Tripeptide Interference with Human Immunodeficiency Virus Type 1 Morphogenesis

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Capsid assembly during virus replication is a potential target for antiviral therapy. The Gag polyprotein is the main structural component of retroviral particles, and in human immunodeficiency virus type 1 (HIV-1), it contains the sequences for the matrix, capsid, nucleocapsid, and several small polypeptides. Here, we report that at a concentration of 100 μM, 7 of 83 tripeptide amides from the carboxyl-terminal sequence of the HIV-1 capsid protein p24 suppressed HIV-1 replication (>80%). The three most potent tripeptides, glycyl-prolyl-glycine-amide (GPG-NH2), alanyl-leucyl-glycine-amide (ALG-NH2), and arginyl-glutaminyl-glycine-amide (RGQ-NH2), were found to interact with p24. With electron microscopy, disarranged core structures of HIV-1 progeny were extensively observed when the cells were treated with GPG-NH2 and ALG-NH2. Furthermore, noddular structures of approximately the same size as the broad end of HIV-1 conical capsids were observed at the plasma membranes of treated cells only, possibly indicating an arrest of the budding process. Corresponding tripeptides with nonamidated carboxyl termini were not biologically active and did not interact with p24.

In all virus particles, the genome is encapsulated in a protein shell which is formed by the condensation of protein subunits in an ordered fashion to form either a helical or icosahedral capsid or, as in the case of human immunodeficiency virus type 1 (HIV-1), a conical capsid structure (21, 28). The assembly of the capsid is thus a potential target for antiviral therapy. HIV-1 assembles at the plasma membrane of infected cells and buds off as immature spherical particles containing an electron-dense shell composed of the Gag and Gag-Pol polyproteins surrounded by the envelope membrane. These particles must undergo maturation to become infectious. This is accomplished by the virus protease present in the immature virus particles, which cleaves the Gag and Gag-Pol polyproteins into individual protein components. The Gag polyprotein is the main structural component of retroviral particles, and in HIV-1, it contains the sequences for the matrix (p17, MA), capsid (p24, CA), nucleocapsid (p7, NC), and p6 proteins and two spacer peptides called spacer peptide 1 (SP1) and spacer peptide 2 (SP2). Upon maturation, p24 is released from the matrix protein and condenses into a conical capsid that encases and stabilizes the ribonucleoprotein complex (24, 27), which also includes the enzymes needed for virus replication (28).

The mature conical capsid of HIV-1 contains 1,500 to 2,000 copies of p24. Capsid protein p24 is composed of approximately 230 amino acids, is highly hydrophobic, and contains two distinct α-helical domains that are connected by a flexible linker (5, 28, 30). The amino-terminal domain (residues 1 to 146) binds cyclophilin A (CyPA) (4, 13, 14) and participates in capsid formation (8, 33, 43). The second carboxyl-terminal domain (residues 148 to 231) is involved in p55 Gag polyprotein assembly and the budding of virus particles (3, 6, 8, 41, 44). Within the carboxyl-terminal domain is a region conserved among lentiviruses known as the major homology region. Deletions in this region of the carboxyl terminal abolish the ability of HIV-1 to form infectious viral particles (8). Furthermore, an interaction between SP1 and the carboxyl-terminal domain of p24 is probably necessary, at least transiently, in order to weaken CA-CA interactions and allow the rearrangement of the capsid shell during virus maturation (20, 46). It has also been shown that extensions at the N terminal of the capsid protein with sequences corresponding to the matrix protein redirect in vitro protein assembly from cylinders to spheres (9, 19, 42). These results together with those showing that capsid cones can be formed in vitro from CA-NC fusion proteins (16) indicate that release of the capsid from the matrix protein is necessary for capsid cone formation. Moreover, the introduction of mutations in the amino-terminal domain of p24 has produced viruses with retained ability to assemble and bud but which developed aberrant capsid morphologies (8, 33, 43). The formation of a conical capsid appears to be essential, because gag mutations that disrupt proper core formation invariably lead to a reduction or loss of viral infectivity (8, 17, 33, 43, 46).

Immature capsids are stable and can be isolated easily, whereas mature capsids are less stable and have only recently been successfully purified from infectious particles by mild detergent treatment and centrifugation (45). Therefore, it appears that the maturation process prepares the virus particle for infection by facilitating the disassembly of the capsid structure upon virus entry into the target cell. Substances that sta-
bilize capsid structures and inhibit uncoating are well known inhibitors of picornavirus replication (2, 18, 32, 34).

Thus, substances that interfere with the proper assembly of p24 could be potential antiretroviral agents. In this regard, it has been shown that a 10-amino-acid-long peptide derived from the p24 amino acid sequence can block virus replication by interfering with capsid formation (31).

Here, we report that tripeptides derived from the carboxyl-terminal sequence of the HIV-1 capsid protein p24 were found to interfere with HIV-1 morphogenesis and to suppress viral replication.

MATERIALS AND METHODS

Peptide synthesis. To manufacture screening peptides, solid-phase peptide synthesis was performed as described previously (25). The peptides were carboxyl-terminal amides (CONH₂), i.e., the hydroxyl group was replaced by an amide group. For all experiments except the initial screening, peptides GPG-NH₂, ALG-NH₂, CQG-NH₂, RQG-NH₂, and ALGPG-NH₂ were obtained by custom order from Bachem Feinchemikalien AG (Bubendorf, Switzerland), as were peptides GPG-OH and ALG-OH, which have normal carboxyl termini (COOH). [1-¹⁴C]glycyl-prolyl-glycine-amide (2.5 mCi/ml with a specific activity of 56 mCi/mmol) was custom ordered from Amersham Pharmacia Biotech (Uppsala, Sweden).

Viruses, cells, and infections. HIV-1 SF-2 stock was prepared from HUT78 cells, and two clinical isolates were prepared from donors’ peripheral blood mononuclear cells (PBMC). Fifty percent tissue culture infectious doses (TCID₅₀) were prepared as described previously (38). All T-cell lines were kindly provided by the NIBSC AIDS Reagent Project, National Institute for Biological Standards and Control, Potters Bar, United Kingdom. HUT78, H9, and ACH-2 (an HIV-1 chronically infected human T-cell line [12]) cells were propagated and maintained in RPMI 1640 medium (GIBCO Laboratories) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (GIBCO Laboratories), penicillin, and streptomycin (100 U/ml; Sigma, St. Louis, Mo.). PBMC were purified by Ficoll-Hypaque density gradient centrifugation and stimulated with phytohemagglutinin (KEBO Lab, Stockholm, Sweden) for 3 days in RPMI 1640 medium supplemented as described above before use.

For peptide screening, 10⁵ H9 cells were infected with HIV-1 SF-2 (25 TCID₅₀) with or without 100 μM of the peptides. After virus adsorption for 1 h at 37°C, the cells were washed three times in RPMI medium and then resuspended in culture medium with or without the peptides. Supernatants were collected on days 4, 7, and 11 postinfection, and the medium was refreshed in the presence (100 μM) or absence of the peptides. HIV-1 p24 antigen (ELISA kit; Abbott Laboratories, North Chicago, Ill.) and reverse transcriptase activity (Lenti RT kit; Cavi AB, Uppsala, Sweden) in growth medium were assayed on the collected supernatants (36). Infectivity assays of the HIV-1 clinical isolates
TABLE 1. ACE analysis of the interaction between tripeptides and HIV-1 p24 and human hemoglobin (used as control protein)*

<table>
<thead>
<tr>
<th>Tripeptide</th>
<th>Migration time ( t_1 ) (min) in the absence of:</th>
<th>Migration time ( t_2 ) (min) in the presence of:</th>
<th>RDM ( \frac{(100 \cdot \frac{t_1 - t_2}{t_1})}{u} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p24 Hemoglobin</td>
<td>p24 Hemoglobin</td>
<td>p24 Hemoglobin</td>
</tr>
<tr>
<td>pH 6.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROQ-NH₂</td>
<td>3.10 ND</td>
<td>3.36 ND</td>
<td>7.75 ND</td>
</tr>
<tr>
<td>GPG-NH₂</td>
<td>3.30 ND</td>
<td>3.43 ND</td>
<td>4.58 ND</td>
</tr>
<tr>
<td>ALG-NH₂</td>
<td>4.28 4.31</td>
<td>4.56 4.32</td>
<td>6.1 0.2</td>
</tr>
<tr>
<td>ALG-OH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPG-OH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 7.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROQ-NH₂</td>
<td>3.76 ND</td>
<td>4.21 ND</td>
<td>10.6 ND</td>
</tr>
<tr>
<td>GPG-NH₂</td>
<td>4.06 3.82</td>
<td>4.50 3.83</td>
<td>9.8 0.3</td>
</tr>
<tr>
<td>ALG-NH₂</td>
<td>5.58 5.38</td>
<td>6.19 5.40</td>
<td>9.8 0.4</td>
</tr>
<tr>
<td>ALG-OH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GPG-OH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pH 8.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ROQ-NH₂</td>
<td>3.69 ND</td>
<td>3.93 ND</td>
<td>6.1 ND</td>
</tr>
<tr>
<td>GPG-NH₂</td>
<td>7.15 ND</td>
<td>7.94 ND</td>
<td>9.9 ND</td>
</tr>
<tr>
<td>ALG-NH₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ALG-OH</td>
<td>6.51 6.41</td>
<td>6.54 6.43</td>
<td>0.5 0.3</td>
</tr>
<tr>
<td>GPG-OH</td>
<td>8.91 8.90</td>
<td>8.93 8.92</td>
<td>0.2 0.2</td>
</tr>
</tbody>
</table>

* The length of the protein zone was 4.7 or 14.0 (for pH 8.2) mm long in each run. The values are averages of two or three measurements in which the run-to-reproducibility was higher than 98%. Note that the series of runs with p24 were performed in one capillary and those with hemoglobin in another. The coating used to suppress electroendosmotic flow and adsorption vary somewhat from one capillary to another. A few percentage variations are expected and tolerable, ND, not determined.

b The equation \((l·E)/(V·r)\) reveals the relationship between the mobility \((u)\) and the migration time \((t)\) \((l\) is the effective length and \(V\) is the total length of the capillary, respectively, and \(r\) is the applied voltage). Using this relationship, it is easy to show that RDM can be written as \((u/r1)-u/r2\). 100.

c Electrophoretic mobility = 0.

were performed as described above in PBMC stimulated for 3 days with 2.5 μg of phytohemagglutinin (Difco, Franklin Lakes, New Jersey) ml and injected at 25 TCD50/2/10 cells with or without different concentrations of peptides GPG-NH₂, ALG-NH₂, and ALGPG-NH₂ in the medium. Medium containing different concentrations of the peptides was refreshed on days 4 and 7 postinfection. The production of p24(37) in the culture supernatants collected on days 7 and 11 was measured to monitor viral replication by use of the Abbott ELISA kit.

Transmission electron microscopy (TEM) of HIV-1 assembly. Virus-infected cells were fixed by freshly made 2.5% glutaraldehyde in phosphate buffer and postfixed in 1% osmium tetroxide. The cells were embedded in Epon and post-stained with 1% uranyl acetate. Sections were made to be approximately 60 nm thick to accommodate the volume of the core structure parallel to the section plane. Duplicate sample preparations were done so that possible volume changes during polymerization and the capacity to withstand electron beam exposure could both be estimated. Specimens were analyzed with a Zeiss CEM 902 electron microscope, equipped with a spectrometer to enhance image contrast, at an accelerating voltage of 80 kV. A liquid nitrogen-cooling trap for the specimen holder was used throughout. Statistical evaluation of morphology was done with a series of electron micrographs to depict different categories of virus morphology, specifically focusing on the packing of the virus core structure. A test for the difference between two population proportions was also included.

Three-dimensional (3D) visualization of the internal structure of HIV was elicited from several TEM projections taken at evenly spaced tilt angles and subjected to computer processing. Duplicate sample preparations were done so that possible volume changes during polymerization and the capacity to withstand electron beam exposure could both be estimated. Furthermore, the Epon sections were covered by an approximately 1-nm-thick carbon layer for protection. Approximately 60-nm-thick sections were cut and mounted on 400-mesh copper grids. The specimens were poststained with lead citrate. Ten-nanometer average-diameter gold particles were applied as fiducary marks to align the images for computer analysis. The specimens were analyzed in a Zeiss CEM 902 system with a goniometer stage at 80 kV. The spectrometer unit was used to improve image quality, especially at high tilt angles. The minimal beam dose technique was employed throughout. Forty projections were obtained in each tilt series taken from −60° to +60°. All tilt series were carefully scrutinized for visible beam damage by comparison of images taken from the tilt series. Series of micrographs demonstrating no visible changes were further digitized with a photoscanner. Image alignment and 3D reconstruction were done essentially as described before (24; A. Höglund, personal communication).

The reconstructed virus models were viewed on a computer-controlled display (Silicon Graphics O2 workstation) as vector models. Volume images that are obtained by 3D reconstructions are important for the evaluation of virus structure. In two-dimensional (2D) micrographs, it is difficult to determine whether two overlapping objects are present, something which is clearly discernible in 3D images. Another topological feature that can be revealed in volume images, but not in 2D micrographs, is the presence of cavities in the particles, i.e., it can be determined whether the particles are solid or hollow. The 3D reconstructions also reveal surface structures of the particles that cannot be observed easily with 2D micrographs. These may be visualized by common rendering techniques (26).

The samples used for immunoocytochemical analysis were fixed in 4% formaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline. The rabbit anti-HIV p24 polyclonal antibody used for primary immunolabeling was obtained from an Abbott p24 ELISA kit (at dilutions of 1:5 and 1:10). For secondary immunolabeling, a goat anti-rabbit gold (5 nm diameter)-conjugated antibody was used (dilutions of 1:500 and 1:1,000; BBI International. Cardiff, United Kingdom). Low image contrast is obtained by this method irrespective of post-staining.

Affinity capillary electrophoresis (ACE). An interaction between the tripeptides and p24 is likely to be attended by a change of the charge of the peptide. Therefore, electrophoresis ought to be the method of choice for the study of these possible interactions, particularly in the capillary mode, since only minute amounts of material are required for a rapid high-resolution analysis. Capillary electrophoresis permits calculation of the electrophoretic mobility of a charged species. The electrophoretic mobility of a given species, which is related to its net surface charge density, can be used to characterize and identify a substance (7).

In this study, we used the partial-filling technique, employing a non-UV-absorbing buffer and injecting a zone of the slow UV-absorbing constituent first and then a zone of the fast UV-absorbing constituent (1, 22, 40), with the HIV-1 capsid protein p24 being the slow component and five tripeptides being the fast components.

Fused silica tubing (inner diameter, 50 μm) was purchased from MicroQuartz (Munich, Germany) and cut to a length of 23 cm (effective length, 18.5 cm). A BioFocus 3000 capillary electrophoresis system (Bio-Rad Laboratories, Hercules, Calif.) was used in all experiments. The internal wall of the capillary was coated prior to use with 5% (wt/vol) linear polyacylamide (23) in order to suppress the electroendosmotic flow and to prevent the adsorption of the proteins onto the capillary wall. Sodium phosphate solutions (0.01 M) at pH values between 6.8 and 8.2 were used as buffers. The polarity was negative (the detection point was closer to the cathode) for GPG-NH₂, ALG-NH₂, and ROQ-NH₂ at pH values between 6.8 and 7.5 (see Fig. 2a) but positive (the detection point was closer to the anode) at pH 8.2, which was also the case for GPG-OH and ALG-OH (see Fig. 2b). The peptides have low UV absorbance and were therefore dissolved in the buffer at a relatively high concentration (0.5 mg/ml) to permit online monitoring by a standard UV detector. The stock solution of p24 was diluted 10-fold with the running buffer to a final concentration of 50 μg/ml. Hemoglobin was prepared from human red blood cells from a healthy donor following the method described by Molteni et al. (29). The normal hemoglobin variants in blood have isoelectric points (pI) between 6.7 and 7.4, with the major component having a pI of 6.9. The concentration of the hemoglobin solution was much higher than that of p24.

The capillary was filled with the buffer. The protein was injected by pressure (10 to 60 lb/in²), and then the peptide was injected (1 lb/in²). Since the electrophoretic migration velocity of the peptide was much higher than that of the protein, the peptide molecules moved through the protein zone more quickly.

**Dialysis of [¹⁴C]GPG-NH₂ versus p24.** Fifty microliters of 10 μM solutions of recombinant protein p24 (NIBSC AIDS Reagent Project) or, as controls, recombinant gp120 (NIBSC AIDS Reagent Project) or bovine serum albumin (BSA; Sigma) was dialyzed, using a 10-kDa cutoff dialysis cassette (Slide-A-Lyzer; Pierce), against 27.5 μM [¹⁴C]GPG-NH₂ in 500 ml of 150 mM NaCl and 50 mM Tris·HCl, pH 7.4, buffer at 4°C. After 2 days. Radioactivity was quantified in a Rackbeta 1218 instrument (LKB-Wallac) after mixing 10 or 5 μl of the protein solutions with 3 ml of ReadySafe (Beckman).
RESULTS

Screening of the peptides corresponding to the C terminus of p24 for antiviral activity. The overlapping tripeptides corresponding to the C-terminal domain of HIV-1 p24 (residues 146 to 231) were tested at a concentration of 100 μM for their ability to inhibit viral replication in H9 cells. The results are shown in Fig. 1a. Besides GPG-NH2, 31 of the 82 tripeptides inhibited HIV-1 SF-2 replication by 50% or more. ALG-NH2 showed a strong inhibitory effect and reduced virus production by more than 95%. In fact, the reduction of viral replication obtained with ALG-NH2 at different concentrations was close to that obtained with GPG-NH2 (Fig. 1). The pentapeptide amide ALGPG-NH2, corresponding to residues 204 to 208, also inhibited HIV-1 replication (Fig. 1), albeit slightly less efficiently than the tripeptides ALG-NH2 and GPG-NH2. ALG-NH2 also inhibited the replication of two clinical isolates of HIV-1 in PBMC in a dose-dependent manner (Fig. 1).

Binding of GPG-NH2 to p24. The amidated peptides are positively charged at and below pH 8.2, except for ALG-NH2, which is not charged and hence does not move at pH 8.2. The two tripeptides with nonamidated carboxyl termini (GPG-OH and ALG-OH) and p24 migrated very slowly in the capillaries at pH 7.0. The isoelectric points of these substances should, therefore, be around 7.

The migration times of the peptides were determined at pH values of 6.8, 7.5, and 8.2 in the absence (t1 values in Table 1) and presence (t2 values in Table 1) of the protein. Figure 2 shows four typical electropherograms. As a measure of the interaction, we used the relative difference between the mobilities (RDM) in the presence and absence of protein \( \frac{(u_1 - u_2)}{u_1} \times 100 \). Note that the parameter RDM is not equivalent to the capacity factor since the peptides do not interact with p24 during the entire migration time. Accordingly, in terms of RDM, GPG-OH and ALG-OH showed almost no affinity to recombinant p24 whereas GPG-NH2, ALG-NH2, and RQG-NH2 interacted with it. Hemoglobin was chosen as a control protein because it has an isoelectric point similar to that of the capsid protein. In addition, hemoglobin (molecular
FIG. 3. Morphological study of the HIV-1 virions produced from the GPG-NH\textsubscript{2}-treated HUT\textsubscript{78} cells from tilt series by TEM and 3D reconstruction. (a) Control virions from untreated cells showing a characteristic dense, conical capsid (middle) and a round, dense disk upon tangential sectioning. (b and c) Dense nodular material is shown protruding from the viral envelope. (c and e) Dislocation is shown, with dense material in the broad end and low density in the narrow part of the viral capsid. A nodule is also shown in panel e (arrow). (d) Round, dense material is shown accommodated outside of an empty conical capsid. (f) Virions from ACH-2 cells pretreated with 100 \(\mu\text{M}\) GPG-NH\textsubscript{2} also showed dislocation of viral capsid material. (g) Evaluation by 3D reconstruction of the control virion from untreated cells. The volume rendering shows a tight, cone-shaped capsid which is attached by its narrow part to the viral envelope. (h) Evaluation by 3D reconstruction of HIV-1 particle from GPG-NH\textsubscript{2}-treated cells. This volume rendering shows tight viral capsid material irregularly condensed in the broad part of the capsid. Bar, 100 nm.
weight, 68,000) has almost three times more amino acid residues than p24, which increases the possibility for unspecific interactions with tripeptides. In the series of experiments where p24 was replaced by hemoglobin, no differences in the migration times of the peptides were observed (Table 1). The three amidated tripeptides at all pH values tested (6.8 to 8.2) gave increased RDM values, indicating an interaction with p24 (Table 1). The two nonamidated tripeptides did not show any interaction with p24 at pH 8.2. We used this pH because the electrophoretic mobilities of these peptides at pH 6.8 and 7.5 are too low to permit interaction studies.

The ability of GPG-NH₂ to bind to the HIV-1 capsid protein was also tested by dialysis experiments. ¹⁴C-labeled tripeptide was added to the dialysis buffer outside the dialysis chamber containing p24 or, as controls, envelope glycoprotein gp120 or BSA. After 2 days of dialysis, the radioactivity of the buffer inside the dialysis chamber did not differ from that found outside in the control experiments (1.7 μCi/ml for both gp120 and BSA). However, the radioactivity of the buffer containing p24 was 13.7 μCi/ml, which was 7.6 times greater than that found in the buffer outside the dialysis chamber (1.8 μCi/ml). After consecutive dialysis against buffer without GPG-NH₂ for 2 days, the p24 solution still contained 30% higher radioactivity than the dialysis buffer. These dialysis experiments, along with the capillary electrophoresis experiments, indicate that GPG-NH₂ binds to p24.

**Evaluation of virus assembly with TEM tilt series.** In the initial experiments with HUT₇₈ cells infected with HIV-1 SF-2, GPG-NH₂ was added at the time of infection. However, too few virus particles were produced to permit statistical analysis. Therefore, TEM was performed on HUT₇₈ cells to which the tripeptides were added 6 days postinfection. The cells were then cultured for an additional 4 days before being fixed for TEM. TEM was also performed on ACH-2 cells, which already carried 1 copy of HIV-1 proviral DNA and which were stimulated to produce virus by the addition of 100 nM phorbol-12-myristate-13-acetate (PMA) to the culture medium. After stimulation for 3 days, the ACH-2 cells were harvested. The results, i.e., changes in the morphology of the virus particles (Fig. 3) (see also below), obtained with ACH-2 cultures pretreated with 100 μM concentrations of the peptides for 4 days prior to stimulation to produce virus (Fig. 3f) were similar to those obtained with cultures to which a 1 mM concentration was added on the day of stimulation. Since HUT₇₈ cells pretreated with GPG-NH₂ or ALG-NH₂ produce too few virus progeny to permit statistical analysis of the changes observed by TEM, the statistical results presented here were all from cultures treated at a peptide concentration of 1 mM. As an internal reference in every experiment, TEM was performed on virus particles and HIV-1-infected cells from infected cell cultures without tripeptides. Sixty-nanometer-thick slices of sample were made to allow the accommodation of viral core structure with a proper section plane.

HIV-1 progeny produced in the presence of GPG-NH₂ showed a completely altered assembly of viral capsids (Fig. 3). In the GPG-NH₂-treated cultures, only 10% of the virus particles counted had a longitudinal sectioning of the capsid with a normal, mature filled appearance compared with 58% of the particles in untreated controls (Table 2). It should be kept in mind that virus in the treated HUT₇₈ cells had been allowed to

**TABLE 2. Statistical evaluation of electron micrographs of sectioned HIV-1 virions prepared from infected HUT₇₈ cells**

<table>
<thead>
<tr>
<th>Type of virion internal capsid packaging</th>
<th>Diagram</th>
<th>% of particles or cells showing each type*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>GPG-NH₂ treated</td>
<td></td>
</tr>
<tr>
<td>Dense conical capsid structure</td>
<td><img src="image" alt="diagram" /></td>
<td>58</td>
</tr>
<tr>
<td>Redistribution of dense material at broad end of viral capsid</td>
<td><img src="image" alt="diagram" /></td>
<td>26</td>
</tr>
<tr>
<td>Dislocation of dense material</td>
<td><img src="image" alt="diagram" /></td>
<td>14</td>
</tr>
<tr>
<td>Capsid devoid of dense material or with nodular, dense material outside</td>
<td><img src="image" alt="diagram" /></td>
<td>0</td>
</tr>
<tr>
<td>Tangential section of empty viral capsid</td>
<td><img src="image" alt="diagram" /></td>
<td>0</td>
</tr>
<tr>
<td>Redistribution of dense material to broad end of viral capsid and with a dense nodular structure outside the envelope or solely with a nodule outside the envelope</td>
<td><img src="image" alt="diagram" /></td>
<td>0</td>
</tr>
<tr>
<td>Two or more capsid structures</td>
<td><img src="image" alt="diagram" /></td>
<td>2</td>
</tr>
<tr>
<td>Nodular structure protruding from the cellular membrane</td>
<td><img src="image" alt="diagram" /></td>
<td>0</td>
</tr>
</tbody>
</table>

* Number of control particles, 107; number of GPG-NH₂-treated particles, 1,402; number of control cells, 170; number of GPG-NH₂-treated cells, 740.
replicate without the peptides for 6 days before treatment. In the GPG-NH₂-treated cultures, capsids with dislocation of material, with a dense assembly in the broad end and low density in the narrow part of the capsid structure (Fig. 3c and e), were observed. Another striking feature of viral cores from peptide amide-treated cells was irregular packaging in variable parts of the core. Occasionally, we also observed such a dense nodular structure attached to the outer part of the viral envelope (Fig. 3b) (7% of the number of virus particles with GPG-NH₂ treatment). In addition, empty viral capsids were obtained with GPG-NH₂ (Fig. 3d). Condensed circular projections located in the middle of the envelope or distributed at the edge of the envelope could represent both normal capsids and capsids that were aberrant in their narrow end and that were only slightly less abundant in the treated cells. The frequencies of misassembled virus cores (Table 2) were 90% for the GPG-NH₂-treated cells (similar results were obtained with ALG-NH₂ treatment) and 42% for the control cells. Further evaluation of the results was accomplished by a 3D computer-modeled reconstruction from the TEM tilt series of HIV-1 particles produced in the presence or absence of GPG-NH₂ (Fig. 3g and h).

**Viral nodules.** A unique feature of nodular structures was observed to be associated with the outer membranes of virus-producing cells (Table 2) (Fig. 4). These structures were found only in cells treated with GPG-NH₂ (Table 2) or ALG-NH₂. Most of the treated cells (74% of GPG-NH₂-treated ACH-2 cells; similar results were obtained with ALG-NH₂-treated cells), which carried virus particles, had these nodular structures. ACH-2 cells that were treated with 1 mM GPG-NH₂ but not stimulated with PMA to produce virus did not show such nodules. Combined treatment of PMA-stimulated ACH-2 cells with GPG-NH₂ (1 mM) and the protease inhibitor ritonavir (2 μM) produced no such nodules. In the latter experiments, only budding virus particles of normal appearance and immature virus particles were seen (data not shown). With a tilt series, it was shown that the dense nodules were protruding from the outer cell membrane. The size of the nodules, i.e., of the dense material of the irregular viral core, was approximately 50 nm.
in the present study, however, con
"our understanding of virion morphogenesis. The observations
results). Further studies on the af
the effect of GPG-NH₂ on HIV-1 probably occurs at a late
TEM tilt series of virus-infected cell cultures with GPG-NH₂
°
interfere with the formation of HIV-1 particles with a normal
core structure. The antiviral effect of tripeptide GPG-
NAH₂ could possibly involve interference with the assembly of
viral core structures in particles that have budded off from the
infected cells. This might be the case if the tripeptide competed
with Gag polyprotein and/or p24 for protein-protein interac-
tion sites. In support of this possibility, it was found in the ACE
experiments that only the amidated tripeptides interacted with
p24. These results were corroborated with the results of the
dialysis experiments with [¹⁴C]GPG-NH₂ and p24. However, it
should be emphasized that the binding of a peptide to a protein
per se does not necessarily mean that the peptide interferes
with a biological function of the protein. Further studies with
other tripeptides and other possible HIV-1 target proteins,
as well as estimations of binding constants, will be of interest.
Also, the internal pH of the virus particle should be deter-
mined since it very likely differs from that of the surrounding
medium. In the present study, it was not known whether the
CA protein p24 in the various experiments was in the form of
monomers or dimers, which are formed under certain condi-
tions (10, 15). It should also be pointed out that whether the
binding of the amidated tripeptides to the capsid protein is in
fact affecting p24-p24 interactions, and thus interfering with
capsid assembly, cannot definitely be deduced from the present
study. Indeed, it remains to be shown that the disarranged
virus particles contain the antiviral tripeptide.

It is possible that the tripeptides bind to another protein
contained in the virus particle, such as CyPA, or that binding of
tripeptides to p24 interferes with its binding to another protein
necessary for capsid formation. Recently, it was reported that
CyPA binds with high affinity to a peptide corresponding to
p24 amino acids 214 to 228 (10) that contains a GP motif
(residues 223 and 224), which is a prerequisite for CyPA bind-
ing (11, 14, 39, 47, 48), that coincides with the site of the
carboxyl-terminal GPG (residues 223 to 224) sequence of p24.
However, it has been shown previously that the dipeptide GP-
NH₂ in contrast to the dipeptide PG-NH₂ does not inhibit
HIV-1 replication (35). Furthermore, preliminary ACE studies
on CyPA−GPG−NH₂ interactions have shown that this protein
binds GPG-NH₂ less well than does p24 (our unpublished
results). Further studies on the affinity of the tripeptides for
both cellular and viral structural proteins might contribute to
our understanding of virion morphogenesis. The observations
in the present study, however, confirm our earlier findings that
the effect of GPG-NH₂ on HIV-1 probably occurs at a late
stage of the viral replication cycle. Additionally, in vitro studies
using electron microscopy and dynamic light scattering have
shown that GPG-NH₂ can hinder the self-assembly of tubular
structures of p24 (D. van der Spoel, C. Hetényi, A. McKenzie-
Hose, L. Ågren, L. Goobar-Larsson, S. Högglund, and A.
Vahlne, submitted for publication).

A striking finding in the present study was the observation of
dense nodular structures at the plasma membrane of HIV-1-
infected cells treated with GPG-NH₂ or ALG-NH₂. No such
structures were seen in untreated infected cells or in GPG-
NH₂-treated ACH-2 cells, which were not stimulated to pro-
duce virus. The nodules contain the capsid protein p24, as
determined by immune electron microscopy. Their size and
density were similar, if not identical, to those of the broad end
of the mature HIV-1 core structure. Possibly, these nodules
represent virus particles that were arrested in their budding. If
so, activation of the HIV-1 protease in such virus structures
arrested at the plasma membrane might have cleaved off the
p17 matrix protein, allowing the remaining polyprotein to con-
dense, generating incomplete capsid structures trapped in the
plasma membrane. Notably, no such nodular structures were
observed after combined treatment with GPG-NH₂ and an
inhibitor of the viral protease.

New classes of antiviral pharmaceuticals are warranted in
the fight against the AIDS epidemic. In the future, small pep-
tides or peptidomimetic molecules derived from the peptides
described in the present paper might prove useful for the
treatment of HIV-infected individuals.

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