VIROLOGY 206, 535–544 (1995)

The Proteins of Lymphocyte- and Macrophage-Tropic Strains of Simian Immunodeficiency Virus Are Processed Differently in Macrophages

EDWARD B. STEPHENS,*,[1] HAROLD M. MCCLURE,† AND OPENDRA NARAYAN*

*Department of Microbiology, Immunology, and Molecular Genetics, University of Kansas Medical Center, Kansas City, Kansas 66160-7424; and †Yerkes Primate Center, Emory University, Atlanta, Georgia 30322

Received August 16, 1994; accepted October 14, 1994

Since the pathogenesis of SIV_{mac} disease complex is thought to be explained by the tropism of the infecting virus for either $CD4^+$ T-lymphocytes or macrophages or both types of cells, we compared the infection in primary macaque macrophages with molecularly cloned, lymphocyte-tropic $SIV_{mac}239$ and a cloned, macrophage-tropic chimeric virus ($SIV_{mac}239$/17E) whose *env* gene was derived from brain of a macaque (17E) dying from SIV-induced encephalopathy. $SIV_{mac}239$/17E caused a productive, syncytial cytopathic infection accompanied by accumulation of virus particles within cytoplasmic vesicles of the macrophages. Pulse–chase and immune precipitation studies showed that both the viral glycoprotein precursor (gp160) and the *gag* precursor (p57) were cleaved into gp120 and p27, respectively, and both were released into the culture medium of infected cells, although most of the p27 remained cell associated. $SIV_{mac}239$ also infected macrophages, but in comparison to $SIV_{mac}239$/17E, minimal virus replication occurred. Immunocytostaining revealed that while occasional syncytia were observed in cultures, the majority of the infected cells were not associated with syncytium formation. Ultrastructural studies did not reveal the accumulation of virions within infected macrophages. Pulse–chase studies showed that both gp160 and p57 were produced but were cleaved inefficiently and only minimal amounts of gp120 and p27 were released into the culture medium, even after prolonged incubation times. The processing of proteins of the two viruses was indistinguishable in lymphocytes. Since these two viruses are identical except for changes within the *env* gene, these results indicate that efficient assembly and release of SIV from blood-derived macrophages is mediated by changes in the envelope glycoprotein.

INTRODUCTION

The simian immunodeficiency viruses (SIVs) comprise a group of nonhuman primate lentiviruses that are related genetically and biologically to human immunodeficiency virus types 1 and 2 (HIV-1 and HIV-2) (Barre-Sinnoussi *et al.*, 1983; Chatrabarti *et al.*, 1987; Clavel *et al.*, 1986; Daniel *et al.*, 1985; Franchini *et al.*, 1987; Guyader *et al.*, 1987). Similar to HIV infection of humans, rhesus macaques (*Macaca mulatta*) infected with SIV_{mac} develop acquired immunodeficiency syndromes (AIDS) characterized by a progressive loss of circulating $CD4^+$ T-lymphocytes and an increased susceptibility to opportunistic infections (Letvin and King, 1990; Daniel *et al.*, 1985; Letvin *et al.*, 1985; Kestler *et al.*, 1990). Based on the similarities of the two systems, the SIV/macaque system represents an excellent model for dissecting the molecular mechanisms by which HIV causes AIDS in humans.

Both HIV-1 and HIV-2 as well as the SIVs share the common property of being able to infect $CD4^+$ T-cells as well as cells of the monocyte/macrophage lineage (Barre-Sinoussi *et al.*, 1983; Gartner *et al.*, 1988; McEntee *et al.*, 1991). While virus entry into these two cell types is mediated by gp120/CD4 interactions at the cell surface, the sites of virus assembly appear to differ within these two cell types (Gendelman *et al.*, 1988; 1989; Orenstein *et al.*, 1988). Within lymphocytes, the envelope glycoprotein precursor, gp160, is synthesized on the rough endoplasmic reticulum as a high-mannose precursor. The gp160 is transported to the Golgi complex where the N-linked oligosaccarides are converted to those of the complex type and is probably cleaved within the trans Golgi or the trans Golgi network (TGN) into the receptor binding component, gp120, and the transmembrane glycoprotein, gp41. The gp120/gp41 complex is then transported via vesicles to the cell plasma membrane where virus assembly occurs (Hunter and Swanstrom, 1990). In contrast, less information is available on the processing of the envelope glycoprotein and virus assembly in macrophages. Electron microscopic studies have shown that HIV is assembled and accumulates within cytoplasmic vesicles of macrophages (Orenstein *et al.*, 1988). Virus has also been observed to be assembled at the cell plasma membrane (Orenstein and Jannotta, 1988).

Using the simian immunodeficiency virus (SIV) model, we have examined the processing of viral proteins following the infection of macrophages with the molecularly cloned $SIV_{mac}239$ (239) and a neuroadapted variant of this virus, $SIV_{mac}239$/17E (17E) (Anderson *et al.*, 1992).

[1] To whom correspondence and reprint requests should be addressed. Fax: (913) 588-5599.

0042-6822/95 $6.00

The parental 239 virus has been reported to replicate poorly in macrophages (Mori *et al.,* 1992), whereas 17E has been reported to replicate well with accompanying cytopathicity in macrophages (Anderson *et al.,* 1992). Our results indicate that viral proteins were synthesized in macrophages infected with either virus but both the *gag* and *env* precursors were processed more efficiently in macrophages infected with 17E than with 239. These results may explain in part why lymphocyte-tropic viruses such as $SIV_{mac}239$ infect but replicate poorly in macrophages.

MATERIALS AND METHODS

Virus strains and cell lines

Two strains of SIV_{mac} were used in this study. The first is the lymphocyte-tropic strain $SIV_{mac}239$ (Regier and Desrosiers, 1990) and the second, $SIV_{mac}239/17E$ (17E), is an interviral recombinant in which the majority of the envelope glycoprotein gene of $SIV_{mac}239$ was removed and replaced with *env* gene that was PCR amplified from the brain of macaque 17E, which had died of SIV-induced encephalitis (Sharma *et al.,* 1992; Anderson *et al.,* 1992). Thus, the two viruses are identical except for the envelope glycoprotein. Virus 17E was shown to cause cytopathology (fusion) in both rhesus macrophages as well as CEMx174 cells (Anderson *et al.,* 1992). Both strains had a premature termination codon in the *nef* gene that was found in the original $SIV_{mac}239$ clone.

Isolation of blood-derived rhesus macrophages (BDM)

Peripheral blood mononuclear cells (PBMC) were isolated from SIV-seronegative rhesus macaques. Blood was collected in EDTA-coated tubes and mononuclear cells were isolated on Ficoll–Hypaque gradients. Approximately 6×10^6 mononuclear cells were seeded into 35-mm dishes in macrophage driving medium (MDM: 75% RPMI containing 10% human serum, 15% conditioned L-cell medium prepared by culturing confluent monolayers of L-929 cells in the presence of EMEM supplemented with 10% fetal bovine serum for 5 days followed by filtration through a 0.22-μm filter, 5.5×10^{-5} *M* β-mercaptoethanol, 10 m*M* HEPES, 5 units macrophage colony-stimulating factor (Genetics Institute), 100 units granulocyte macrophage colony-stimulating factor (Genetics Institute), and 10 μg/ml of gentamicin) and incubated for 3 days at 37° with 5% CO_2. At 3 days, the cultures were refed with fresh medium. Cultures were washed to remove nonadherent cells and refed with fresh MDM every 3 days for 2 weeks prior to inoculation with SIV_{mac}.

Virus infections and immune precipitation analyses

The biosynthesis and processing of SIV proteins were analyzed by immune precipitation analyses similar to that described previously (Stephens *et al.,* 1991). BDM in 35-mm dishes were infected with 10^3 $TCID_{50}$'s of either 17E or 239 for 1 hr at 37°. At 1 hr postinfection, an additional 2 ml of medium was added and the cells were incubated at 37° for 3 days. Cells were refed at 3 days postinfection. At 5 days postinfection, the medium was removed and infected cells were incubated in methionine-free Dulbecco's modified Eagle's medium (DMEM) for 2 hr. The cells were then radiolabeled for 1 hr with 200 μCi of ^{35}S-Translabel (^{35}S-methionine and cysteine, ICN) and the radiolabel was chased for various periods of time in DMEM containing 100× unlabeled methionine. SIV proteins were immune precipitated from the cell culture medium and infected cell lysates using plasma pooled from several rhesus monkeys infected previously with 239 (Joag *et al.,* 1994). Briefly, the cell culture medium was clarified (16,000 *g*) for 2 min. The supernatant was transferred and made 1× with respect to cell lysis buffer (50 m*M* Tris–HCl, pH 7.5; 50 m*M* NaCl; 0.5% deoxycholate; 0.2% SDS; 10 m*M* EDTA) and SIV proteins were immune precipitated with 10 μl of the monkey plasma. For immune precipitation of cell-associated SIV proteins, cell lysis buffer was pipeted into dishes and cell lysates were obtained after incubation of dishes on ice for 30 min. Lysates were centrifuged in a microfuge to remove nuclei prior to the addition of antibody. Cell lysates and culture medium were incubated with antibody for 16 hr at 4°.

All immune precipitates were collected on Protein A–Sepharose, the beads washed four times with RIPA buffer, and the samples resuspended in sample reducing buffer (Laemmli, 1970). Samples were boiled and the SIV-specific proteins analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were then visualized by standard fluorographic techniques.

Preparation of SIV-infected macrophages for ultrastructural studies

Rhesus blood-derived macrophages were infected with either the 239 or 17E for 5 days. Cells were washed 3× with RPMI without serum and fixed for electron microscopy with 2% glutaraldehyde in phosphate buffer (pH 7.4) overnight at 4°. The cells were washed in phosphate-buffered saline, scraped from the plastic surface, and transferred into a microfuge tube. After postfixation with 1% osmium tetroxide, cells were dehydrated in a series of graded ethanols and embedded in LR-White resin. Thin sections were stained with uranyl acetate and lead nitrate and examined on a Joel 100CX electron microscope.

Infectivity of virus released from 239- and 17E-infected cells

We analyzed the infectivity of the virus produced from macrophage cultures using a modified polymerase chain

reaction/infected cell assay (PCR/ICA) described by Joag *et al.* (1994). Macrophage cultures were infected with either stock virus (DNase treated with 1 μg/ml of DNase for 30 min at 37°) for 5 days at 37°. At 5 days, the culture medium from infected cells was harvested, clarified by centrifugation (1200 rpm for 10 min), and then treated with DNase. The clarified medium, DNase-treated medium, was filtered through a 0.22-μm filter and a series of 10-fold dilutions (undiluted to 10^{-8}) used to infect CEMx174 cells. Five days later, inoculated CEMx174 cells from each virus dilution were harvested, pelleted by centrifugation, and resuspended in 0.1 ml of RPMI medium. DNA extracted from each cell pellet was used for PCR amplification (Saiki *et al.,* 1985; 1988) using a nested set of oligonucleotide primers that will amplify a 240-bp fragment of the 239 or 17E *gag* gene. As an internal control for the PCR amplification, a nested set of oligonucleotide primers was used to amplify the cytoplasmic β-actin gene (Nakajima-Iijima *et al.,* 1985). The procedures for PCR amplification have been previously described (Joag *et al.,* 1994). The advantage of using this modified PCR/ICA technique over conventional virus titration assays was that it detected non-syncytium-forming virus produced in CEMx174 cells. Infections with heat-inactivated virus (56° for 60 min) were also included as a control to detect possible proviral carryover.

p27 Assays

Determination of the amount of p27 core protein synthesized by virus-infected macrophages and released into the culture medium was performed using the core antigen capture assay (Coulter Corp., Hialeah, FL).

Immunocytostaining procedures

Macrophage cultures were established on coverslips in 35-mm plates using the procedures described above. Cells were inoculated with either $SIV_{mac}239$ or $SIV_{mac}239$/17E or remained uninfected. At 5 days postinfection, cultures were washed once with sRPMI and fixed in a solution of Zn^{+2}–formalin. Cells on coverslips were pretreated with 0.3% H_2O_2/0.1% NaN_3 for 30 min to remove endogenous peroxidase activity, washed with PBS, and then blocked using 5% goat serum for 60 min at room temperature. Cells were reacted with a mouse monoclonal antibody directed against the p27 core antigen (FA-2) overnight at room temperature. Cells were washed three times with PBS/0.1% Triton X-100, reacted with a biotinylated goat anti-mouse antibody for 30 min, and washed again with PBS/0.1% Triton X-100. Cells were reacted with ABC horseradish peroxidase for 30 min at room temperature and then reacted with DAB substrate. Cells were counterstained with hematoxylin, dehydrated, and then mounted onto glass slides.

RESULTS

Both $SIV_{mac}239$ and $SIV_{mac}239$/17E infect and synthesize proteins in rhesus blood-derived macrophages

We first determined whether the two strains of SIV could infect and synthesize viral proteins within infected macrophages. Macrophage cultures were inoculated with either virus at a similar m.o.i., as determined by titration in CEMx174 cells. Culture media were collected and cell lysates prepared at various times (0–12 days) following inoculation. The results shown in Fig. 1A indicate that the level of p27 released into the culture medium peaked at 6 days postinoculation for both viruses. However, the levels of p27 released into the culture medium of $SIV_{mac}239$/17E-infected macrophages were three- to five- fold higher than those observed for $SIV_{mac}239$-infected macrophages. Examination of infected cell lysates also revealed that the biosynthesis of p27 peaked at 3 days following inoculation (Fig. 1B). Comparison of the amount of p27 in the culture medium with the amount remaining cell associated revealed that only a small fraction (<1%) of the total synthesized p27 protein was released from infected cells. Sequential sampling indicated that peak production of p27 occurred between Days 5 and 6. Immunocytochemical analyses of the cultures at this time showed that whereas uninfected macrophage cultures did not have p27 antigen, cultures inoculated with $SIV_{mac}239$/17E developed extensive syncytial cytopathology with greater than 50% of the cells in culture staining for the presence of p27 antigen (Figs. 2B and 2C). In contrast, $SIV_{mac}239$-infected macrophage cultures revealed that only about 10% of the cells in culture had p27 (Fig. 2D). In addition, while an occasional infected syncytial cell was observed, the majority of the $SIV_{mac}239$-infected macrophages expressing p27 were not associated with syncytia (Fig. 2E). The failure of infected cells to recruit neighboring cells into syncytia formation suggested that the viral glycoprotein was functionally impaired, possibly due to inefficient transport to the cell surface or inefficient cleavage of the precursor gp160. These results indicate that while $SIV_{mac}239$ will infect macrophages, the low frequency of syncytia formation suggest that the $SIV_{mac}239$ glycoprotein was transported inefficiently to the surface of infected cells.

The glycoprotein of $SIV_{mac}239$ is transported inefficiently to the surface of infected macrophages

Immune precipitation analyses were used to determine what SIV proteins were released from infected cells. Macrophage cultures were inoculated with either virus and 5 days later cells were radiolabeled with [^{35}S]-methionine for 24 hr. SIV proteins were immune precipitated from the culture media with anti-SIV antibodies. Similar to the results of the p27 assays, the number of

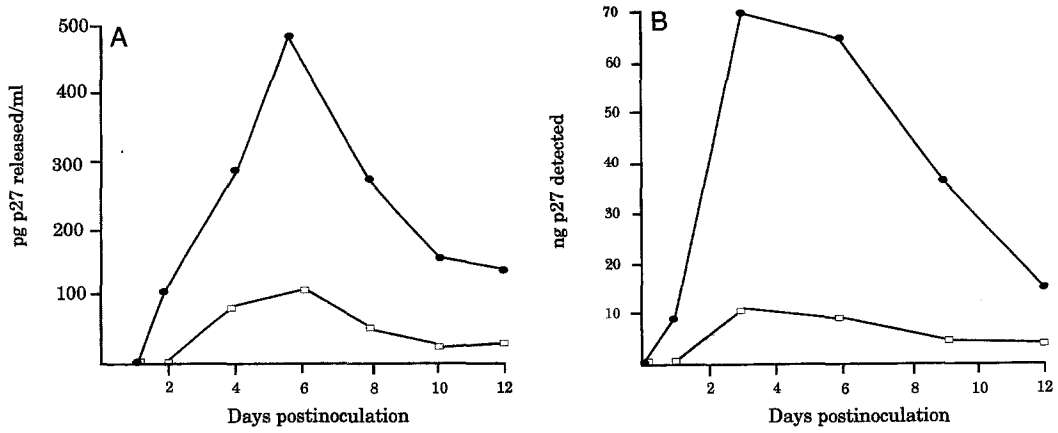


FIG. 1. Quantitation of p27 levels from macrophage cultures infected with either $SIV_{mac}239$ or $SIV_{mac}239/17E$. Rhesus macrophages (in 35-mm dishes) were infected with either virus 239 or virus 17E for 2 hr at 37°. At 2 hr postinfection, the inoculum was removed, washed three times with RPMI medium without serum (sRPMI), and then refed with 2 ml of medium. One-half of the culture medium was changed at 4-day intervals. For quantitating levels of cell-associated p27 protein, infected macrophage cultures were scraped into 1 ml of sRPMI, freeze-thawed, and assayed for p27 according to the manufacturer's instructions using appropriate uninfected cultures. For assaying levels of p27 released into the culture medium, samples were collected and centrifuged in a microfuge prior to assay. (A) Time course of p27 release from $SIV_{mac}239$ (□)- and $SIV_{mac}239/17E$ (●)-infected macrophages. (B) Time course of cell-associated p27 in $SIV_{mac}239$ (□)- and $SIV_{mac}239/17E$ (●)-infected macrophages.

immune-precipitable counts from $SIV_{mac}239/17E$-infected cells were always four- to fivefold higher than for $SIV_{mac}239$-infected cells (data not shown). The results demonstrated that following a 24-hr labeling period, both the major glycoprotein and gag cleavage products, gp120 and p27, respectively, of 17E virus were detectable in the culture medium (Fig. 3). These results contrasted with those obtained for 239-infected macrophages in which p27 but little or no gp120 could be immune precipitated from the culture medium after a 24-hr labeling period (Fig. 3).

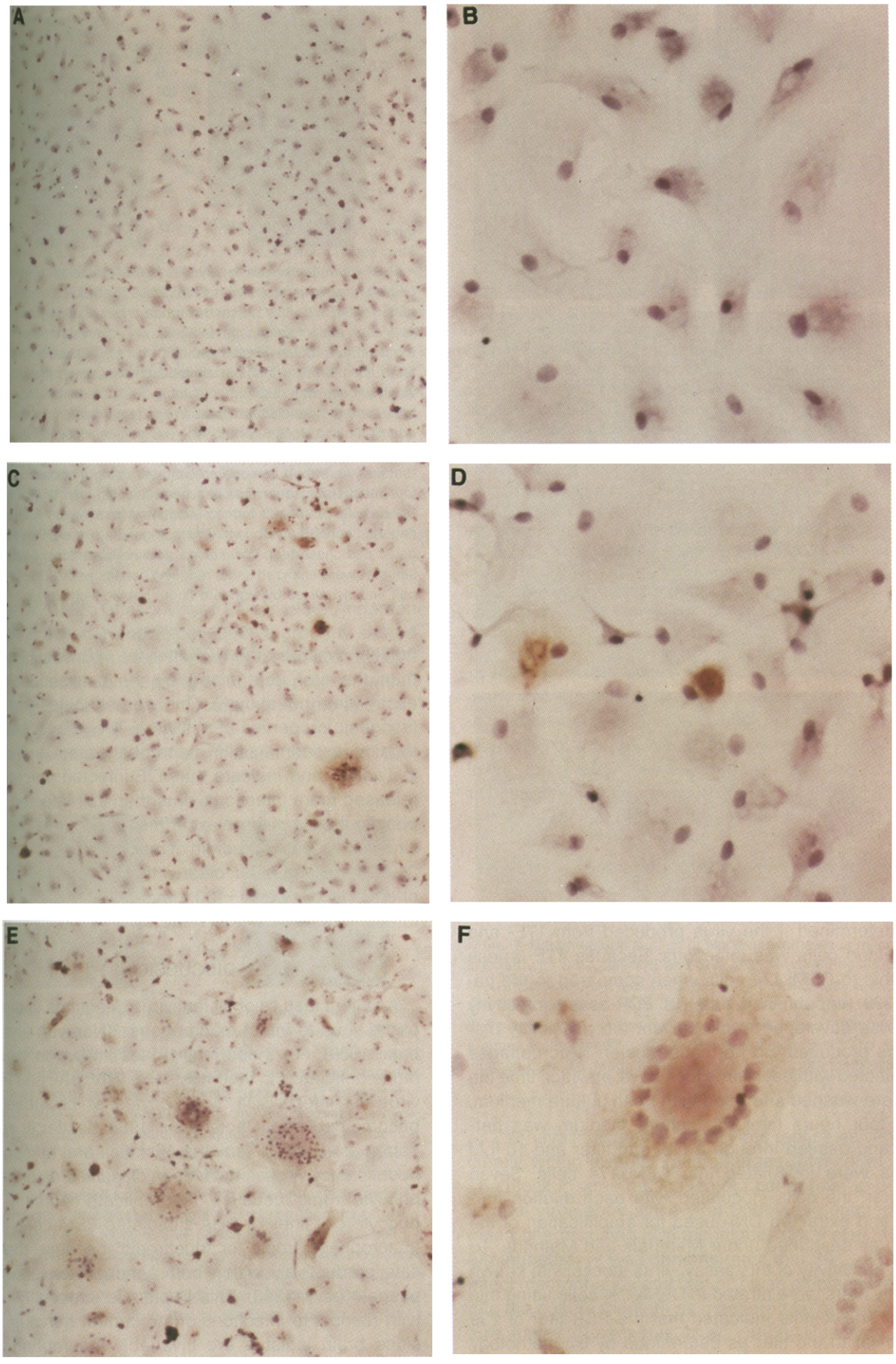
Pulse–chase analyses of CEM cells and rhesus macrophages infected with either $SIV_{mac}239$ or $SIV_{mac}239/17E$

To determine if the amount of gp120 released into the culture medium might be due to differential processing of the viral glycoprotein precursor, pulse–chase analyses were performed on CEMx174 cells or rhesus macrophages infected with either virus Figs. 4–6). The results of pulse–chase analyses of CEMx174 cells infected with 17E are shown in Fig. 4. At the 0 hr chase period, the glycoprotein precursor species with a M_r of 160,000 (gp160) and the *gag* precursor with a M_r of 57,000 (p57) were observed. By the 1-hr chase period, gp160 was cleaved into a protein species with a M_r of 120,000 (gp120) which increased in amount with longer chase periods (Fig. 4A). Similarly, the *gag* precursor (p57) was cleaved into the major *gag* cleavage product (p27) starting at the 1-hr chase period. Immune precipitation of SIV-specific proteins from the culture medium revealed that both gp120 and p27 were released from cells beginning at the 3-hr chase period and increased through the 7-hr chase period (Fig. 4B). The results for virus 239-infected cells were indistinguishable from those for virus 17E (data not shown). However, results were discordant in infected macrophage cultures (Figs. 5 and 6). Whereas the proteins of 17E were processed by the first hour of the chase period, proteins of 239 were processed much less efficiently in macrophages. Cleavage of the glycoprotein precursor into gp120 and gag precursor into p27 was much less efficient when compared to 17E (Fig. 6A). The apparent M_r of the glycoprotein precursor also appeared to decrease in size with increasing chase periods. This may be due to processing of the carbohydrate side chains. This inefficient processing of the glycoprotein and gag precursors cleavage was also reflected in the amount of gp120 and p27 released into the culture medium (Fig. 6B).

Ultrastructural analyses of SIV-infected macrophages

Since the protein labeling studies indicated that differences in the processing of the *env* and *gag* precursors, ultrastructural studies were performed to determine if assembly of viral particles occurred within infected cells. Examination of macrophages infected with 17E revealed

FIG. 2. Immunocytostaining of macrophage cultures infected with $SIV_{mac}239$ or $SIV_{mac}239/17E$. Macrophage cultures were infected with either $SIV_{mac}239$ or $SIV_{mac}239/17E$ or were uninfected. At 5 days following inoculation, cells were washed, fixed, and stained for p27 antigen as described under Materials and Methods. (A) Uninfected macrophage culture, 100×. (B) Uninfected macrophage culture, 400×. (C) Macrophage culture infected with $SIV_{mac}239$, 100×. (D) Macrophage culture infected with $SIV_{mac}239$, 400×. (E) Macrophage culture infected with $SIV_{mac}239/17E$, 100×. (F) Macrophage culture infected with $SIV_{mac}239/17E$, 400×.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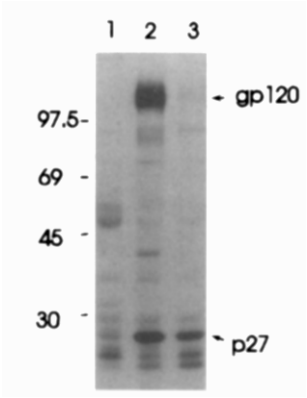

FIG. 3. The glycoprotein of $SIV_{mac}239$ is inefficiently released from macrophages. Rhesus macrophages were prepared and infected with either $SIV_{mac}239$ or $SIV_{mac}239/17E$ viruses. At 5 days postinfection, cells were starved for methionine and radiolabeled with 500 μCi of [^{35}S]-methionine and cysteine for 24 hr. The culture medium was collected and cell lysates prepared as described under Materials and Methods. SIV-specific proteins were then immune precipitated using a pooled sera from four rhesus macaques that had been infected with SIV for 6 months. Gels were loaded with equivalent numbers of immune-precipitated counts. Lane 1, SIV proteins immune precipitated from uninfected cells (negative control). Lane 2, SIV proteins immune precipitated from $SIV_{mac}239/17E$-infected macrophages. Lane 3, SIV proteins immune precipitated from $SIV_{mac}239$-infected macrophages. All samples were analyzed under reducing conditions by SDS–PAGE (8.5% gel) and visualized by standard fluorographic techniques. Sizes of the viral proteins are based on comparison with protein standards of known size.

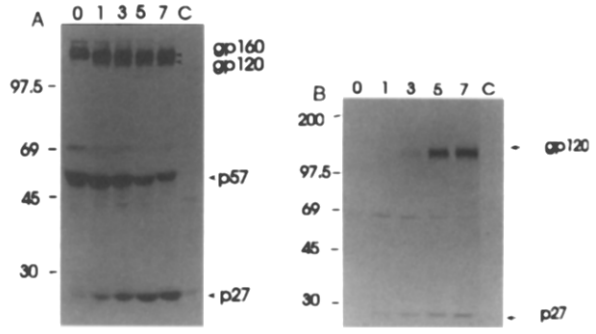

FIG. 4. Pulse–chase analyses of SIV proteins synthesized in CEMx174 cells infected with $SIV_{mac}239/17E$. CEMx174 cells were infected with $SIV_{mac}239/17E$ as described under Materials and Methods. At 3 days postinfection, infected cells were starved for methionine for 2 hr and then pulse-labeled with 100 μCi of [^{35}S]methionine and cysteine for 1 hr. The medium containing the radiolabel was removed, washed once with medium containing 100× cold methionine and cysteine, and the radiolabel chased in the same medium for various periods of time (0, 1, 3, 5, and 7 hr). The culture medium was collected and cell lysates were prepared as described in the text. SIV-specific proteins were then immune precipitated using a pooled plasma from four rhesus macaques that had been infected with SIV for 6 months. SIV-specific proteins immune precipitated from the cell lysates (A) and culture media (B) are shown. Uninfected rhesus macrophages, radiolabeled and chased for 7 hr, served as a negative control (lanes C). All samples were analyzed under reducing conditions by SDS–PAGE (8.5% gel) and visualized by standard fluorographic techniques.

numerous virus particles budding into cytoplasmic vacuoles (Figs. 7A–7C). No viral particles were observed maturing at the surface of infected cells. In contrast, exhaustive examination of cells infected with 239 did not reveal any virus particles budding or maturing into intracellular structures or at the surface of infected cells (Fig. 7D).

Infectivity of virus released from infected macrophages

We determined if the virus produced from 17E- and 239-infected cells was infectious. Because 17E is less cytopathic to CEMx174 cells when compared to the parental 239 (unpublished data), a PCR-based infectivity assay was developed to circumvent the possibility that the virus produced did not cause fusion. Macrophages were infected with each virus for 4 days, at which time the cells were washed and refed with fresh culture medium. Twenty-four hours later the culture medium was harvested and passed through a 0.22-μm filter and 10-fold dilutions were inoculated into CEMx174 cells. Seven days later, the cells inoculated with each dilution harvested and extracted DNA used for amplification of the SIV *gag* as described under Materials and Methods. To test the sensitivity of this assay, virus stocks with known infectivity titers were titrated in CEMx174 cells using this method. The results indicated that the PCR titer of 239 was similar to the titer as determined by cytopathology (fusion), whereas the PCR titer for 17E was 10× higher than the titer determined by the conventional fusion assay (data not shown). This was attributed to the less fusogenic properties of this virus. The results from three titration experiments in macrophages are summarized in Table 1 and indicate that 17E-infected macrophages released more infectious virus than 239-infected macrophages. In two of three experiments, we could not detect virus in the culture medium of macrophages infected with 239. This suggested clearly that 17E-infected macrophages produced 10–100× more virus than similar cultures infected with 239 (Table 1).

DISCUSSION

A salient feature of all lentivirus infections is the ability of the virus to infect macrophages (Narayan and Clements, 1989). For those lentiviruses known to cause immune deficiency in their hosts (i.e., HIV, SIV, and FIV), the virus also infects $CD4^+$ lymphocytes, which subsequently become depleted (Daniel *et al.*, 1985; Kestler *et al.*, 1990; Torten *et al.*, 1991). Isolates of simian and human immunodeficiency viruses have been classified as either "lymphocyte tropic" because they replicate optimally in lymphocyte cell lines or PHA-stimulated PBM cells and the "macrophage tropic" or "dualtropic" because they replicate productively within both lymphocytes and macrophages (Levy, 1993). This tropism is especially relevant with respect to infections of the central nervous system since brain isolates are invariably macrophage tropic

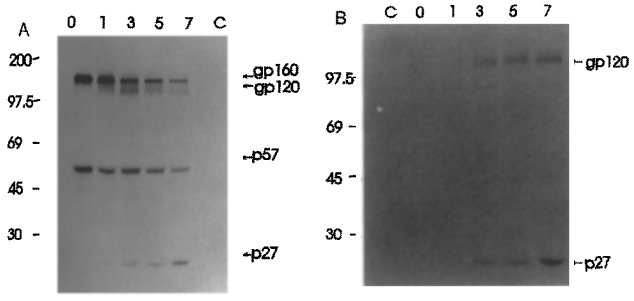

FIG. 5. Pulse–chase analyses of SIV proteins synthesized in rhesus macrophages infected with SIV_{mac}239/17E. Rhesus macrophages were prepared and infected with SIV_{mac}239/17E as described under Materials and Methods. At 5 days postinfection, infected cells were starved for methionine for 2 hr and then pulse-labeled with 100 μCi of [^{35}S]-methionine and cysteine for 1 hr. The medium containing the radiolabel was removed, washed once with medium containing 100× cold methionine and cysteine, and the radiolabel chased in the same medium for various periods of time (0, 1, 3, 5, and 7 hr). The culture medium was collected and cell lysates were prepared as described in the text. SIV-specific proteins were then immune precipitated using a pooled plasma from four rhesus macaques that had been infected with SIV for 6 months. SIV-specific proteins from the cell lysates (A) and culture medium (B) are shown. Uninfected rhesus macrophages, radiolabeled and chased for 7 hr, served as a negative control (lanes C). All samples were analyzed under reducing conditions by SDS–PAGE (8.5% gel) and visualized by standard fluorographic techniques.

(Price *et al.*, 1988; Cheng-Mayer *et al.*, 1989; Anderson *et al.*, 1992; Spencer and Price, 1992), a property shared with ungulate lentiviruses. However, no studies have critically examined the processing of the viral proteins within macrophages infected with macrophage-tropic and lymphocyte-tropic strains of SIV.

Previous studies with the molecularly cloned SIV-

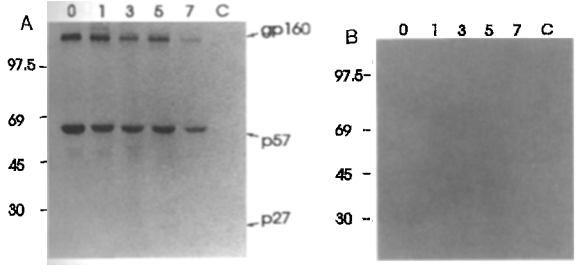

FIG. 6. Pulse–chase analysis of SIV proteins synthesized in rhesus macrophages infected with SIV_{mac}239. Rhesus macrophages were prepared and infected with SIV_{mac}239 as described under Materials and Methods. At 5 days postinfection, infected cells were starved for methionine for 2 hr and then pulse-labeled with 100 μCi of [^{35}S]methionine and cysteine for 1 hr. The medium containing the radiolabel was removed, washed once with medium containing 100× cold methionine and cysteine, and the radiolabel chased in the same medium for various periods of time (0, 1, 3, 5, and 7 hr). The culture medium was collected and cell lysates prepared as described in the text. SIV-specific proteins were then immune precipitated using a pooled sera from four rhesus infected with SIV for 6 months. SIV-specific proteins from the cell lysates (A) and culture medium (B) are shown. Uninfected rhesus macrophages, radiolabeled and chased for 7 hr, served as a negative control (lanes C). All samples were analyzed under reducing conditions by SDS–PAGE (8.5% gel) and visualized by standard fluorographic techniques.

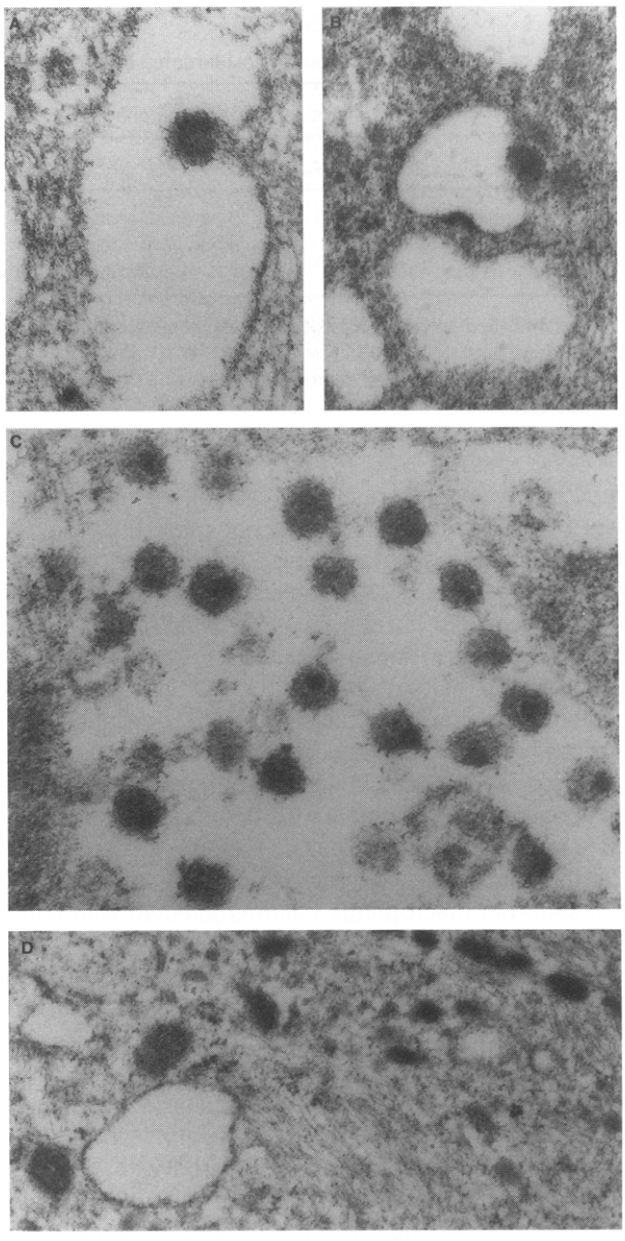

FIG. 7. Ultrastructural studies of blood-derived rhesus macrophages infected with SIV_{mac}239/17E and SIV_{mac}239 viruses. Rhesus blood-derived macrophages were prepared and infected with SIV_{mac}239/17E and SIV_{mac}239 viruses. At 5 days postinfection, the cells were washed and prepared for electron microscopy as described in the text. Shown are the results from SIV_{mac}239/17E-infected cells (A–C) and SIV_{mac}239-infected cells (D). Magnification for A–C: ×93, 100; for D: ×57,000.

$_{mac}$239 have indicated that this virus replicates very inefficiently within alveolar macrophages (Mori *et al.*, 1992). The block in virus replication did not appear to be at the level of virus entry since viral DNA was detected within macrophages using PCR techniques (Mori *et al.*, 1993). In another study, it was demonstrated that SIV_{mac}239 would infect macrophages and that p27 was efficiently released into the culture medium as measured by an antigen capture assay (Brinkmann *et al.*, 1993). However, in this

TABLE 1

TITRATION OF INFECTIOUS SIV RELEASED FROM INFECTED MACROPHAGES

Experiment	Titer of virus produced from macrophage cultures[a]		
	$SIV_{mac}239$	$SIV_{mac}239/17E$	H.I $SIV_{mac}239/17E$[c]
1	$<10^{b}$	100	<10
2	≤10	100	<10
3	$<10^{b}$	100	<10

[a] Titration based on the PCR detection as described under Materials and Methods.

[b] Lower limits of assay, not detected.

[c] Virus stock heat-inactivated for 60 min at 56°.

study, p27 was not released into the culture medium until Day 9 postinoculation and did not peak until Day 15 postinoculation. Our studies also indicate that p27 was released from $SIV_{mac}239$-infected macrophage cultures (Figs. 1A and 1B) but the kinetics of p27 release were very different from those reported by Brinkmann *et al.* (1993). In our studies, release of p27 into the culture medium was first detectable at Day 2 postinoculation and peaked by Day 6. The observed differences in the kinetics of p27 release in these two studies may be due to the source of the primary macrophages and the methods used to cultivate the cells.

In this study, we also compared the amounts of p27 released from macrophage cultures infected with either $SIV_{mac}239$ or the macrophage-tropic $SIV_{mac}239/17E$. Our results indicate that p27 was released from $SIV_{mac}239/17E$-infected macrophages at five times the level observed for $SIV_{mac}239$-infected macrophage cultures, despite inoculation of cultures with an equivalent m.o.i. These results correlate with the results from immunocytostaining which revealed that only 10% of the cells were infected with $SIV_{mac}239$ compared with >50% of the cells infected with $SIV_{mac}239/17E$. Immunocytostaining also revealed differences in the cytopathology of infected macrophage cultures. Whereas extensive syncytial cytopathology was consistently observed in $SIV_{mac}239/17E$ cultures, p27-expressing cells associated with syncytia were observed rarely in $SIV_{mac}239$-infected cultures. The rare occurrence of syncytia in $SIV_{mac}239$-infected macrophage cultures was suggestive that the glycoprotein of this virus may not be efficiently transported to the surface and/or qualitative differences exist in the fusogenicity of the envelope glycoprotein.

We have demonstrated in this study, using molecularly cloned viruses that differ only in their *env* genes, that both lymphocyte-tropic (239) and macrophage-tropic (17E) strains infected and synthesized the viral *gag* or *env* precursor proteins within blood-derived macrophages, albeit at reduced levels in 239-infected macrophages (Fig. 3). Following a 24-hr pulse labeling, we observed that the capsid protein and only trace amounts the glycoprotein could be immune precipitated from the medium of $SIV_{mac}239$-infected macrophages (Fig. 3). These results suggested that the capsid protein (p27) (and in all likelihood virus particles) were being released from 239-infected cells in absence of the virus glycoprotein. It is well established that release of retroviral core particles is not dependent on the presence of the glycoprotein (Wills and Craven, 1991). Thus, most of the particles released from 239-infected macrophages may be replication defective. These results were also reflected in the low infectious titer of virus recovered from cells using PCR-based assay (Table 1). These results may be important from a diagnostic view because, were only a p27 ELISA assay used, the results may be construed as evidence of "productive" replication of the virus in macrophages, whereas in fact the particles may be glycoproteinless particles containing p27.

Our results also indicated that while viral structural proteins such as *gag* and *env* precursors are proteolytically processed with similar kinetics in CEMx174 cells, important differences were found in the processing of the *gag* and *env* precursor proteins in blood-derived macrophages (Figs. 5 and 6). As might be expected for a productive infection, both the *env* and *gag* precursors were synthesized and processed efficiently within 17E-infected macrophages (Fig. 5A). This was also reflected in the amounts of gp120 and p27 released into the culture medium as well as infectivity of the virus produced (Fig. 3). In contrast, we observed that the glycoprotein precursor of 239 was processed inefficiently in macrophages and actually appeared to be degraded with time (Fig. 6A). Several explanations might account for the observed results. Previous studies with HIV have demonstrated that amino acid changes within the gp120, particularly the V3 domain, alter the tropism for various lymphocyte and macrophage cell lines (Cheng-Mayer *et al.*, 1990, 1991; Liu *et al.*, 1990; Milich *et al.*, 1993). Because our data indicate that the glycoprotein precursor of virus 239 is degraded with time, it is possible that amino acid changes at critical positions might alter the ability of the glycoprotein to form oligomeric structures. Oligomerization within the RER has been shown to be essential for the transport of many viral glycoproteins from the RER to the surface of infected cells (Earl *et al.*, 1990, 1991; Doms *et al.*, 1993). The end result of such amino acid changes might be an envelope glycoprotein that is transported inefficiently from rough endoplasmic reticulum to the Golgi complex and intracytoplasmic vesicles where virus assembly of visna-maedi, HIV, and SIV occurs in macrophages (Narayan *et al.*, 1982; Orenstein *et al.*, 1988). Additionally, while we observed release of p27 into the culture medium after prolonged labeling periods (and labeled with higher concentrations of [^{35}S]-methionine and cysteine), it appeared that less *gag* precursor was cleaved into p27 in pulse–chase analyses of 239-infected macrophages. Although a consistent find-

ing, it is perplexing considering that the two *gag* genes are identical.

While the primary site of lentivirus assembly in T-lymphocytes infected with HIV/SIV (Gelderblom, 1991) or sheep fibroblasts (Dubois-Dalcq *et al.,* 1979) with visnamaedi virus is the cell plasma membrane, the principal site of assembly in macrophages are within intracytoplasmic vacuoles (Narayan *et al.,* 1982; Orenstein *et al.,* 1988). Because of the differences in protein processing of the viruses in this study, electron microscopy was used to examine the site of SIV assembly within infected macrophages (Fig. 7). We observed virus particles budding into intracytoplasmic vesicles of virus 17E-infected macrophages but not from the cell plasma membrane. These results were also reflected in the p27 assays which revealed that only a small percentage (<1%) of the synthesized p27 was actually released from infected cells (Figs. 1A and 1B). The results from electron microscopy are similar to those reported for HIV-infected macrophages (Orenstein *et al.,* 1988) and suggest a common mechanism in which lentiviruses preferentially assemble at intracytoplasmic membranes in macrophages. In contrast, we did not observe virus maturing within cytoplasmic vesicles or at the cell surface in 239-infected macrophages even though the number of infectious units used to inoculate cultures were equivalent and infections allowed to proceed for 5 days prior to examination. This suggested that either the 239 infection did not spread throughout the macrophage culture and thus detection of 239 particles was below the limits of electron microscopy or that 17E was more efficient at assembling viral particles. These results are also supported by infectivity assays which indicated that 17E produced up to 100× more virus from macrophage cultures than 239 (Table 1).

Other studies with lymphocyte-tropic strains of HIV suggest that the block in viral replication in macrophages occurs at a stage in the viral lifecycle following integration of the viral DNA. Schmidtmayerova *et al.* (1992) showed that lymphocyte-tropic viruses (LAV and NDK) could infect blood-derived macrophages but less than 1% of the infected cells produced RNA as determined by *in situ* hybridization. Similarly, Huang *et al.* (1993) reported that infection of macrophages with lymphocyte-tropic strains resulted in arrest of the virus life cycle following DNA synthesis. Because our results showed that lymphocyte-tropic strains of SIV such as 239 could infect macrophages and synthesize viral proteins that were inefficiently processed, it suggests that the mechanisms involved in the restriction of replication of lymphocyte-tropic virus in macrophages may occur at different stages of the virus life cycle following establishment of proviral DNA in the nucleus of the cell.

ACKNOWLEDGMENTS

This work was supported by Grants AI-29382, NS-32203, and RR-06753 from the National Institutes of Health. We thank David Galbreath and Istvan Adany for excellent technical assistance.

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