Constitutive Production of Nonenveloped Human Immunodeficiency Virus Type 1 Particles by a Mammalian Cell Line and Effects of a Protease Inhibitor on Particle Maturation

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A stable cell line encoding the sequences of all the human immunodeficiency virus type 1 proteins, with the exception of the gp160 envelope glycoprotein, was derived from transfection of monkey COS-7 cells. This cell line, referred to as CH-1, produces active viral protease that correctly processes its natural substrates and yields capsid particles. These particles contain reverse transcriptase activity and packaged viral RNA but are noninfectious. The level of expression of viral proteins is not toxic to the cells, yet it is comparable to that observed for chronically infected lymphocytes. These constitutively synthesized viral proteins provide a consistent system for the analysis of potential inhibitors of late viral functions. The lack of gp160 increases the biosafety of this assay system, while it allows the measurement of the effects on the production and release of capsid particles. A human immunodeficiency virus type 1 protease inhibitor was used to confirm the viral polyprotein maturation pathway in this system. Particles from cells treated with this protease inhibitor contain unprocessed p55^{gag} precursor and have the same density as the mature particles. These immature particles contain viral RNA, but reverse transcriptase activity is significantly reduced. This cell line may serve to identify compounds that are able to affect viral assembly and maturation as well as to identify the interactions between the viral and cellular proteins involved in these essential processes.

The life cycle of the human immunodeficiency virus (HIV) can be divided into two phases. The early phase begins with receptor-mediated binding of virus to the cell surface and culminates with the integration of the viral genome in the cellular chromosome. The late phase includes all postintegration steps leading to viral protein production, particle assembly, maturation, and budding. The search for anti-HIV agents has focused on both the early and the late events of the viral life cycle. Inhibitors of early functions such as viral reverse transcription would prevent the establishment of infection, while inhibitors of late functions such as proteolytic processing would block the production of viral particles by already infected cells.

The assembly of lentiviruses such as HIV type 1 (HIV-1) is characterized by the formation of capsid particles at the plasma membrane prior to maturation and budding of the enveloped virus (31). The capsid particles consist of a ribonucleoprotein core surrounded by a capsid shell (4). The major structural and enzymatic protein components of the capsid particles, encoded by the *gag* and *pol* genes, are first synthesized as polyprotein precursors (2). Details of the stepwise manner of assembly or proteolytic maturation and the likely interplay with cellular proteins have not been elucidated. The virus-encoded protease is essential for the proper cleavage of the p55^{gag} and p160$^{gag-pol}$ polypeptides and thus is the focus of much attention as a potential antiviral target. Proper processing of the p55^{gag} precursor produces the structural proteins p17 (MA), p24 (CA), p9 (NC), and p6, while processing of the

p160$^{gag-pol}$ precursor provides, in addition to the structural proteins, the enzymatic activities protease, reverse transcriptase, and integrase. Proviruses which contain mutations at the active site of the viral protease produce unprocessed, noninfectious particles (15). Compounds that specifically inhibit the viral protease and prevent the formation of mature infectious viral particles have been identified (for a review, see reference 34). Several of these compounds are now entering human drug trials and offer hope for the identification of a new class of HIV-1 antiviral agents.

The identification of effective protease inhibitors requires preliminary screening in cells that produce the viral protease and its natural substrates p55^{gag} and p160$^{gag-pol}$. Several systems have been devised to produce viral particles and test for a reduction in p55^{gag} precursor processing upon the addition of protease inhibitors. T lymphocytes chronically infected with laboratory strains of HIV-1 have been used in many instances (1, 21, 29), as have acutely infected peripheral blood lymphocytes (17). However, the infectious particles produced by these means require handling in a biological containment level 3 (BCL3) facility. Other methodologies involve infections with vaccinia virus constructions (20), infection with baculovirus vectors (25), transient expression of HIV-1 partial *gag* and *pol* constructs (22), or the use of chimeric vectors containing sequences from other retroviruses (14). These methods are less hazardous but are more artificial than the expression of infectious HIV-1.

A system that closely resembles the expression of HIV-1 proteins during a chronic infection but that produces noninfectious particles is described in this report. The stable cell line, referred to as CH-1 (COS HIV-1), continuously produces all HIV-1 proteins with the exception of the envelope glycoprotein gp160. These cells secrete capsid particles containing reverse transcriptase activity and viral RNA. Their maturation is inhibited in a concentration-dependent manner by the viral

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protease inhibitor U75875. Viral protein expression is transcriptionally regulated by the HIV-1 long terminal repeat promoter and requires no induction or stimulation of the cells. This system, by virtue of lacking the *env* gene, does not produce infectious HIV-1 particles, precluding the need for a BCL3 facility. It therefore expedites the testing of potential antiviral compounds that target steps of viral maturation following genomic integration. Such inhibitor analyses could also help to delineate the steps of viral assembly that occur prior to budding and the roles of cellular components.

MATERIALS AND METHODS

Cells and plasmids. COS-7, SW480, HeLa, and HeLa T4 cells were maintained in Dulbecco's modified Eagle medium H21 (DMEM) supplemented with 10% fetal calf serum (dialyzed, refiltered)–100 U of penicillin per ml–100 μg of streptomycin per ml. SW480, HeLa, and COS-7 cells were obtained from the American Type Culture Collection. HeLa T4 cells were obtained through the AIDS Research and Reference Reagent Program, from R. Axel. The HIV-gpt plasmid consists of the HIV-1 HXB2 sequences (the *env* gene, nucleotides 6402 to 7620, was replaced by the drug-selectable *Escherichia coli gpt* gene) cloned into the pBS (Bluescript; Stratagene) vector; plasmid HXB2-env encodes the gp160 sequences (nucleotides 5999 to 8896) cloned into a simian virus 40 (SV40) expression vector (26). CH-1 cells, which were derived from transfected COS-7 cells as described below, were maintained under selection for guanine phosphoribosyltransferase expression by supplementing the medium with 250 μg of xanthine per ml, 14 μg of hypoxanthine per ml, and 50 μg of mycophenolic acid per ml (Calbiochem).

Protease inhibitor. The peptidomimetic compound U75875 (1-naphthoxyacetyl-histidyl-[(5S)-amino-6-cyclohexyl-(3R,4R)-dihydroxy-(2R)-isopropylhexanoyl]-valyl-isoleucyl-2-amino-methylpyridine) was kindly provided by Alfredo Tomasselli, Upjohn, Kalamazoo, Mich. (1). U75875 was used at concentrations of 0.05 to 5.0 μM in a 0.5% final concentration of dimethyl sulfoxide to ensure solubility in the culture medium and improved cellular penetration. Cytotoxicity was measured by the MTT assay (24), and an approximate 50% lethal dose of 50 μM was determined for 48-h incubations.

Transfections. SW480, HeLa, or COS-7 cells were transfected by the calcium phosphate procedure (8) with the following modifications. Twenty micrograms of each plasmid DNA (purified by polyethylene glycol precipitation) was used per 10-cm dish of approximately 50% confluent cells, and 100 μM chloroquine was added to maximize transfection efficiency (19). After 18 h, fresh medium was added to the cultures and cells were allowed to grow for 48 h. At this point, selection for stable plasmid integration was initiated. The medium was replaced by DMEM containing 10% fetal calf serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 250 μg of xanthine per ml, 14 μg of hypoxanthine per ml, and 50 μg of mycophenolic acid per ml. The medium was changed every 3 to 4 days until drug-resistant colonies appeared (12 to 15 days). Under these selection conditions, 100% of untransfected COS-7 cells were killed within 3 days. Single colonies of surviving cells were removed by using cloning cylinders and were transferred to 24-well plates. Upon reaching confluency, aliquots of the culture supernatant were tested by enzyme-linked immunosorbent assay (ELISA; NEN/Dupont) for production of p24 capsid protein. Confluent monolayers producing more than 10 ng of p24 per ml within 24 h were expanded gradually over a period of 2 weeks. The infectivities of the viral particles secreted into the medium were determined by infec-

tion of HeLa T4 cells and quantitation of drug-resistant colonies as described by Page et al. (26).

Isolation of capsid particles by sucrose gradient fractionation. Culture supernatants were cleared of cellular debris by filtration through 0.45-μm-pore-size filtration units. Sucrose cushions in phosphate-buffered saline (PBS) containing 0.1 g of $CaCl_2$ per liter and 0.1 g of $MgCl_2$ per liter were prepared by layering 3 ml of 20% (wt/vol) sucrose over 75 μl of 60% sucrose. Particles were concentrated from 10 ml of culture supernatant by centrifugation for 1.5 h at 35,000 rpm and 4°C in a Beckman rotor model SW41. The bottom 0.5 ml was collected, diluted threefold in PBS, and then layered over an 11.5-ml continuous gradient of 20 to 45% sucrose in PBS. Following centrifugation for 2 h at 35,000 rpm (SW41 rotor) at 4°C, fractions of approximately 0.75 ml were collected from the bottoms of the tubes. The linearity of the gradients was established by determining the percentage of sucrose in each fraction with a refractometer.

Metabolic labeling of cultures. Cell cultures in 10-cm dishes were grown to approximately 75% confluency in the presence or absence of protease inhibitor. The cultures were washed with warm PBS and were then starved by incubation with DMEM deficient in methionine for 20 min. Cells were labeled for 5 h by using 0.1 mCi of [^{35}S]Translabel (70% methionine and 30% cysteine; specific activity, 1,000 Ci/mmol; ICN) per ml in 5 ml of DMEM deficient in methionine and supplemented with 3% dialyzed fetal calf serum. A 1-h chase with DMEM–10% fetal calf serum followed. The supernatant was then harvested and the cells were collected by scraping as described below.

p24 ELISA. The concentration of p24 protein in samples of culture supernatant or isolated particles was determined by an ELISA (NEK-060 kit; NEN/Dupont). This assay permits the detection of mature p24 with virtually no reaction with the unprocessed precursor proteins p55^{gag} and p160$^{gag-pol}$. To determine the total amount of p24 protein present in the samples used to quantitate viral RNA, exogenous purified HIV-1 protease was added to digest the precursor molecules. Aliquots of capsid samples from untreated cells and U75875-treated cells were digested with 100 ng of HIV-1 protease in the presence of 0.1% Triton X-100–5 mM EDTA. The digested samples were then analyzed by p24 ELISA.

Immunoprecipitations. To obtain whole-cell extracts, confluent monolayers were washed with ice-cold PBS and were then scraped in 1.2 ml of ice-cold PBS containing 5 mM EDTA and 1% Triton X-100; this was followed by centrifugation at 30,000 × g for 10 min at 4°C in a microcentrifuge. Protein A-Sepharose beads (Pharmacia) were initially incubated with rabbit polyclonal anti-p24 (HIV-1) antibody (American Bio-Technologies) for 1 h at 4°C. The beads were then added to aliquots of cellular extracts from metabolically labeled cells and were incubated for 2 h at 4°C. All reactions were done in 0.5-ml volumes with PBS containing 0.5% Tween 20 and 1% bovine serum albumin (BSA) as the diluent. Aliquots of unlabeled cellular extract from COS-7 cells were added to reduce nonspecific binding to beads. The bound proteins were extracted from beads by boiling for 10 min in 2× Laemmli buffer (17) and were separated on sodium dodecyl sulfate (SDS)–12.5% polyacrylamide gels and were then autoradiographed.

Immunofluorescence. Cells were seeded at 50% confluency on glass chamber slides (Nunc). After 24 h, the medium was removed and the cells were washed three times with PBS at 37°C. Cells were fixed at 25°C by using either a 1:1 mix of acetone-methanol for 10 min or a solution of 1% formaldehyde in PBS for 10 min; this was followed by the addition of

neat methanol for 10 min. For all subsequent incubations, PBS containing 0.5% Tween 20 and 3% BSA (Sigma IV) was used as the diluent for all washes. Rabbit anti-p24 (HIV-1) polyclonal antibody (American BioTechnologies) was used at a 1:500 dilution for 1 h of incubation at 37°C. A fluorescein-conjugated goat anti-rabbit immunoglobulin G (heavy and light chains; Pierce) was used as secondary antibody at a 1:25 dilution for a 30-min incubation at 37°C. Following extensive rinses with PBS, the slides were viewed by oil immersion at ×60 magnification.

Electron microscopy analysis. CH-1 cells were grown to near confluency in 100-mm culture plates in the absence or presence of the protease inhibitor U75875. The cells were gently scraped while in the growth medium and were transferred to conical centrifugation tubes. The cells were collected by low-speed centrifugation and were fixed with a solution containing 3% glutaraldehyde, 1% paraformaldehyde, and 0.067 M sodium cacodylate (pH 7.5) for 1 h at 25°C. The cells were postfixed in Veronal-buffered 1% osmic acid containing 7% sucrose for 1 h at 25°C, dehydrated in graded ethanol, and embedded in Epon Em-bed-812. Thin sections (60 to 80 nm) were stained for 10 min in aqueous saturated uranyl acetate, contrasted with Reynolds lead stain, and examined with a Jeol 1200 EX electron microscope operating at 80 kV.

Reverse transcriptase assay. Sucrose gradient fractions and culture supernatants were analyzed for reverse transcriptase activity by the procedure of Tan et al. (33). Aliquots (50 μl) were incubated for 2 h at 37°C in a 100-μl assay mixture containing Mg^{2+}, [^{3}H]TTP, and 4:1 ratios of poly(A) (Pharmacia) to oligo(dT)$_{12-18}$ (Midland Certified Reagents). Quantitation of radioactivity was obtained by scintillation counting in glass microfiber filters (Whatman) following trichloroacetic acid precipitation of the reaction products.

RNA analysis. Aliquots of capsid samples from sucrose gradients were mixed in equal ratios with a denaturation solution [0.4% SDS, 4 M formaldehyde, 50% formamide, 80 mM 3-(morpholino)propanesulfonic acid (pH 7)], and the mixtures were heated to 65°C for 15 min. The samples were then applied to nitrocellulose (Schleicher & Schuell) by using a slot blot apparatus produced by the same manufacturer. A ^{32}P-labeled probe was made by random-primed synthesis by using a 900-bp BclI-SpeI HIV-1 fragment. Hybridization conditions were essentially those described by Pepin et al. (27). After autoradiography, the band intensities were quantitated by densitometry by using a Bio-Rad video densitometer (model 620) and the 1D Analyst II software program.

RESULTS

Expression of viral proteins. The cell lines SW480, HeLa, and COS-7 were cotransfected with the HIV-gpt vector (sequences coding for gp160 deleted) and the HXB2-env vector encoding the gp160 sequences (26). Stable integration was assayed by selection for the gpt gene encoded by the HIV-gpt vector. Cotransfections with the HIV-gpt and HXB2-env vectors yielded stable transfectants secreting p24 protein in the SW480 cells only. The transfection of 3×10^7 cells yielded approximately 50 colonies. Ten colonies were expanded and infectious particles were detected in their supernatants by viral titer analysis on HeLa T4 cells. A titer of approximately 100 to 500 infectious particles per ml was obtained from the supernatants of confluent monolayers of one initial SW480 clone. After five passages, the viral titer from the culture supernatant decreased dramatically. The particles secreted were analyzed by Western blotting (immunoblotting), which showed that less

than 10% of the capsid precursor p55 protein was correctly processed to p24.

Transfection of 3×10^7 cells with the HIV-gpt vector alone yielded approximately 50 colonies of SW480 cells, 100 colonies of HeLa cells, and 500 colonies of COS-7 cells. Approximately 20 clones from each cell type were isolated and further analyzed. Upon expansion, these cells were tested for production of capsid protein p24 by ELISA of culture supernatants. Detectable p24 production (>20 pg/ml) was observed for more than 90% of the cells tested, but COS-7-derived cells consistently produced the largest amounts (1 to 100 ng/ml). One of these clones, designated CH-1, was selected for further characterization. CH-1 cells, which lack expression of gp160, efficiently produced 10 to 100 ng of mature p24 protein per ml and maintained the same level of expression after more than 200 passages. The cells have an increase in number and size of processes, and at times they show irregular multilobed nuclei, but their growth rate is similar to that of the untransfected parent cells. The noninfectious nature of the particles obtained from this cell line was determined by the HeLa T4 cell assay, which yielded no drug-resistant colonies (data not shown). Since our goal was to establish a stable cell line that produced functional viral protease and its natural substrates, the lack of the gp160 envelope in the CH-1 cells was not deleterious.

There are several advantages to stably introducing the HIV-1 genome into cells by using the HIV-gpt vector only. Since this vector lacks the env sequences, it produces noninfectious, nonenveloped particles, thus allowing propagation of CH-1 cells in a BCL2 facility. The presence of the gpt gene encoded within the same vector permitted selection for stable integration as well as maintenance of the cell line. Expression of the gpt gene is regulated by the simian virus 40 early promoter in this vector. The success in generating a stable cell line suggests that spontaneous replication from the SV40 ori did not occur. This could result from either a mutation in the SV40 ori within the vector's promoter or the selection of COS-7 cells carrying a mutant T antigen (6, 7). The presence of either mutation was not investigated. The presence of the HIV-1 long terminal repeats provided the packaging signals for RNA. The viral regulatory proteins Tat and Rev and the Rev responsive element in the HIV-gpt vector ensured the authentic expression and processing of the viral products. Particle production did not require stimulation. No effect was observed following treatment of CH-1 cells with phorbol-12-myristate-13 acetate at concentrations of 10^{-10} to 10^{-8} M (data not shown), which were used to induce HIV-1 transcription in infected T lymphocytes (30).

Immunofluorescence analysis of CH-1 cells was performed following 50 to 100 continuous passages (Fig. 1) to confirm the continued expression of HIV-1 proteins. Cells were grown to 50% confluency and were fixed with either acetone (Fig. 1A) or formaldehyde (Fig. 1B) prior to reaction with a polyclonal antibody that recognizes the HIV-1 p24 capsid protein and its precursor, p55. For comparison, untransfected COS-7 cells were fixed with acetone and were similarly reacted with anti-p24 antibody (Fig. 1C). The presence of p24 protein in these cells was visualized by reacting them with fluorescein-conjugated secondary antibody. Nearly all CH-1 cells were stained, albeit to different extents. Both fixative protocols lead to strong cytoplasmic staining patterns for the CH-1 cells. Untransfected COS-7 cells showed no fluorescent staining.

Detection of viral protease gene. To establish that the correct sequence of the HIV-1 protease was present in the CH-1 cells, PCRs on whole-cell lysates were carried out. The oligonucleotide primers used corresponded to 20 bases flanking the 5′ end (sense, GAACTGTATCCTTTAACTTC) and
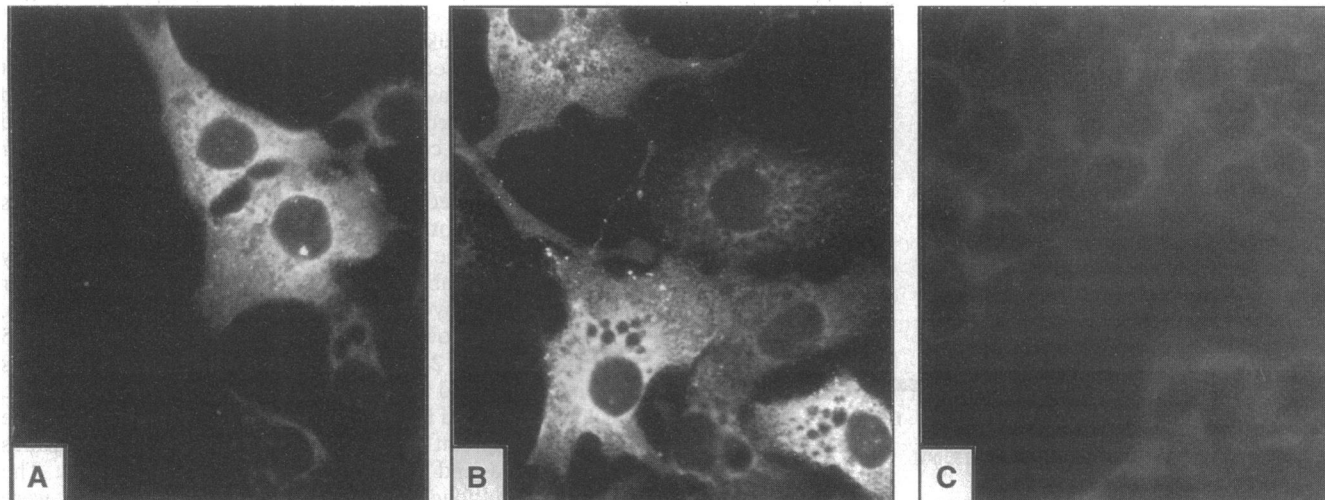
FIG. 1. Detection of p24 capsid protein expression by indirect immunofluorescence staining. Fixed cells were probed with a polyclonal antibody against p24 and then with a fluorescein-conjugated secondary antibody. (A) Acetone-fixed CH-1 cells; (B) formaldehyde-fixed CH-1 cells; (C) acetone-fixed COS-7 cells.

3' end (antisense, GGGTAATCGGGATAACTCTG) of the 297-bp protease gene. The resulting amplified DNA fragments in the PCR mixtures were analyzed by agarose gel electrophoresis and ethidium bromide staining. A fragment with the expected size of 357 bp was detected. This fragment was excised from the gel and was sequenced by the CircumVent procedure (New England Biolabs) by using either one of the oligonucleotide primers used in the PCR. The sequence obtained is identical to that found in the HXB2 strain encoded in plasmid HIV-gpt.

Responses of cells to viral protease inhibitor. To show that the response of the CH-1 cell line to previously characterized protease inhibitors matched that observed in other cell-based assays, the peptidomimetic inhibitor U75875 was used. This dihydroxyethylene isostere has been shown to completely block HIV-1 replication in human peripheral monocytes and effectively inhibits $p55^{gag}$ processing in H9 cells chronically infected with the human T-cell lymphotropic virus type IIIB isolate of HIV-1 at concentrations of 1 μM (1). A time course of incubation of CH-1 cells with various concentrations of this inhibitor is shown in Fig. 2A. The time- and dose-dependent effects on the amount of p24 protein detected by ELISA from cell culture supernatants were comparable to those observed previously (1). The ability of this compound to function intracellularly was also confirmed by immunoprecipitation of whole-cell extracts by using antibodies that recognize the capsid protein p24 and its precursors (Fig. 2B). In the absence of inhibitor, bands corresponding to the p24, p25, p39, p41, and p55 proteins can be detected. At low inhibitor concentrations (0.1 μM), the p24 and p25 capsid proteins and the p41-p39 intermediates are still readily detected. As the inhibitor concentration is increased to 1.0 μM, the p24, p25, and p39 protein bands appear much diminished. With a 5 μM concentration of inhibitor, the p24 and p25 bands are undetectable. A similar dose-dependent block in p55 processing was observed when cytoplasmic extracts were analyzed (data not shown).

The p39 and p41 intermediates arise from partial cleavages of the precursor p55 polypeptide (23). The persistence of these intermediates in cell extracts of cells treated with viral protease inhibitors has been observed by others (26). The presence of these proteins in cellular fractions but not in virions secreted

from the same cells may be due to the fact that host cell proteases act on labile bonds in the accumulated p55 molecules. The p24 and p25 proteins are proteolytically processed forms of the major capsid protein which differ by a short C-terminal extension in the longer protein (23). While both forms are found in the cytoplasm, only the p24 polypeptide is found in mature virions (13).

Morphological analysis of secreted particles by electron microscopy. Thin-section electron microscopy was performed on CH-1 cells grown to confluency alone or in the presence of 2.5 μM U75875 (Fig. 3). The cells were harvested by scraping and were gently pelleted (see Materials and Methods) to retain fully budded and cellularly associated capsid particles. Capsid particles displaying condensed cores are secreted by CH-1 cells (Fig. 3A and B). A variety of viral morphologies can be observed in these budded particles, as noted previously for chronically infected H9 cells (3). All particles show a density within the core; some cores are cone shaped and others are tubular, and several cores within a single virion were also found. Intracellular particles were only rarely observed, suggesting that particle secretion is efficient. All of the cells identified as secreting capsid particles also displayed a multilobed irregular nucleus (note two such lobes in Fig. 3A). The cells grown in the presence of 2.5 μM U75875 produced only immature capsid particles. These particles can be seen budding from the cell (Fig. 3C) and being secreted into the supernatant (Fig. 3D). The immature particles lack a condensed nuclear core, consistent with the absence of mature capsid protein as observed in Fig. 2.

Characterization of capsid components. The ability of gag proteins to form particles in the absence of envelope protein has been well documented in transient expression systems (5, 14). We isolated capsid particles from the CH-1 culture supernatant by sucrose gradient sedimentation, and aliquots of the fractions were tested for p24 capsid protein by ELISA and for reverse transcriptase activity in vitro (Fig. 4). The peaks of both p24 protein and reverse transcriptase activities overlap at approximately 30 to 32% sucrose (1.14 ± 0.02 g/ml) for untreated CH-1 cells. This value is similar to the one obtained for capsids isolated after the transient expression of HIV-gpt in COS-7 cells (26) and for infectious virions isolated from
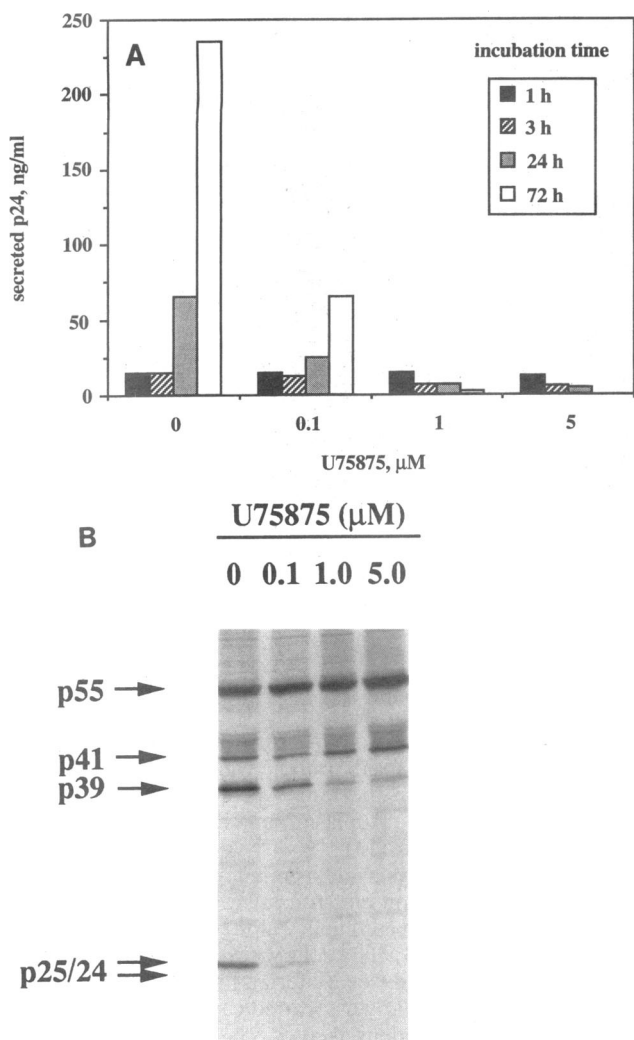
FIG. 2. Detection of capsid proteins in culture supernatants and cell extracts of the CH-1 cell line in response to treatment with increasing concentrations of a peptidomimetic protease inhibitor. (A) Time course and dose-response of extracellular p24 from CH-1 cells treated with the inhibitor U75875. Culture supernatants were assayed for core p24 protein by ELISA. (B) Immunoprecipitation of intracellular core proteins from cell extracts of drug-treated CH-1 cells (following a 24-h incubation). Extracts were treated with polyclonal antibodies to p24 that had been preadsorbed to protein A-Sepharose beads.

infected T cells (28). The presence of p24 and reverse transcriptase at the top of the gradient indicates that a fraction of these viral proteins is not encapsidated. Measurement of the total p24 protein and reverse transcriptase activities in culture supernatants shows that approximately 50% of the p24 protein and 25% of the reverse transcriptase activity are found in the soluble, nonencapsidated form. Transient transfection of COS-7 cells with HIV-1 subviral constructs has yielded ca. 50% of secreted p24 protein in the soluble, nonencapsidated form (27). Treatment of CH-1 cells for 24 h with U75875 at a concentration of 1 μM resulted in a decrease of more than 80% in both mature p24 protein and reverse transcriptase activities in peak fractions. The detection of some reverse transcriptase activity in the fractions treated with 1 μM U75875 may reflect the low reverse transcriptase activity of the

gag-pol precursor as reported previously for defective HIV-1 (27) and avian leukosis virus virions (32). The p24 ELISA (Dupont/NEN) used in the present study detected almost exclusively the cleaved p24 protein but not the unprocessed polyproteins. Nevertheless, a limited reactivity toward p41 and p39 occurs, which could explain the p24 signal observed for some fractions from drug-treated cells. The unprocessed precursors not detected by p24 and reverse transcriptase assays were observed by other methods (see below).

The nature of the capsid proteins packaged and secreted by CH-1 cells in the absence and presence of protease inhibitor was investigated by metabolic labeling of cultures (Fig. 5). CH-1 cells were grown in the presence of 0 μM (Fig. 5A), 0.25 μM (Fig. 5B), or 2.5 μM (Fig. 5C) U75875 for 24 h. This incubation included a pulse-label with [^{35}S]methionine for 5 h, which was followed by a 1-h cold chase in the presence of the appropriate amount of inhibitor. The culture supernatant was then collected and fractionated on sucrose gradients. The fractions were loaded onto polyacrylamide gels and were observed by autoradiography. The peaks of gag proteins p24, p25, p39, p41, and p55 all cosedimented at a sucrose density of 1.14 ± 0.02 g/ml, indicating similar densities for the processed and unprocessed capsid particles. The relative concentrations of each protein were quantified by laser densitometry, which showed that more than 80% of the gag proteins are in fractions 5 to 7 in all gradients. It is also worth noting that the amounts of p55, p41, and p39 precursors isolated as immature particles (Fig. 5B and C) are greater than the amount of mature p24 isolated as mature particles (Fig. 5A). The p160 polyprotein could be readily detected in samples from cells treated with 2.5 μM U75875 (Fig. 5C), and the ratio of gag-pol to gag polypeptide was approximately 1:25. This ratio agrees with the expected rate of translational frameshifting reported previously for HIV-1 (11).

The abilities of these particles to incorporate viral RNA were also investigated. Since the density of the capsid particles is not affected by the lack of gag protein processing, we looked at the effect of the protease inhibitor on the ability to encapsidate RNA. Figure 6 shows the results of slot blot analysis of fractions taken from sucrose gradients. The hybridization probe used is a SpeI-BclI DNA fragment of approximately 900 bp encompassing a region of gag and pol. The amount of RNA detectable increased as the concentration of the protease inhibitor added to the cells increased (0, 0.25, and 2.5 μM U75875). Quantitation by laser densitometry showed approximately fourfold more RNA for the fractions isolated from cells treated with 2.5 μM U75875 than from untreated cells.

To relate the amount of packaged RNA to the number of capsid particles isolated, the concentration of total p24 protein was measured. Exogenous purified HIV-1 protease was used to digest precursor molecules from capsids isolated from the same number of cells. This treatment released about 3.5-fold more p24 protein from the U75875-treated cells than from untreated cells (data not shown). This result also agrees with the laser scanning of p55 and p24 proteins isolated by sucrose gradient fractionation (Fig. 5), in which an approximately fourfold greater intensity was observed for the p55 protein in unprocessed capsids than for the p24 protein in processed capsids. Therefore, the ratio of RNA to total capsid protein is maintained in capsid particles regardless of the level of proteolysis.

DISCUSSION

We report the identification of a stable cell line that continuously produces HIV-1 capsid particles, thus facilitating the
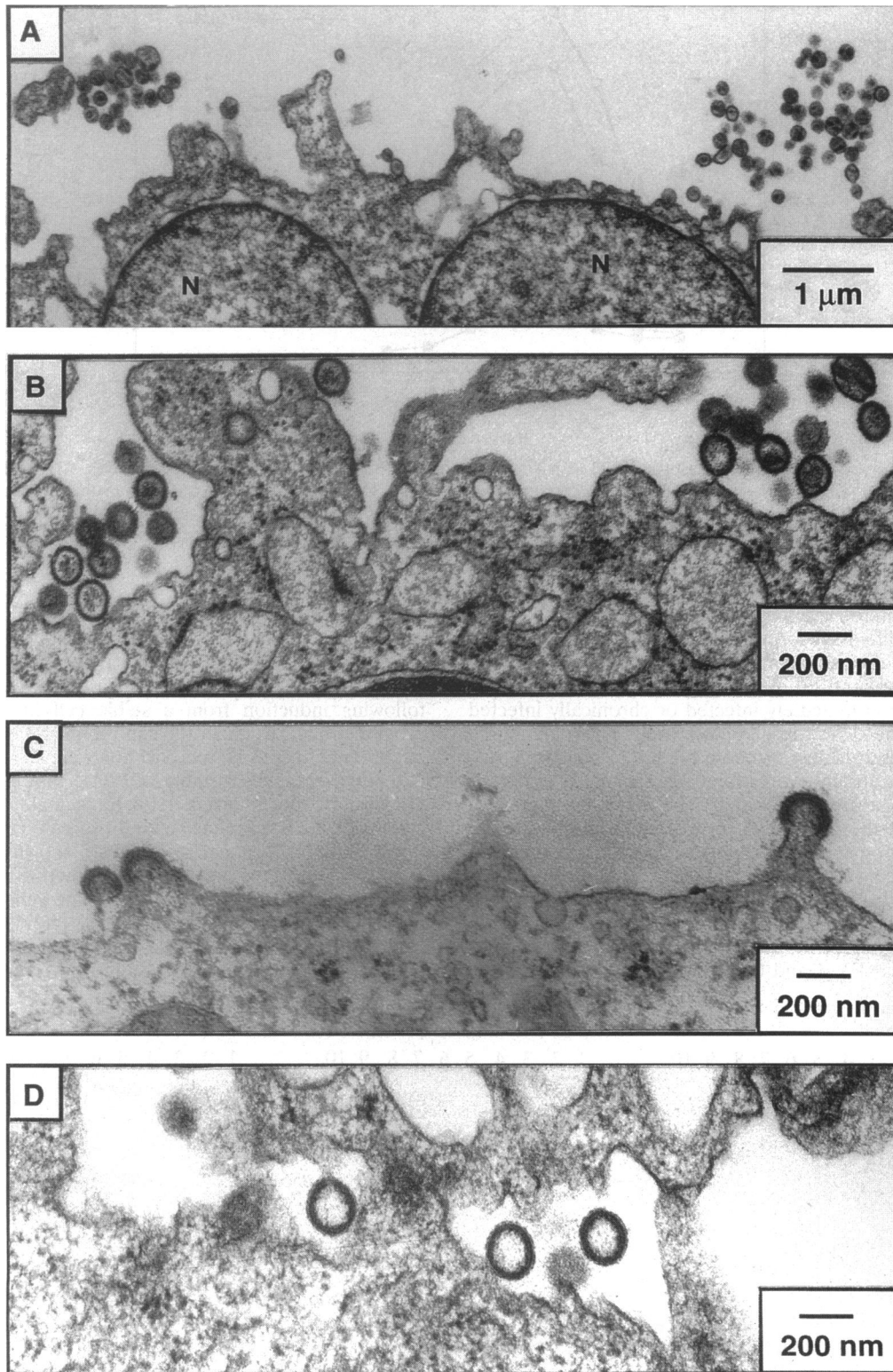
FIG. 3. Thin-section electron micrographs of CH-1 cells. (A and B) Cell grown in the absence of inhibitor. (C and D) Cell grown in the presence of 2.5 μM U75875. N, nucleus of irregular lobed shape, where connections are out of the plane of the electron microscopy section.
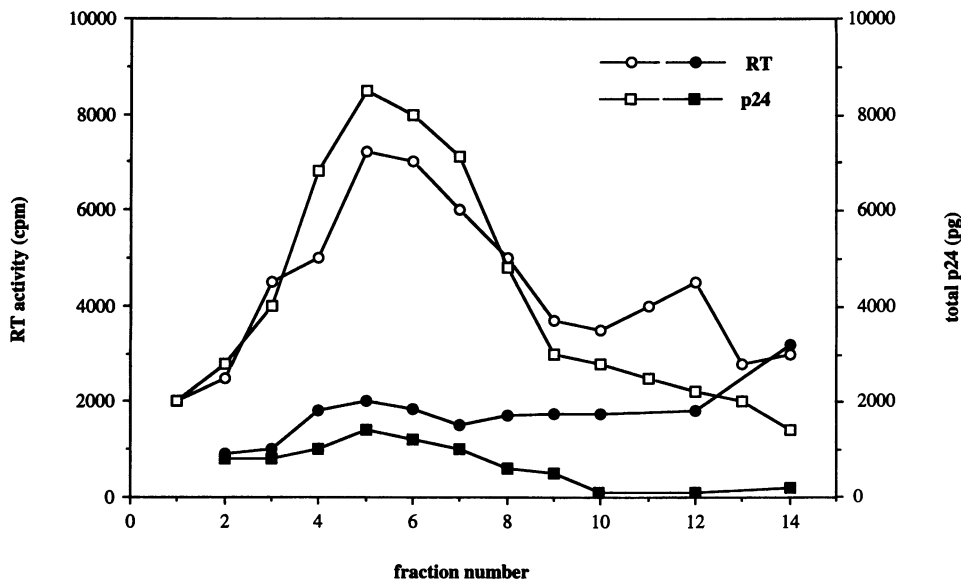
FIG. 4. Measurement of reverse transcriptase (RT) activity and p24 protein profile from isolated capsid particles. CH-1 cells were allowed to grow untreated (open symbols) or were treated with 1 μM compound U75875 (closed symbols) for 24 h. Culture supernatants were subjected to sucrose gradients, and fractions were assayed for reverse transcriptase activity and p24 content as described in Materials and Methods. Fraction 1 corresponds to the bottom of the gradient.

assay of inhibitors of late viral functions. At present, several systems are used to assay for inhibitors of HIV-1 replication in cell culture. The most commonly used assays measure the efficacies of compounds at blocking viral replication and viral protein production in acutely infected or chronically infected cells and offer the closest resemblance to an in vivo situation. Other systems used to test protease inhibitors produce virus-like particles by transient expression from vaccinia virus (20) or baculovirus (25) expression vectors. The major limitations of these systems are the variability in expression levels inherent to various transfection and infection efficiencies and the pleiotropic effects of the vectors on the host cells. Cell lines that produce noninfectious virus-like particles have also been used to analyze viral protease inhibitors. For example, a "safe system" that continuously produces chimeric particles from cells that express HIV-1 and Moloney murine leukemia virus

sequences has been described previously (15). Although these cells produced active HIV-1 protease, the particles lacked detectable reverse transcriptase activity and could not package viral RNA. Noninfectious virus-like particles were produced following induction from a stable cell line that expressed proviral sequences under the control of a human promoter (9), but no inhibitors of HIV replication were tested in this system. Attempts to establish stable cell lines that express infectious HIV-1 have been unsuccessful, presumably because of the cytotoxic effects of an active viral protease (16).

We have chosen cells previously shown to support the expression of HIV-1 proteins to transfect with a vector encoding all HIV-1 genes except the envelope gene in the presence or absence of an envelope-encoding vector. Only the SW480 cotransfection yielded cells that produced infectious particles, but production decreased markedly after several passages. This
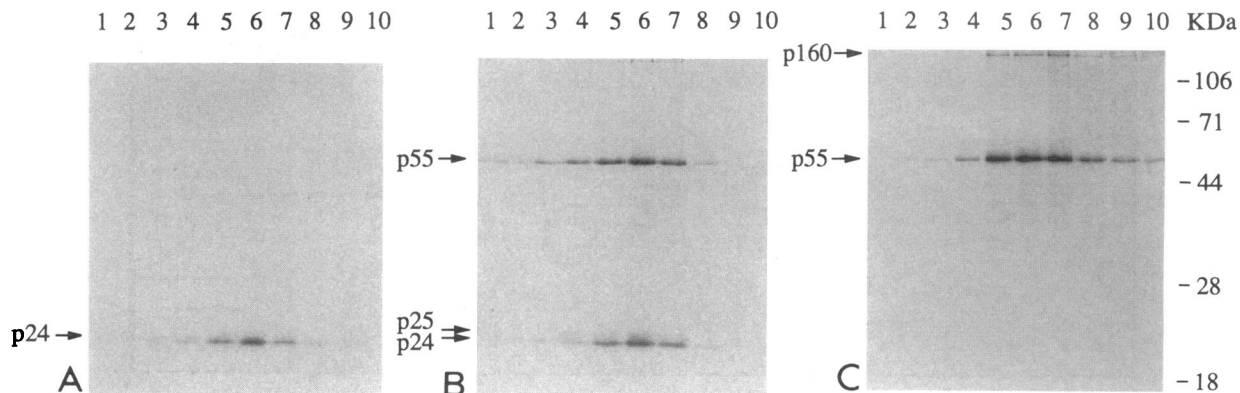

FIG. 5. Isolation of capsid particles and identification of major protein components. CH-1 cells were grown untreated (A), treated with 0.25 μM U75875 (B), and treated with 2.5 μM U75875 (C) for 24 h and were labeled with [³⁵S]methionine for 6 h prior to harvest. Culture supernatants were fractionated on sucrose gradients to isolate capsid particles. The collected fractions (lanes 1 to 10 containing fractions 1 to 10, respectively) were subjected to SDS-polyacrylamide gel electrophoresis, and the labeled proteins were visualized by autoradiography.

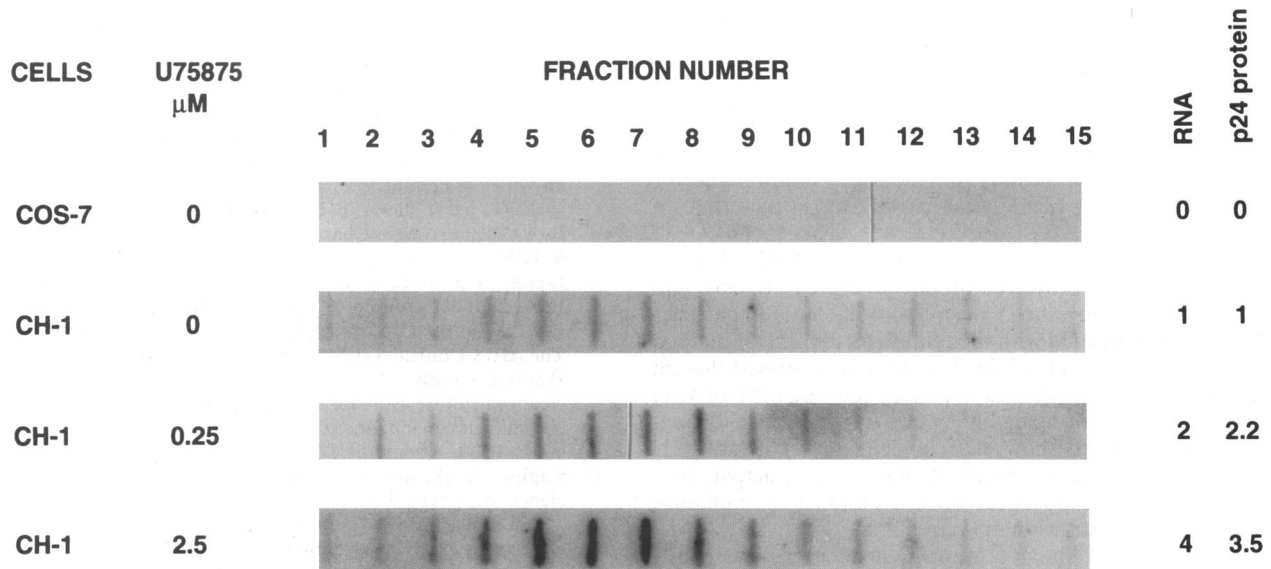| CELLS	U75875 μM	FRACTION NUMBER															RNA	p24 protein
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15		
COS-7	0																0	0
CH-1	0																1	1
CH-1	0.25																2	2.2
CH-1	2.5																4	3.5

FIG. 6. Identification of viral RNA in purified capsid particles. The secreted particles isolated by sucrose gradient fractionation were transferred to nitrocellulose and were probed with a ^{32}P-labeled HIV-1 *gag-pol* DNA fragment. The amount of radioactivity incorporated was visualized by autoradiography. For the experiment, CH-1 cells were grown for 48 h untreated or in the presence of 0.25 or 2.5 μM U75875. The amount of total p24 protein present in the peak fractions (fractions 3 to 7) of each gradient was determined by p24 ELISA as described in Materials and Methods, and the amount of RNA in the same fractions was determined by laser scanning of the RNA autoradiographs. Values are expressed relative to that for untreated cells, which was equal to 1.0.

loss of infectivity was concomitant with a reduction in the amount of p24 protein and an increase in the amount of p55 precursor observed in whole cells and secreted particles. The inability to maintain stable cell lines that produce infectious viral particles may reflect a cytotoxic effect of the virus on cellular function. Cotransfections of COS-7 cells did not yield virus-producing stable clones, but in the absence of the HIV-1 envelope gene a large number of clones that secreted the p24 protein were obtained. Interestingly, previous transient expression studies with these vectors to produce viral particles also showed that a higher yield of p24 protein could be obtained in the absence of envelope production (26).

The COS-7-derived cell line, designated CH-1, produced the highest levels of capsid protein p24 from the onset and was the subject of the present study. This is the first system reported to constitutively express active HIV-1 protease and all of its natural substrates under the control of the viral long terminal repeat promoter, resulting in the secretion of capsid particles containing properly processed gag and gag-pol proteins and viral RNA. CH-1 cells can serve as a tool for analyzing the events following integration and clarifying the steps in the assembly process that do not involve interactions with the HIV-1 envelope glycoprotein. The effect on viral maturation of a previously described viral protease inhibitor (U75875) was dependent on incubation time and concentration, as expected. Inhibition of the viral protease resulted in the production and packaging of the unprocessed p55 precursor and the p41 and p39 intermediates. Capsids assemble and sediment with the same density regardless of protease processing. The inhibition of polyprotein processing at the same concentrations of the compound U75875 in these cells as was reported for T lymphocytes (1) suggests that the amounts of viral protease and substrates are comparable between the two systems.

The CH-1 cells produce approximately 50 ng of p24 protein per ml in 24 h of growth of 10^7 cells. This level is comparable to that obtained for chronically infected cells (12). Approxi-

mately 50% of the p24 protein secreted into the medium is in the form of capsids, which is comparable to a previous report of p24 shedding from infected cells (18). Mature p24 can be detected in both cytoplasmic and plasma membrane fractions, indicating that the viral protease is active throughout these cells. It has been suggested that the expression of active viral protease in cells is toxic (9, 16). One possible explanation for the lack of toxicity in this cell line could be a protease mutation which reduces its activity. We used PCR to confirm that the wild-type sequence of the HIV-1 protease was maintained in this cell line. The amounts of viral protein produced may be sufficient for the processing of the capsid proteins observed in cytoplasmic and membrane fractions as well as in the secreted material but may be low enough to prevent a cytotoxic effect. The pattern of viral protein processing and secretion in CH-1 cells resembles that observed for chronically infected T lymphocytes and not lytically infected cells (19). We have observed by electron microscopy that CH-1 cells that express large numbers of capsid particles display nuclei with an irregular lobed shape. This aberrant morphology is absent from CH-1 cells treated with the protease inhibitor U75875. Honer et al. (10) noted changes in nuclear morphology after microinjection of HIV-1 protease into human fibroblasts. This effect was correlated with the proteolytic cleavage of vimentin and cytoskeletal proteins. The proteolysis of matrix proteins may explain the abnormal morphologies of untreated CH-1 cells and the normal morphologies following treatment with U75875.

Quantitation of packaged viral RNA and total capsid proteins shows that the presence of the protease inhibitor U75875 leads to the isolation of approximately four times more capsids. This result was confirmed by comparison of the amount of encapsidated p55 and p24 proteins detected from pulse-labeled cells. The increase in the number of capsids isolated from the same number of cells may reflect an increase in the number of particles released. More likely, however, it could reflect an increase in the stabilities of these capsids rendered

by the polyprotein nature of their components. Immature virions produced by protease-defective Rous sarcoma virus were shown to remain morphologically stable after a nonionic detergent treatment that completely disrupted wild-type cores (32). These results suggest that unprocessed capsid particles may not dissociate as readily as the processed particles. Thus, correct polyprotein processing may be a key requirement for the proper uncoating of the viral capsid after it penetrates a newly infected cell.

Transient expression of subviral constructs of HIV-1, without an active viral protease, produced uncleaved Gag and Gag-Pol polyproteins which efficiently assembled into particles but remained associated with the plasma membrane or budded into intracellular vacuoles (22). The authors proposed that an active viral protease may play a role in the targeting of viral proteins to the site of assembly by cleaving cytoplasmic components. Our results show that in cells that constitutively produce viral proteins, the block of viral protease activity by a potent inhibitor has no effect on the budding of immature particles into the extracellular space.

Identification of cellular proteins that affect viral assembly and budding could point to new targets for antiviral intervention. Analysis of the antiviral effects of compounds known to regulate the functions of cellular proteins may serve to locate such proteins and dissect their modes of action. Besides applications such as screening of new viral protease inhibitors, other compounds that affect late events in capsid maturation and release could be identified by this system. Antiviral assays that use this cell line bypass the strict containment requirements of infectious virus production while they maintain a reliable method for quantifying the effects on HIV particle formation.

ACKNOWLEDGMENTS

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