromclade B HIV-1_{HXB2}. Frequency of amino-acid substitution (% of total viral isolates) is given in parentheses.

been completed; moreover, the possibility that a cellular HIV-1 PR inhibitor is incorporated in the viral particles concomitant with viral budding is low. Furthermore, the proteolytic processing of Gag and Gag-Pol precursors occurs in an orderly fashion with the primary, secondary, and tertiary sites being sequentially cleaved by HIV-1 PR (*6*, *12*, *15–17*). These studies revealed that the p2^{gag} peptide was finally cleaved from the C-terminus of the p24^{gag} protein. Therefore, we investigated whether or not the p2^{gag} peptide inhibited HIV-1 PR activity *in vitro* (*3*). The results suggest that the p2^{gag} peptide markedly inhibits HIV-1 PR activity. It is reasonable to hypothesize that the mechanism involves functional inhibition, in which HIV-1 PR converts the substrates, Gag and Gag-Pol precursors, into the reactive inhibitor, the p2^{gag} peptide, which immediately abplishies its catalytic activity.

To test this hypothesis, we studied the effect of the p2^{gag} peptide on the inactivation of HIV-1 PR activity in the virion. The results obtained suggest that the p2^{gag} peptide inhibits the Gag precursor processing by HIV-1 PR in the virion (Fig. 1). Furthermore, the Fusion-p2^{gag} peptide was prepared since methods for the delivery of exogenous proteins into living cells with the help of membrane-permeable carrier peptides such as HIV-1 Tat-(48–60) and antennapedia-(43–58) have been developed (*18–29*). Consequently, a dose-dependent decrease in the amount of HIV-1 p24^{gag} was also observed after at least passage 3 when CEM/LAV-1 cells were treated with the fusion peptide (Fig. 3B). It seems that the effect of the Fusion-p2^{gag} peptide did not appear immediately since the Fusion-p2^{gag} peptide in these cells effectively penetrated into the cells, but was not sufficiently incorporated into the virion due to its nuclear localization ability.

Our previous study suggested that the nona p2^{gag} peptide (AEAMSQVTN) possesses wild-type-like inhibitory activity (*3*). Therefore, it can be assumed that this segment of the p2^{gag} peptide contains all of the structural elements needed for HIV-1 PR inhibition. The progressive substitution and deletion series showed a major decrease in inhibitory activity that occurred at three major sites, M4, T8 and N9 (Table 1). The replacement of M4 or T8 by Ala resulted in a major decrease in inhibitory activity. This contribution of the side chains exhibits some similarity to the results obtained with the full-length p2^{gag} peptide. Interestingly, M4 of the sequence is highly conserved among HIV-1 subtypes A to K isolates (Table 2). Analyses of the structure-activity relationships of the p2^{gag} peptide showed that the nine *N*-terminal amino acids are the major contributors to the inhibition of HIV-1 PR activity. In particular, M4, T8 and N9 play equally crucial roles in the p2^{gag} peptide. These side chains probably contribute to p2^{gag} peptide-HIV-1 PR interactions. Other side chains within the p2^{gag} peptide may be involved in effective binding by inducing a conformational change in HIV-1 PR that is necessary for its inhibition.

Overall, it is concluded that the p2^{gag} peptide consequently inhibits HIV-1 PR activity to block the autolysis of the HIV-1 virion after sequential processing and rearrangement of the virion core.

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