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Effect of Retroviral Proteinase Inhibitors on Mason-Pfizer Monkey Virus Maturation and Transmembrane Glycoprotein Cleavage

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Mason-Pfizer monkey virus (M-PMV) is the prototype type D retrovirus which preassembles immature intracytoplasmic type A particles within the infected cell cytoplasm. Intracytoplasmic type A particles are composed of uncleaved polyprotein precursors which upon release are cleaved by the viral proteinase to their constituent mature proteins. This results in a morphological change in the virion described as maturation. We have investigated the role of the viral proteinase in virus maturation and infectivity by inhibiting the function of the enzyme through mutagenesis of the proteinase gene and by using peptide inhibitors originally designed to block human immunodeficiency virus type 1 proteinase activity. Mutation of the active-site aspartic acid, Asp-26, to asparagine abrogated the activity of the M-PMV proteinase but did not affect the assembly of noninfectious, immature virus particles. In mutant virions, the transmembrane glycoprotein (TM) of M-PMV, initially synthesized as a cell-associated gp22, is not cleaved to gp20, as is observed with wild-type virions. This demonstrates that the viral proteinase is responsible for this cleavage event. Hydroxyethylene isostere human immunodeficiency virus type 1 proteinase inhibitors were shown to block M-PMV proteinase cleavage of the TM glycoprotein and Gag-containing precursors in a dose-dependent manner. The TM cleavage event was more sensitive than cleavage of the Gag precursors to inhibition. The infectivity of treated particles was reduced significantly, but experiments showed that inhibition of precursor and TM cleavage may be at least partially reversible. These results demonstrate that the M-PMV aspartyl proteinase is activated in released virions and that the hydroxyethylene isostere proteinase inhibitors used in this study exhibit a broad spectrum of antiretroviral activity.

Mason-Pfizer monkey virus (M-PMV), defined as the prototype type D retrovirus, exhibits pathogenicity in the genus *Macaca* of nonhuman primates. Although previously isolated from a mammary tumor (5), M-PMV is not oncogenic; instead, infected primates succumb to a severe and often fatal immunosuppressive disease (3, 10) which is distinct from those associated with lentiviruses such as human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus. Members of the type D virus subgroup of oncoviruses are characterized by the formation of intracytoplasmic type A particles (ICAPs) within the infected cell cytoplasm which migrate to the plasma membrane and are released by budding. This is in contrast to the type C oncoviruses, which assemble and bud simultaneously at the plasma membrane. M-PMV belongs to an enlarging family of exogenous type D simian retroviruses which can be distinguished from one another by their neutralization characteristics. This has necessitated a new nomenclature based on the viral serotype. M-PMV is therefore synonymous with simian retrovirus type 3.

The genomic organization of M-PMV consists of four genes in the order 5′ *gag-pro-pol-env* 3′ (24), where *gag* encodes the structural genes, *pro* encodes the viral proteinase, *pol* encodes the viral polymerase (reverse transcriptase [RT]), and *env* encodes the envelope glycoproteins. Expression of the *gag* gene results in the biosynthesis of a polyprotein precursor, Pr78, in which the viral structural proteins are arranged as H_2N-p10(MA)-pp24/16-p12-p27(CA)-p14(NC)-p4-COOH. To express the *pro* gene product, a ribosomal frameshift into the −1 reading frame must occur near the end of *gag* to form the Gag-Pro precursor Pr95. The *pol* gene product is similarly expressed as Pr180 after two frameshifting events, one at the end of *gag* and one at the end of *pro*. The envelope gene, in contrast, is translated from a spliced mRNA to yield a glycosylated precursor protein, Pr86. This precursor is cleaved to the surface glycoprotein (SU) gp70 and the transmembrane glycoprotein (TM) gp22 by a cellular endopeptidase within the Golgi complex of the cell.

It is the self-assembly of the Gag, Gag-Pro, and Gag-Pro-Pol precursors within the cytoplasm that results in ICAP formation. During or shortly after particle release, the viral proteinase is activated and cleaves the precursor polypeptides to their constituent proteins. This results in a morphological change in the virus particle, and the electron-lucent core of the ICAP is converted to the dense, rod-shaped core of the mature virion. This proteolytic event and the morphological change, described as maturation, are irreversible and are required in other retroviruses for viral infectivity.

The M-PMV proteinase is defined as an aspartyl proteinase because it contains the motif Asp-Thr-Gly as do other retroviral proteinases (24). The proteinase-coding domain, shown in Fig. 1, encodes a protein of 314 amino acids which is cleaved by the viral proteinase; the N-terminal region has dUTPase activity, and the C-terminal region of 154 amino

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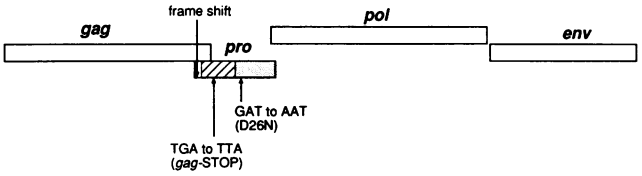

FIG. 1. Genomic organization of M-PMV. *gag* encodes the structural genes, *pro* encodes the viral proteinase, *pol* encodes the RT, and *env* encodes the envelope glycoproteins. The proteinase-coding domain is composed of two regions which are cleaved apart on maturation; the N-terminal region (striped) has dUTPase activity, while the C-terminal region (shaded) has proteinase activity. The location of each point mutation is shown by arrows.

acids has proteinase activity (26). Amino acids 26 to 28 in the C-terminal cleavage product contain the Asp-Thr-Gly motif. It has been shown for both HIV-1 and Rous sarcoma virus that the aspartic acid within this motif is critical for proteinase activity (11, 14, 25). Retroviral proteinases have been shown to be autocatalytically cleaved from precursors when expressed in heterologous systems, including bacterial, yeast, and insect cells (15, 21), but in the infected cell, this is normally a late event in the virus assembly process. In M-PMV, the activation process is particularly tightly controlled, since proteinase precursors preassembled into ICAPs are not activated while the capsid remains in the cytoplasm (23), unlike the proteinase of HIV-1, which can be activated intracellularly (11, 12).

Mutagenesis of the active-site residues in several type C viruses has been shown to abrogate the activity of the proteinase (11, 14, 22), rendering mutant virions noninfectious. We sought to determine the role of the M-PMV proteinase in viral assembly and morphogenesis by using oligonucleotide-directed point mutagenesis to convert aspartic acid residue 26 to an asparagine. We also investigated whether the proteinase and polymerase precursor proteins were required in the morphogenesis of M-PMV by inserting a stop codon at the end of the *gag* gene so that, after the frameshifting event, the proteinase gene could not be expressed. In this way, only ICAPs composed of Pr78 could be generated. To explore further the process of proteolysis, we have investigated whether peptide analogs, designed to mimic the transition state of the reaction catalyzed by the HIV-1 proteinase and shown to potentially inhibit that enzyme, could interfere with M-PMV precursor proteolysis. For these studies, we used two synthetic inhibitors containing a hydroxyethylene isostere replacement of the sissile bond (8, 9, 18).

The results of these studies demonstrate that the product of the *gag* gene alone is sufficient for immature capsid assembly-budding and that the viral proteinase is responsible for not only cleavage of the Gag-containing precursors, but also processing of the cytoplasmic domain of the M-PMV TM glycoprotein. Moreover, inhibitors designed for the HIV-1 proteinase can be used to efficiently block processing of both *gag* and *env* gene products of M-PMV, opening up the possibility of in vivo studies of their effect on simian retrovirus-induced immunosuppression.

MATERIALS AND METHODS

Cells. Uninfected African green monkey kidney cells, COS-1 (ATCC CRL 1650), and human osteosarcoma cells (HOS) (ATCC CRL 1543) were grown in Dulbecco minimal essential medium supplemented with 10% fetal bovine serum. Rhesus monkey CMMT cells (ATCC CRL 6299), a cell line chronically infected with M-PMV derived by cocultivating the original mammary tumor with rhesus monkey placental cells, were grown in RPMI medium supplemented with 10% fetal bovine serum, 5% tryptose phosphate broth, and 0.05% sodium bicarbonate. Cells were maintained at 37°C in 5% CO_2 incubators.

Mutagenesis. Oligonucleotides of 22 and 24 nucleotides were designed to convert the active-site aspartic acid (proteinase residue 26) to asparagine (GAT to AAT) and to generate a termination codon within the −1 reading frame immediately after the end of *gag* (TGA to TTA) by mutagenesis. These mutants will be referred to as D26N and *gag*-STOP, respectively. A 2.7-kb *Sac*I-to-*Pst*I fragment, corresponding to nucleotides 1163 to 3660 of the viral genome, was cloned into M13mp19 from the full-length infectious molecular clone pSHRM15 and used as a substrate for mutagenesis. The method was based on that of Kunkel (17), in which recombinant M13mp19 DNA containing the fragment of interest was transformed into *Escherichia coli* CJ236 (F′ *ung dut*) cells and single-stranded DNA was isolated. After DNA synthesis with the mutagenic primers, *E. coli* DH5αF′ (*dut*$^+$ *ung*$^+$) cells were transformed and single-stranded DNA was prepared from each plaque. Mutant recombinant M13 was identified by DNA sequencing. The mutated *Sac*I-to-*Pst*I fragment was then subcloned back into pSHRM15.

Proteinase inhibitors. Two peptides, SKF107461 (molecular weight [MW], 711) and SKF109026 (MW, 511), have been shown to inhibit precursor processing in HIV-1-infected T lymphocytes at concentrations of 10 to 20 and 1 μM, respectively (8, 20). The control peptide GBD 16737-160 was pepstatin A (MW, 686), previously shown to be inactive in inhibiting precursor processing in HIV-1-infected T cells (20). The structure of each peptide is as follows: SKF107461, Cbz-Ala-Ala-Phe-Psi[CH(OH)-CH2]Gly-Val-Val-OCH_3; SKF109026, Boc-Phe-Psi[(S)-CH(OH)-CH_2]-Phe-Val-NH_2. The peptides were dissolved in 100% dimethyl sulfoxide (DMSO) to 10 mM and subsequently diluted in culture medium to the concentrations described later in the text.

Transfection. COS-1 cells were transfected with either the infectious genome of M-PMV in a plasmid that contained a simian virus 40 origin of replication (pSHRM15) or pSHRM15 into which the mutations had been subcloned. The transfection protocol used was based on that of Chen and Okayama (4).

Pulse-chase immunoprecipitation. Duplicate plates of cells transfected or infected with M-PMV were pulse-labeled with 100 μCi of [^{3}H]leucine (200 Ci/mM; Dupont, NEN) as described previously (1). In addition, the relevant concentrations of inhibitor (SKF109461 or SKF109026), control peptide (pepstatin A), or DMSO were present throughout.

Infectivity assays. The culture medium from cells transfected with either of the site-directed mutants was harvested on the third day posttransfection. An RT assay was performed with 3 ml of the supernatant to give counts per minute per milliliter of culture medium. Equivalent counts per minute were added in a volume of 2 ml to human HOS cells that had been seeded the day before on 100-mm-diameter tissue culture dishes and were approximately 60% confluent. The infection was allowed to proceed at 37°C with rocking for 2 h before 5 ml of medium was added, and the cells were incubated for 2 days at 37°C before being passaged. The cells were maintained and passaged every fourth day, with the

supernatant being harvested at each passage, and were stored at −100°C for later RT assays.

In order to study the effect of proteinase inhibitors on M-PMV infectivity, COS-1 cells transfected with pSHRM15 were incubated continuously in 0, 20, or 100 μM concentrations of SKF109026 at 24 h posttransfection and inhibitor-containing medium was replenished each day. The virus-containing culture medium, corresponding to 48 to 72 h posttransfection, was removed and incubated with HOS cells for 2 h and then replaced with inhibitor-free medium. The cells were passaged every fourth day, and cell supernatants harvested at these times were stored at −100°C.

RT assay. Three-milliliter volumes of infected cell culture supernatant were clarified by centrifugation at 8,000 rpm for 20 min (4,000 × *g*) before being assayed for RT activity as described previously (23).

M-PMV proteolytic processing in virions following the removal of inhibitor. Cells were transfected with pSHRM15 as previously described. At 72 h posttransfection, the cells were starved of leucine and pulse-labeled in the presence of 0, 20, and 100 μM SKF109026. Cells transfected with the proteinase mutant D26N were used as a control. Following a 4-h chase period in the presence or absence of inhibitor, particles were pelleted by centrifugation at 80,000 rpm for 30 min in a TL100 rotor of the Beckman tabletop centrifuge and resuspended in serum-free medium to remove the inhibitor. One aliquot was immunoprecipitated immediately (time 0), and additional aliquots were immunoprecipitated following 6 and 18 h of incubation at 37°C. After immunoprecipitation, the samples were electrophoresed on a 12% polyacrylamide gel and their protein profiles were determined.

Electron microscopy. Cells transfected with pSHRM15 were treated with a 100 μM concentration of inhibitor for 24 h. The cells were fixed in glutaraldehyde and postfixed with osmium tetroxide. The samples were then dehydrated and embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate.

RESULTS

Mutagenesis of the M-PMV proteinase. Both the aspartate-to-asparagine mutant and the *gag*-STOP mutant, shown in Fig. 1, were subcloned into the full-length infectious molecular clone of M-PMV, pSHRM15. This plasmid contains a simian virus 40 origin of replication so that after transfection into COS-1 cells there is transient high-level expression of viral proteins. Figure 2 shows the protein profiles in cells expressing wild-type, D26N, and *gag*-STOP mutant genomes. During a 20-min pulse in wild-type transfected cells, all three Gag-containing precursors (Pr78, Pr95, and Pr180) and the glycoprotein precursor (Pr86) were labeled. After a 4-h chase, significant processing of the precursors was observed, and cell-associated cleavage products p27(CA), p14(NC), p12, and p10(MA) were found (Fig. 2, lane C of WILD TYPE). During the chase, the envelope glycoprotein precursor was processed to gp70 and gp22. The released virions contained the cleaved Gag products as well as gp70(SU) and gp20(TM). The TM glycoprotein of M-PMV is initially synthesized as a cell-associated gp22 which is cleaved to gp20 found on released virions, as has been shown previously by using a monoclonal antibody to gp20 (2).

The proteinase mutant, D26N, showed a protein profile similar to that of the wild type in immunoprecipitates of pulse-labeled cells (Fig. 2, lane P of D26N), but no Gag cleavage products could be seen after a 4-h chase. Nevertheless, significant amounts of all three Gag-containing precursor proteins are found in released virus particles after the chase (Fig. 2, lane M of D26N), demonstrating that immature particles can be efficiently released in the absence of proteolysis. We also observed that cleavage of gp22 to gp20 does not occur in the proteinase mutant virions, demonstrating that it is the viral proteinase itself and not a cellular endopeptidease that is responsible for this event.

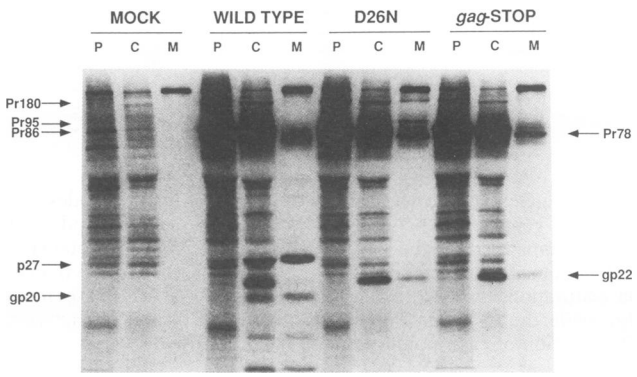

FIG. 2. Pulse-chase immunoprecipitation of proteinase mutants after transfection into COS-1 cells. For wild-type M-PMV, polyprotein precursors are labeled during the 20-min pulse (lanes P) and are cleaved to their constituent proteins following a 4-h chase (lanes C), and the protein profile of released virions is shown in the chase medium (lanes M). For the D26N mutant, after the 4-h chase, the precursors remain uncleaved and virions comprised of uncleaved precursors are present in the medium. The *gag*-STOP mutant shows particles composed only of uncleaved Pr78 precursors. gp22(TM) is similarly not cleaved to gp20(TM) in mutant virions.

In the *gag*-STOP mutant, efficient production of virions containing only uncleaved Pr78 (along with gp70 and gp22) was observed. Unlike with the proteinase mutant, no Pr95 or Pr180 was evident in these mutant virions. Also, because no proteinase was present in these particles, no conversion of gp22 to gp20 was seen.

The infectivities of both mutants were assayed by determining RT activities released from HOS cells inoculated with transfection supernatants of each mutant. RT activity increasing over time was observed only for wild-type virus, whereas RT levels for both mutants remained at the level of that for the mock-infected control (Fig. 3). These results show that a functional proteinase enzyme is critical for virus infectivity.

Assessing the effects of different proteinase inhibitors on M-PMV expressed from COS-1 and CMMT cells. CMMT cells, chronically producing M-PMV, and COS-1 cells transfected with M-PMV were pulse-labeled in the presence of 20 μM concentrations of each peptide analog to determine which served as a more potent inhibitor of M-PMV proteolytic processing. Figure 4A shows the results of a pulse-chase experiment in the presence and absence of each peptide analog in COS-1 cells transfected with the wild-type M-PMV genome. Cleavage products in DMSO-treated cells and in cells treated with the control peptide were observed; p27(CA), gp70(SU), and gp20(SU) could be detected in released virions (Fig. 4A, lanes M of DMSO and Control Peptide). Immunoprecipitates of transfected cells and released virions labeled in the presence of peptide analogs SKF107461 and SKF109026 showed significantly reduced levels of p27 and the presence of an incomplete cleavage product, p45, that is not seen in untreated cells. A doublet

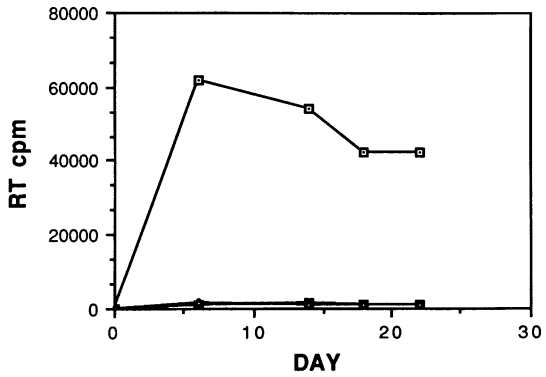

FIG. 3. Human HOS cells were inoculated with supernatants from COS-1 cells transfected with wild-type (⊡) and mutant (◆ [D26N] and ◘ [*gag*-STOP]) M-PMV. RT activity was measured over time. Only wild-type M-PMV showed an increase in RT activity with time, whereas, as with the mock-infected control (◆), there is negligible RT activity for either mutant with time, showing that both mutants are noninfectious.

corresponding to Pr78 could be seen comigrating with the diffuse gp70 band in inhibitor-treated virions.

This result suggested that at 20 μM concentrations of these inhibitors, there was incomplete processing of the Gag precursors. Immunoprecipitation with an anti-p27 polyclonal antibody showed that the p45 product contained p27 epitopes (data not shown). Antisera to individual *gag* gene products, currently not available, will be required to determine which other *gag* gene product is fused to p27 in this p45 protein. Interestingly, inhibition of gp22 cleavage appeared to be complete at this concentration of inhibitor, suggesting that this cleavage event was more sensitive to inhibition than that of the *gag* gene precursor proteins.

When CMMT cells were pulse-labeled in the presence of 20 μM concentrations of both SKF107461 and SKF109026 (Fig. 4B), both p27 and incomplete cleavage products in addition to p45 are evident (Fig. 4B, lanes C and M of SKF-107461 and SKF109026), suggesting that either the peptide-based inhibitors had a faster turnover rate in this cell line or that the cells were not as permeable to the inhibitors as COS-1 cells. Nevertheless, gp22 cleavage was completely blocked by both inhibitors in this experiment (Fig. 4B). Although both SKF107461 and SKF109026 appeared to be equally active against the M-PMV proteinase, SKF109026 was arbitrarily chosen for further studies because of its potent activity against the HIV-1 proteinase (8).

In order to determine the concentration of inhibitor at which complete inhibition of the M-PMV proteinase could be observed, pulse-chase experiments were carried out in the presence of increasing concentrations of inhibitor. Figure 4C shows immunoprecipitates of the culture medium from CMMT cells after a 4-h chase period in the presence of 1, 5, 10, 20, 40, 60, and 100 μM concentrations of inhibitor SKF109026. To completely inhibit gp22 cleavage, 20 μM concentrations of inhibitor were required (Fig. 4C), while 100 μM concentrations were required to completely inhibit production of the p27 cleavage product. Interestingly, in CMMT cells it was not possible to inhibit cleavage of the Gag precursors completely (higher concentrations of inhibitor were insoluble), whereas in COS-1 cells, a concentration of 100 μM SKF109026 completely inhibited cleavage of M-PMV Gag precursors (see Fig. 5).

Analysis of SKF109026 inhibition of proteolysis with time. In order to more carefully analyze the incorporation of uncleaved precursor proteins into virions, a pulse-chase experiment in which COS-1 cells transfected with pSHRM15 were treated with a concentration (100 μM) of SKF109026 which completely blocked precursor cleavage was carried out. Cells were labeled for 20 min and then chased for 2, 4, or 6 h in the absence or presence of inhibitor. Viral proteins released into the culture medium were immunoprecipitated and separated on a sodium dodecyl sulfate–12% polyacrylamide gel. The results summarized in Fig. 5 show that virions released in a 2-h chase from DMSO-treated cells contained essentially equal levels of gp22 and gp20. The gp20/gp22 ratio increased with time, with gp22 cleavage completed after a 6-h chase. In contrast, in virus from inhibitor-treated cells, no Gag cleavage products could be detected at 2 h and particles were composed of the three protein precursors, Pr78, Pr95, and Pr180. Moreover, there was complete inhibition of gp22 cleavage. At 4- and 6-h chase times, faint bands corresponding to intermediate cleavage products as well as p14 and p10 could be seen associated with the released virus, but no p27 was observed. Even at these later chase times, no cleavage of gp22 was seen.

The virions released from COS-1 cells treated with 100 μM inhibitor possessed the morphology of immature particles with electron-lucent cores (Fig. 6E) and were indistinguishable from the virions released from mutant D26N or *gag*-STOP transfected cells (Fig. 6C and D) or immature untreated M-PMV (Fig. 6A). No mature particles with the electron-dense core typical of those released from untreated cells (Fig. 6B) could be seen in the mutant or treated cultures.

Infectivity of particles treated with 20 and 100 μM concentrations of SKF109026. Infection of HOS cells with the culture medium from DMSO-treated, pSHRM15-transfected COS-1 cells resulted in detectable RT activity by 6 days postinfection (Fig. 7). In contrast, no significant RT activity from cells infected with inhibitor-treated virions could be detected until day 14. Viruses treated with a 20 μM concentration of SKF109026 showed a rise in RT earlier than that of particles treated with 100 μM concentrations. These results suggest that some virions, which were not detectable on immunoprecipitation gels, had escaped the effects of the inhibitor or that a reversible interaction of the inhibitors with the viral proteinase allowed the emergence of infectious virions.

M-PMV polyprotein processing in released virions after removal of inhibitor. Since the infectivity assays raised the possibility that proteinase inhibition might be reversible, the protein profiles of particles released from cells treated with 20 and 100 μM SKF109026 were determined after incubation in the absence of inhibitor. Figure 8 shows that after 6 and 18 h at 37°C and after the removal of 20 μM SKF109026, there was a progressive emergence of p27 with time. Also, low levels of gp20 in addition to gp22 were detected after 18 h. In particles treated with 100 μM concentrations, a variety of incomplete cleavage products were observed after 6 h of incubation, but no significant levels of p27 were observed even after 18 h, suggesting that in the presence of higher concentrations of SKF109026, significant activity of the proteinase was not observed in the time frame of this experiment. In similar experiments in which released virions were monitored over time without the inhibitor being removed, a similar pattern of proteolytic processing was observed (data not shown). The proteinase mutant particles contained only the precursor proteins even after 18 h,

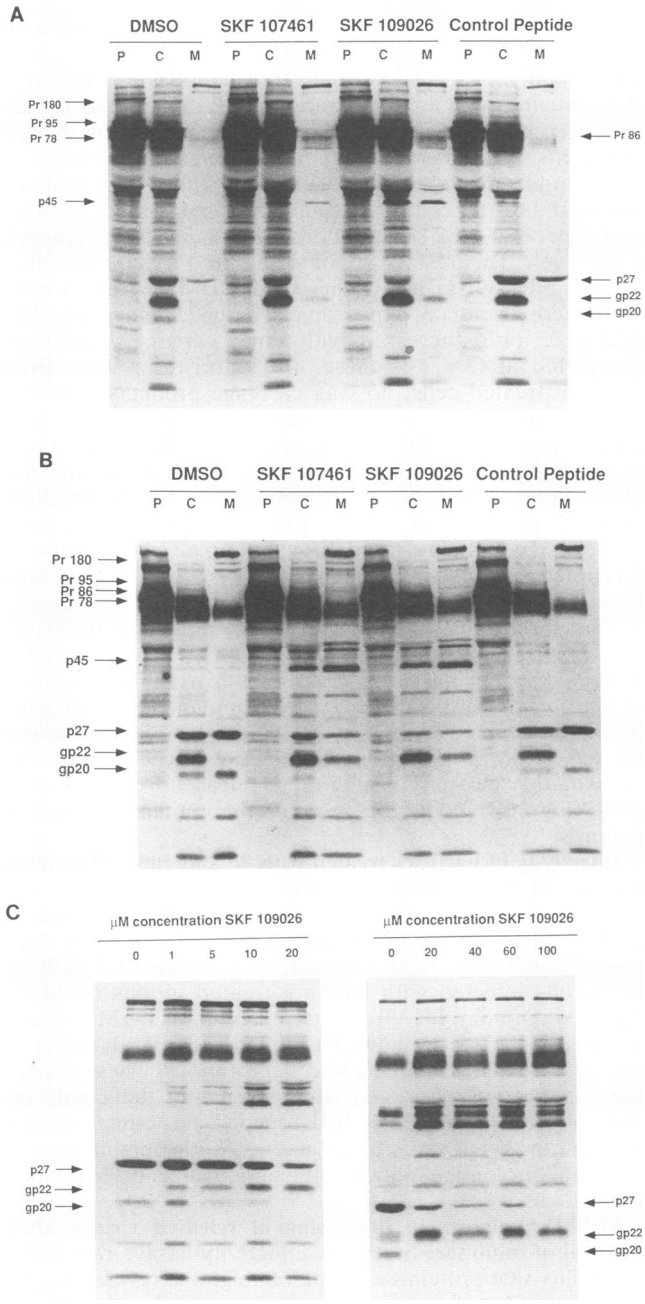

FIG. 4. Immunoprecipitations of COS-1 cells, CMMT cells, and virus particles. (A) Immunoprecipitation with polyclonal antiserum to M-PMV of COS-1 cells transfected with the full-length infectious molecular clone of M-PMV pSHRM15 and treated with 20 μM concentrations of each inhibitor and control peptide. Lanes: P, 20-min pulse; C, 4-h chase period; M, medium harvested from the chase dish. For DMSO-treated cells and cells treated with the control peptide (pepstatin A), M-PMV processing is normal. Inhibition of gp22(TM) cleavage to gp20(TM) is complete at this concentration for both inhibitors. Gag cleavage remains incomplete with the emergence of a p45 incomplete cleavage product. (B) Immunoprecipitation of CMMT cells chronically infected with M-PMV and treated with 20 μM concentrations of proteinase inhibitors precipitated with a goat anti-M-PMV antibody. Proteolytic processing within DMSO- and control peptide-treated virions is complete; however, p45 and other incomplete *gag* cleavage products can be seen in virions treated with inhibitors SKF109026 and SKF107461. Inhibition of gp22(TM) cleavage to gp20(TM) is also complete at this concentration. Lanes are as described in the legend to panel A. (C) Immunoprecipitation of virus particles from CMMT cells treated with increasing concentrations of inhibitor SKF109026. It is evident that 20 μM concentrations of inhibitor are required to completely inhibit TM glycoprotein cleavage, as at more than this concentration, particles contain only the uncleaved gp22. As the level of the p27 cleavage products decreases, there is an increase in incomplete Gag cleavage products not present in untreated virions (lane 0).

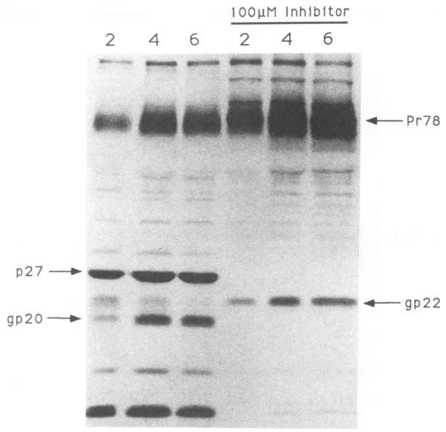

FIG. 5. Immunoprecipitation of virions harvested from COS-1 cells transfected with pSHRM15 over 2-, 4-, and 6-h chase periods in the absence and presence of 100 μM concentrations of inhibitor SKF109026. It is evident that, in untreated virions, TM cleavage is a late event, as released particles initially contain both gp22 and gp20. With time, cleavage is completed and virions contain predominantly gp20. Inhibition of both TM and Gag cleavage is complete in this system with 100 μM concentrations of inhibitor, and particles composed of all Gag precursors and only uncleaved TM proteins are obtained even after a 4-h chase period.

confirming that activation of the viral proteinase is absolutely required for precursor cleavage and that nonspecific degradation of polyproteins was not occurring. Particles from untreated, wild-type infected cells showed a mature protein profile that did not change significantly with time. Thus, it appears that SKF109026-mediated inhibition of precursor cleavage was either partially reversible with an increasing time of incubation in the absence of drug or, alternatively, that the protease enzyme active sites were not saturated even at 100 μM concentrations of inhibitor, resulting in a low level of proteolytic processing that continues whether or not inhibitor was present. We do not know, however, whether released virus particles are permeable to exogenous inhibitor.

DISCUSSION

Proteolytic cleavage events appear to be essential control mechanisms that are utilized by a number of different virus groups in the process of virus assembly and release. A proteolytic cleavage event results in an irreversible change in the virion and commits the particle to an infectious cycle rather than merely a reversal of the assembly process itself (16).

By using oligonucleotide-directed mutagenesis, we have inhibited both the expression and the activity of the M-PMV proteinase and demonstrated that this resulted in the release of noninfectious immature particles. In addition, these ex-

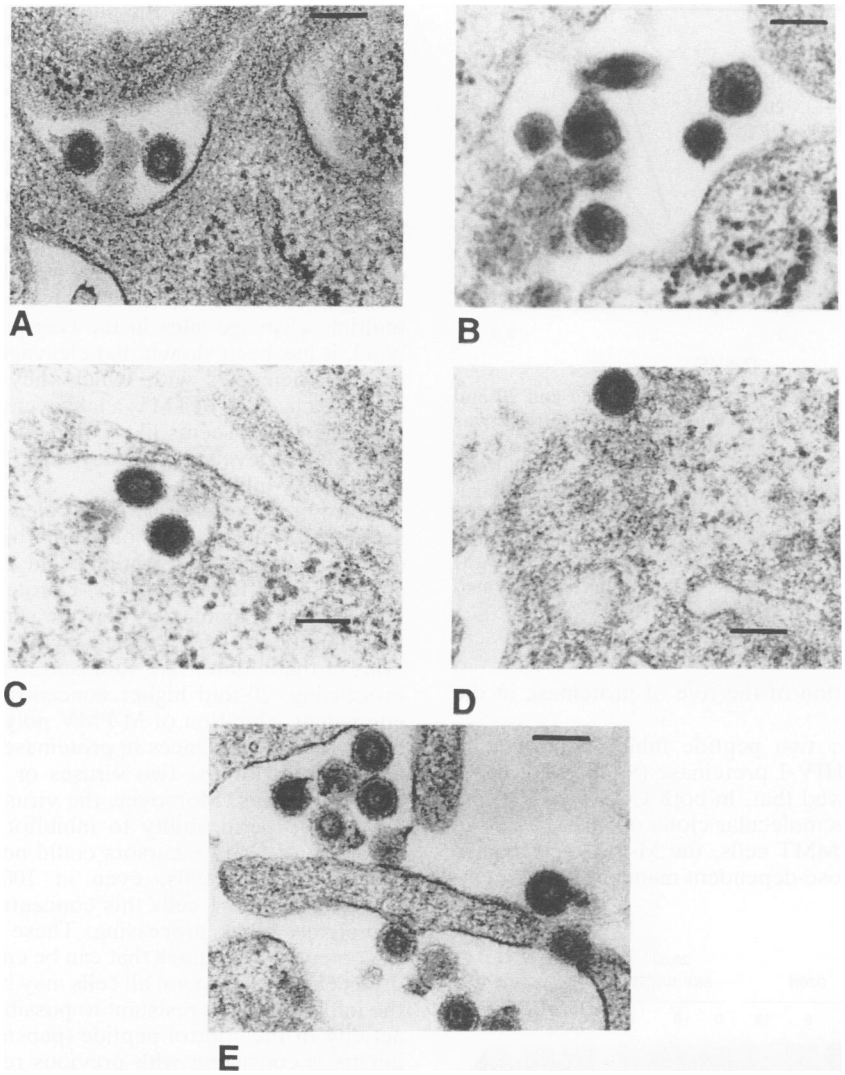

FIG. 6. Electron micrograph of M-PMV. Bars, 130 nm. (A) Immature untreated particles having electron-lucent cores released into the supernatant. (B) Mature untreated M-PMV particles demonstrating the morphological change accompanying proteolysis. The cores have become electron dense. (C) *gag*-STOP mutant particles composed only of Pr78 precursors having immature morphology. (D) Morphology of the D26N mutant of M-PMV, from which only immature virus particles are released. (E) Morphology of M-PMV particles treated with 100 μM concentrations of inhibitor SKF109026. Only immature particles with electron-lucent cores were observed.

periments brought to light a new substrate for the M-PMV proteinase. Retroviral proteinases are predominantly associated with the cleavage of the Gag-containing polyprotein precursors, but in addition, the M-PMV proteinase cleaves the viral TM glycoprotein from a predominantly cell-associated gp22 to a predominantly virus-associated gp20. This cleavage resembles that described for murine leukemia virus, in which the viral proteinase has been shown to cleave p15E(TM) to p12E(TM) (6, 13). The biological significance of this cleavage event has been investigated further and will be discussed in more detail elsewhere (2).

Expression of the M-PMV proteinase can be prevented by introducing a termination codon within the proteinase reading frame at the end of the *gag* gene so that the Pr95 and Pr180 precursors are not synthesized. Nevertheless, in the absence of these products, capsids can assemble within the cytoplasm and are efficiently released from the plasma membrane. As might be expected, neither the *gag*-STOP nor the D26N mutation had an effect on Pr86 precursor cleavage to gp70(SU) and gp22(TM), since this is carried out by a cellular endopeptidase in the Golgi. While cleavage of the envelope glycoprotein precursor is required for retroviral infectivity, it is not sufficient, since the proteinase mutant virions are noninfectious. As in other retroviruses (13, 14), cleavage of the type D virus capsid precursors is also a prerequisite for virus infectivity.

Mutagenesis of the proteinase of the prototype type D retrovirus, M-PMV, formally demonstrates that, as with the proteinases of the type C oncoviruses and lentiviruses, the M-PMV enzyme is an aspartyl proteinase in which aspartic acid residue 26 is critical for activity. This was also supported by the sensitivity of the M-PMV enzyme to peptide-analog inhibitors that were designed to inhibit the HIV-1 aspartyl proteinase. Because mutagenesis provides an irreversible and complete block to proteinase activity, we have explored the use of inhibitors which have the potential of

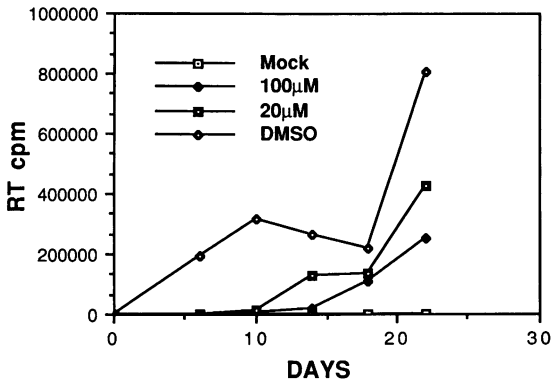

FIG. 7. Infectivity of particles treated with DMSO and 20 and 100 μM concentrations of inhibitor SKF109026. RT activity was monitored over time. The level of RT activity present in supernatants from infections with DMSO-treated virus increased with time. Supernatants from cells infected with inhibitor-treated virus particles had negligible RT activity until day 14 postinfection, after which RT activity was seen to increase. RT activity increased more rapidly for cells inoculated with virus that had been treated with 20 μM concentrations of inhibitor SKF109026 than for particles treated with the higher dose of 100 μM SKF109026.

providing a finer dissection of the role of proteinase in the replication process.

In the present study, two peptide inhibitors originally designed to inhibit the HIV-1 proteinase (8, 18) were used. These experiments showed that, in both COS-1 cells transfected with an infectious molecular clone of M-PMV and in productively infected CMMT cells, the M-PMV proteinase could be inhibited in a dose-dependent manner. The proteinase was confirmed as the site of action of these inhibitors because virions produced from transfected COS-1 cells in the presence of 100 μM concentrations of inhibitor had the same protein profile as the M-PMV proteinase mutant D26N. Interestingly, cleavage of the TM protein from gp22 to gp20 appeared to be more sensitive to inhibition by SKF109026 than was cleavage of the Gag-containing precursors; at a 20 μM concentration of drug, inhibition of gp22 cleavage was complete, while cleavage of the *gag* gene products was incompletely blocked. The enhanced sensitivity of TM cleavage to inhibitor could have been because there was only a single sissile bond in the TM glycoprotein, compared with multiple cleavage sites in the Gag precursor. On the other hand, it has been shown that cleavage sites for HIV-1 differ in the efficiencies with which they are cleaved (7), and because cleavage of TM is a late event that often follows Gag cleavage (2), it seems likely that the site in TM is cleaved with a lower level of efficiency than those in Gag and is thus more easily inhibited.

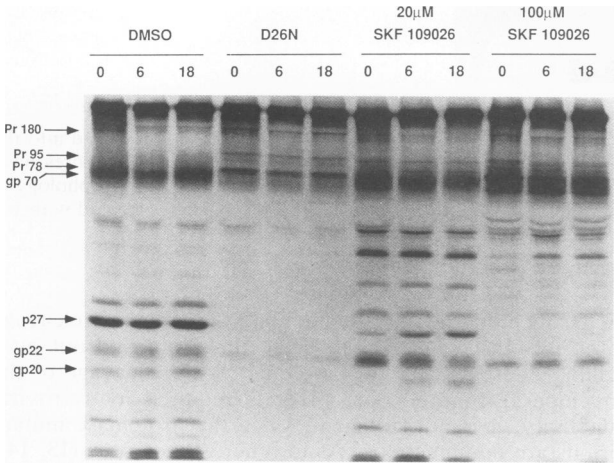

FIG. 8. Immunoprecipitation of particles from COS-1 cells transfected with pSHRM15 harvested after a 4-h chase period in the presence of 0, 20, and 100 μM concentrations of SKF109026. Virions were pelletted and resuspended in inhibitor-free medium, and particles were immunoprecipitated either immediately (0) or after 6 or 18 h of incubation at 37°C. The mutant D26N was used as a control. There was no degradation of wild-type particles, as p27 remained stable over time; the D26N precursors also remained stable. Virions treated with 20 μM concentrations of inhibitor at time 0 showed incomplete cleavage products which were cleaved over time. Similarly, virions treated with 100 μM concentrations of inhibitor initially were composed of uncleaved precursors (time 0). At 6 and 18 h, incomplete cleavage products were evident.

Retroviral proteinases do not cleave at a specific peptide sequence in all proteins but show high specificity for their own native precursor proteins. The structures of the active sites in the M-PMV and HIV-1 proteinases must, however, be sufficiently similar so that the same synthetic inhibitors will inhibit both. Nevertheless, while micromolar concentrations of the inhibitor are sufficient to block HIV-1 precursor processing, 20-fold higher concentrations are required for equivalent inhibition of M-PMV polyprotein cleavage. This could reflect differences in proteinase structure or substrate accessibility for the two viruses or their distinct morphogenic pathways. Moreover, the virus-infected cell type and its relative permeability to inhibitor may also be factors. Cleavage of Gag precursors could not be completely inhibited in CMMT cells, even at 100 μM concentrations, whereas in COS-1 cells this concentration was sufficient to completely block processing. These observations highlight the potential difficulties that can be encountered in vivo with drug delivery, since not all cells may be equally permeable to the inhibitors or as resistant to possible toxicity. The lack of activity of the control peptide (pepstatin A) in these experiments is consistent with previous results with HIV-1 (20), which may also be a reflection of the permeability of cells to pepstatin A.

The proteinase inhibitor SKF107461 used in the experiments described here was shown to abrogate the infectivity of HIV-1 released from treated cells when assayed over a 10-day period (18). Similar results were obtained when M-PMV virions released from COS-1 cells treated with either 20 or 100 μM SKF109026 were assayed for infectivity; RT activity in cells infected with virions from treated cultures was not detectable for up to 10 days postinfection. However, a rapid increase in RT activity was observed by day 14 in cultures infected with medium from cells treated with 20 μM inhibitor, and a similar increase was seen after day 16 with the 100 μM supernatants. These results suggested that a small fraction of particles had escaped the effects of the inhibitor or that the inhibitory effect of SKF109026 on the M-PMV proteinase was incomplete. The latter possibility was supported by our finding that the precursor proteins packaged in virions released from inhibitor-treated cells underwent progressive cleavage when the particles were incubated in the absence or presence of drug. In virions released from cells treated with 20 μM concentrations of SKF109026, significant levels of p27 were present after 6 h of incubation in the absence of inhibitor, and processing of gp22 to gp20 could be seen. Even in virions

released from cells treated with 100 μM drug, intermediate processing of precursor proteins could be observed at 6 and 18 h. We cannot distinguish at this time between reversal of inhibition and an incomplete saturation of protease active sites (even at 100 μM concentrations of inhibitor), since we do not know whether released virions are permeable to exogenous inhibitor. Other investigators have demonstrated reversibility of protease inhibition following a 3-h incubation of HIV-1 in the absence of drug (19).

M-PMV was the first primate retrovirus to be isolated, and it is associated with an immunosuppressive disease in macaques. As it is the activity of the viral proteinase that commits the virus to an infectious life cycle, these inhibitors provide a useful tool to analyze the relevance of proteinase inhibitors in antiviral therapy as well as to dissect critical stages in the viral life cycle. As these inhibitors have been shown to be effective in vitro, this system might be useful for further studies regarding the administration and efficacy of these inhibitors in vivo.

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REFERENCES

1. **Bradac, J., and E. Hunter.** 1984. Polypeptides of Mason-Pfizer monkey virus. I. Synthesis and processing of the gag gene products. Virology **138:**260–275.
2. **Brody, B., S. S. Rhee, M. A. Sommerfelt, and E. Hunter.** 1992. A viral protease-mediated cleavage of the TM protein of M-PMV can be modified by mutations within the MA protein. Proc. Natl. Acad. Sci. USA **89:**3443–3447.
3. **Bryant, M. L., M. B. Gardner, P. A. Marx, D. H. Maul, N. W. Lerche, K. G. Osborn, L. J. Lowenstein, A. Bodgen, L. O. Arthur, and E. Hunter.** 1986. Immunodeficiency in rhesus monkeys associated with the original Mason-Pfizer monkey virus. J. Natl. Cancer Inst. **77:**957–965.
4. **Chen, C., and H. Okayama.** 1987. High-efficiency transformation of mammalian cells by plasmid DNA. Mol. Cell. Biol. **7:**2745–2752.
5. **Chopra, H. C., and M. M. Mason.** 1970. A new virus in a spontaneous mammary tumor of a rhesus monkey. Cancer Res. **30:**2081–2086.
6. **Crawford, S., and S. P. Goff.** 1985. A deletion mutation in the 5′ part of the *pol* gene of Moloney murine leukemia virus blocks proteolytic processing of the *gag* and *pol* polyproteins. J. Virol. **53:**899–907.
7. **Darke, D. L., R. F. Nutt, S. F. Brady, V. M. Garsky, T. M. Ciccarone, C.-T. Leu, P. K. Lumma, R. M. Freidinger, D. F. Veber, and I. S. Sigal.** 1988. HIV-1 protease specificity of peptide cleavage is sufficient for processing of gag and pol polyproteins. Biochem. Biophys. Res. Commun. **156:**297–303.
8. **Dreyer, G. B., D. M. Lambert, T. D. Meek, I. J. Carr, T. A. Tomaszek, Jr., A. V. Fernandez, H. Bartus, E. Cacciavillani, A. M. Hassell, M. Minnick, S. R. Petteway, Jr., B. W. Metcalf, and M. Lewis.** Hydroxyethylene isostere inhibitors of human immunodeficiency virus-1 protease; structure-activity analysis using enzyme genetics, X ray crystallography and infected T cell assays. Biochemistry, in press.
9. **Dreyer, G. B., B. W. Metcalf, T. A. Tomazek, Jr., T. J. Carr, A. C. Chandler III, L. Hyland, S. A. Fakhoury, V. W. Magaard, M. L. Moore, J. E. Strickler, C. Debouck, and T. D. Meek.** 1989. Inhibition of human immunodeficiency virus 1 protease in vitro: rational design of substrate analogue inhibitors. Proc. Natl. Acad. Sci. USA **86:**9752–9756.
10. **Fine, D. L., R. J. Landon, M. T. Pienta, M. T. Kubicek, M. J. Nalerio, W. F. Loeb, and H. C. Chopra.** 1975. Responses of infant rhesus monkeys to inoculation with Mason-Pfizer monkey virus materials. J. Natl. Cancer Inst. **54:**651–658.
11. **Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine.** 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. USA **86:**5781–5785.
12. **Kaplan, A. H., and R. Swanstrom.** 1991. Human immunodeficiency virus type 1 Gag proteins are processed in two cellular compartments. Proc. Natl. Acad. Sci. USA **88:**4528–4532.
13. **Katoh, I., Y. Yoshinaka, A. Rein, M. Shibuya, T. Odaka, and S. Oroszlan.** 1985. Murine leukaemia virus maturation: protease region required for conversion from immature to mature core form and for virus infectivity. Virology **145:**280–292.
14. **Kohl, N. E., E. A. Emini, W. A. Schleif, L. J. Davis, J. C. Heimbach, R. A. F. Dixon, E. M. Scolnick, and I. S. Sigal.** 1988. Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl. Acad. Sci. USA **85:**4686–4690.
15. **Kräusslich, H.-G., H. Schneider, G. Zybarth, C. A. Carter, and E. Wimmer.** 1988. Processing of in vitro-synthesized *gag* precursor proteins of human immunodeficiency virus (HIV) type 1 by HIV proteinase generated in *Escherichia coli*. J. Virol. **62:**4393–4397.
16. **Kräusslich, H. G., and E. Wimmer.** 1988. Viral proteinases. Annu. Rev. Biochem. **57:**701–754.
17. **Kunkel, T. A.** 1985. Rapid and efficient site-specific mutagenesis without phenotypic selection. Proc. Natl. Acad. Sci. USA **82:**488–492.
18. **Lambert, D. M., S. R. Petteway, Jr., C. E. McDanal, T. K. Hart, J. J. Leary, G. B. Dreyer, T. D. Meek, P. J. Bugelski, D. P. Bolognesi, B. W. Metcalf, and T. J. Matthews.** 1992. Human immunodeficiency virus type 1 protease inhibitors irreversibly block infectivity of purified virions from chronically infected cells. Antimicrob. Agents Chemother. **36:**982–988.
19. **McQuade, T. J., A. G. Tomasselli, L. Liu, V. Karacostas, B. Moss, T. K. Sawyer, R. L. Heinrikson, and W. G. Tarpley.** 1990. A synthetic HIV-1 protease inhibitor with antiviral activity arrests HIV-like particle maturation. Science **247:**454–456.
20. **Meek, T. D., D. M. Lambert, G. B. Dreyer, T. J. Carr, T. A. Tomaszek, Jr., M. L. Moore, J. E. Strickler, C. Debouck, L. J. Hyland, T. J. Matthews, B. W. Metcalf, and S. J. Petteway.** 1990. Inhibition of HIV-1 protease in infected T-lymphocytes by synthetic peptide analogues. Nature (London) **343:**90–92.
21. **Overton, H. A., Y. Fujii, I. R. Price, and I. M. Jones.** 1989. The protease and gag gene products of the human immunodeficiency virus: authentic cleavage and post-translational modifications in an insect cell expression system. Virology **170:**107–116.
22. **Peng, C., B. K. Ho, T. W. Chang, and N. T. Chang.** 1989. Role of human immunodeficiency virus type 1-specific protease in core protein maturation and viral infectivity. J. Virol. **63:**2550–2556.
23. **Rhee, S. S., and E. Hunter.** 1987. Myristilation is required for intracellular transport but not for assembly of D-type retrovirus capsids. J. Virol. **61:**1045–1053.
24. **Sonigo, P., C. Barker, E. Hunter, and S. Wain-Hobson.** 1986. Nucleotide sequence of Mason-Pfizer monkey virus: an immunosuppressive D-type virus. Cell **45:**375–385.
25. **Stewart, L., G. Schatz, and V. M. Vogt.** 1990. Properties of avian retrovirus particles defective in viral protease. J. Virol. **64:**5076–5092.
26. **Strop, P. (Czechoslovakian National Academy of Sciences, Prague).** 1992. Personal communication.