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Processing of the Glycoprotein of Feline Immunodeficiency Virus: Effect of Inhibitors of Glycosylation

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The processing and transport of the envelope glycoprotein complex of feline immunodeficiency virus (FIV) in the persistently infected Crandell feline kidney (CRFK) cell line were investigated. Pulse-chase analyses revealed that the glycoprotein is synthesized as a precursor with an M_r of 145,000 (gp145) and is quickly trimmed to a molecule with an M_r of 130,000 (gp130). Treatment of gp130 with endoglycosidase H (endo H) resulted in a protein with an M_r of 75,000, indicating that nearly half the weight of the gp130 precursor consists of endo H-sensitive glycans during biosynthesis. Chase periods of up to 8 h revealed intermediates during the further processing of this glycoprotein precursor. Initially, two minor protein species with apparent M_rs of 100,000 and 90,000 were detected along with gp130. At later chase times these two species appeared to migrate as a single dominant species with an M_r of 95,000 (gp95). Concomitant with the appearance of gp95 was another protein with an M_r of approximately 40,000 (gp40). Chase periods of up to 8 h revealed that approximately half of the precursor was processed into the gp95-gp40 complex within 4 h. gp95 was efficiently transported from the cell into the culture medium by 1 to 2 h after labeling, whereas gp40 was not observed to be released from infected CRFK cells. Analysis of the processing in the presence of monensin, castanospermine, and swainsonine also suggests the existence of these intermediates in the processing of this lentivirus glycoprotein. As with human immunodeficiency virus, virus produced in the presence of glucosidase inhibitors had reduced infectivity for T-lymphocyte cultures.

Feline immunodeficiency virus (FIV) is a T-lymphotropic lentivirus that was initially isolated from a group of cats with an immunodeficiencylike syndrome (25, 27). The disease in cats is characterized by lymphadenopathy, an alteration of the CD4/CD8 T-lymphocyte ratio, and susceptibility to opportunistic pathogens (26, 27, 47). FIV has a cell tropism similar to that of human immunodeficiency virus (HIV), having been demonstrated to infect macrophages, astrocytes, and the CD4 subset of lymphocytes (2, 5, 26). Because FIV infection of cats has many similarities to HIV-induced AIDS, it has been suggested that FIV may provide a useful animal model for AIDS (11).

The glycoproteins encoded by retroviruses are of considerable interest because they (i) determine the cell tropism of the virus through interaction with cell receptors on the cell surface, (ii) exhibit membrane fusion activity involved in virus penetration, and (iii) act as targets of neutralizing antibodies and cytotoxic T cells in the immune response directed against retrovirus infections (3, 13, 16, 17, 37). Replication-competent retroviruses encode a single glycoprotein that is synthesized as a high-mannose precursor on the rough endoplasmic reticulum (32, 36, 38, 43). The glycoprotein precursor is subsequently transported from the rough endoplasmic reticulum to the Golgi complex, where it is proteolytically cleaved by a cell-encoded protease into a heterodimeric complex. Cleavage of the precursor is concomitant with the acquisition of resistance to endoglycosidase H, suggesting that this event probably occurs after transport to the Golgi apparatus (46). The heterodimer complex has two components: (i) an outer membrane protein that is heavily glycosylated and is responsible for binding to cell receptors and (ii) a transmembrane protein that is probably responsible for virus-cell membrane fusion as well as for anchoring the glycoprotein in the viral membrane (14, 18). In addition to containing N-linked glycans, the outer membrane glycoprotein of some retroviruses has been reported to have O-linked glycans added during its biosynthesis (29). The cleaved heterodimer may be linked by disulfide bonds, as in the gp70/p15E of murine leukemia virus, or may not be linked by disulfide bonds, as with the gp120/gp41 of HIV (28, 45). Previous studies of the glycoproteins encoded by FIV suggest the existence of three glycoprotein species within infected cells (6, 19, 22, 35). We report here on the synthesis and processing of the FIV-encoded glycoprotein and the effect of various inhibitors of asparagine-linked glycosylation. We also report on the infectivity of virus made in the presence of these inhibitors. Our results suggest that, as found for HIV, inhibitors that affect specific enzymes of the glycosylation pathway have differential effects on the processing and transport of the FIV glycoprotein. The data suggest that specific inhibitors of asparagine-linked glycosylation alter the infectivity of FIV in a manner similar to that in HIV, reinforcing the potential of FIV as an animal model for lentiviruses that induce immunodeficiency in humans.

MATERIALS AND METHODS

Cells, viruses, and antisera. Both uninfected Crandell feline kidney (CRFK) cells and those persistently infected with the Petaluma strain of FIV were used throughout this study. Cell lines were maintained in a mixture of 45% L-15 medium and 45% Dulbecco minimal essential medium, which was supplemented with 10% fetal bovine serum and 10 μg of gentamicin per ml. Antiserum to FIV was obtained from a specific-pathogen-free cat that was infected with

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purified FIV and was maintained at the College of Veterinary Medicine, University of Florida. This cat was seronegative for feline leukemia virus and feline syncytial virus but became seropositive for FIV.

Inhibitors and enzymes. Monensin was obtained from Sigma Chemical Co., and stocks were prepared in methanol. Tunicamycin, castanospermine, and swainonsine were obtained from Boehringer Mannheim Biochemicals and were freshly prepared in Dulbecco minimal essential medium without methionine and filter sterilized just before use. The concentration of the inhibitors used in this study were (i) 3 mM castanospermine, (ii) 300 ng of swainsonine per ml, (iii) 0.01 to 10 μM monensin, and (iv) 1 μg of tunicamycin per ml. Endo H (endo-β-*N*-acetylglucosaminidase H) and endo F (endo-β-*N*-acetylglucosaminidase F) were obtained from Boehringer Mannheim Biochemicals.

Analysis of radiolabeled FIV proteins. Glycoproteins were identified by labeling infected or uninfected cultures with 100 μCi of [^{3}H]glucosamine per ml for 20 h at 37°C. For pulse-chase analyses, confluent monolayers of CRFK cells infected with FIV or uninfected CRFK cells were incubated for 2 h in methionine-free Dulbecco minimal essential medium containing the appropriate concentration of the inhibitor. Cells were then radiolabeled with 100 μCi of [^{35}S]methionine per ml for either 5 or 30 min. Following the labeling period, the label was removed and chased for various periods in fresh Dulbecco minimal essential medium containing a 100× concentration of cold methionine. Glycosylation inhibitors were present throughout both the labeling and chase periods. At appropriate times following labeling, both culture medium and infected cells were analyzed for the presence of FIV-specific proteins. Culture medium was removed and centrifuged at 16,000 × *g* for 5 min. The medium was made 1× with respect to RIPA buffer (50 mM Tris-HCl, [pH 7.5], 0.15 M NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], 20 mM EDTA) and reacted with FIV-positive or FIV-negative antiserum for 16 h at 4°C. For immunoprecipitation of cell-associated polypeptides, monolayers were washed with cold phosphate-buffered saline (PBS) and lysed in 1 ml of RIPA buffer. Samples were vigorously vortexed and centrifuged at 16,000 × *g* for 15 min to remove the cell debris and reacted with FIV-positive or FIV-negative antiserum overnight at 4°C. Immunoprecipitates were collected on Sepharose-protein A, washed four times with cold RIPA buffer, and resuspended in sample buffer containing 2-mercaptoethanol. Samples were boiled for 10 min and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (15). The protein bands were visualized by standard fluorographic techniques.

Endo H and endo F digestions. For endo H digestions, immunologically precipitated material was resuspended in 0.1% SDS–0.01 M sodium citrate (pH 5.5) containing 0.01 U of endo H and digestions were allowed to proceed at 37°C for 16 h. Endo F digestions were performed on immunologically precipitated samples resuspended in 0.1 M sodium phosphate buffer (pH 6.0) containing 50 mM EDTA, 1% Nonidet P-40, 0.1% SDS, 1% 2-mercaptoethanol, and 2 U of endo F. Digestions were allowed to proceed for 16 h at 37°C. Samples were analyzed by SDS-PAGE as described above. Endo H and endo F digestions contained both 0.01 U of aprotinin and 1 mM phenylmethylsulfonyl fluoride to inhibit proteolytic activity.

Infectivity studies. To determine whether virus grown in the presence of glycosylation inhibitors was infectious, FIV-infected CRFK cells were treated with each inhibitor for 6 h at 37°C. At 6 h, the medium was removed and replaced with fresh medium containing the same concentration of the inhibitor. The cells were incubated for an additional 18 h at 37°C. Companion cultures were labeled with [^{35}S]methionine and immunoprecipitated with a mouse monoclonal antibody to the FIV p28 *gag* antibody to confirm that the amount of virus released from each treated culture was similar to that released from untreated cultures (data not shown). The virus-containing supernatant was collected, centrifuged at 1,200 × *g*, and filtered through a 0.45-μm-pore-size filter. The filtered medium was then used to infect 10^6 FL-74 cells (a cat T-lymphoid cell line susceptible to FIV) in the presence of 1 μg of Polybrene per ml. Cells were incubated for an additional 5, 10, 15, 20, or 25 days, at which time the levels of Mg^{2+}-dependent reverse transcriptase activity were determined. The conditions used to assay for reverse transcriptase activity were as follows: 0.1 M Tris-HCl (pH 8.0), 8 mM $MgCl_2$, 150 mM KCl, 0.05% Triton X-100, 1 μg poly(A)-oligo$(dT)_{12-18}$ template, 1 mM dithiothreitol, 10 μCi of [^{3}H]TTP. All reactions were carried out at 37°C for 2 h, at which time the trichloroacetic acid-precipitable counts were determined by standard methods.

RESULTS

Processing of the FIV glycoprotein. The results of labeling FIV (Petaluma)-infected CRFK cells with [^{35}S]methionine and [^{3}H]glucosamine, followed by immunoprecipitation analysis, are shown in Fig. 1. When infected cells were labeled with [^{35}S]methionine, six major virus-specific proteins with M_rs of 130,000, 95,000, 47,000 (Pr^{gag}), 40,000, 28,000, and 17,000 (not shown) were observed. The M_r 47,000, 28,000, and 17,000 proteins represent the *gag* precursor (p47) and its major cleavage products (p28 and p17), respectively (25, 39). Using the same antiserum from an FIV-positive cat, we detected three specific glycoproteins associated with FIV-infected CRFK cells; these proteins had apparent M_rs of 130,000, 95,000, and 40,000. Pulse-chase analyses were performed on CRFK cells infected with FIV to determine the precursor-product relationships of the FIV-specific glycoproteins (Fig. 2). The results of pulse-labeling infected cells for short periods (5 min) followed by chasing the synthesized proteins for periods of up to 25 min are presented in Fig. 2. When the radioactive label was chased for 5 min and then analyzed for FIV-specific polypeptides, a labeled protein with an apparent M_r of 145,000 was found that was gradually trimmed to a species with an M_r of 130,000 by the 20-min chase period (Fig. 2). Pulse-chase analyses were then performed in which cells were labeled for 30 min and chase periods extended for up to 8 h (Fig. 3). At the 0 h chase period we observed one major protein species with an M_r of 130,000 and two minor proteins with apparent M_rs of approximately 100,000 and 90,000. By the 2-h chase period, the minor proteins with M_rs of 100,000 and 90,000 had disappeared and two major proteins had appeared with M_rs of 95,000 (gp95) and 40,000 (gp40) (Fig. 3A). Immunoprecipitation of FIV-specific proteins from the culture medium revealed that gp95 was released from cells as early as the 2-h chase period and that significant amounts of gp95 had accumulated in the medium by the 4-h chase period. In contrast, gp40 was not released into the culture medium of infected cells during chase periods of up to 6 h (Fig. 3B). Extension of the chase periods up to 24 h failed to detect gp40 in the culture medium (data not shown).

Glycosylation of gp130 and gp95. The level of glycosylation

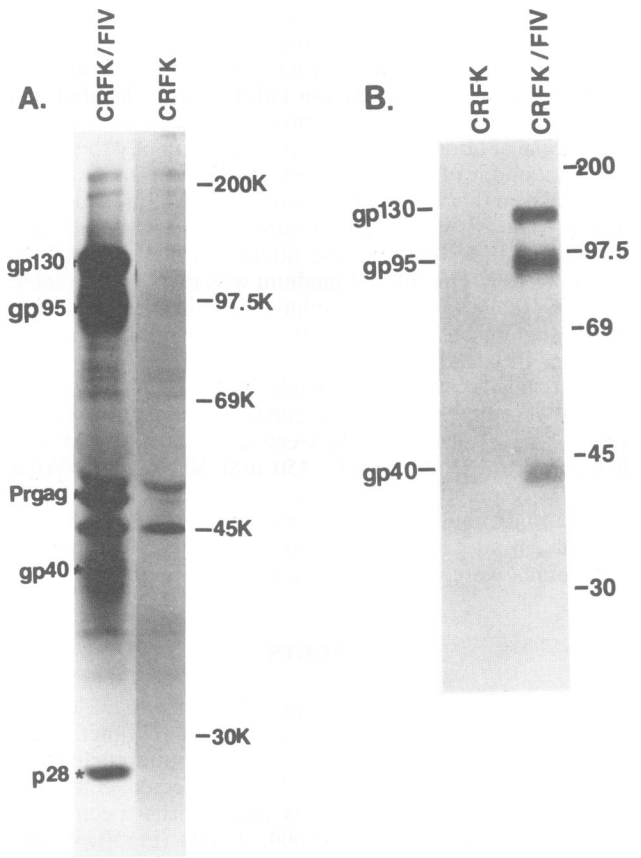

FIG. 1. SDS-PAGE analysis of FIV-specific proteins and glycoproteins. CRFK cells infected with FIV (CRFK/FIV) and uninfected CRFK cells were radiolabeled with 50 μCi of [^{35}S]methionine (A) or 200 μCi of [^{3}H]glucosamine (B) for 20 h and FIV-specific proteins were immunoprecipitated by using an FIV-positive, feline leukemia virus-negative antiserum as described in Materials and Methods. All precipitated proteins were analyzed under reducing conditions by SDS-PAGE (10% gel) and visualized by standard fluorographic techniques.

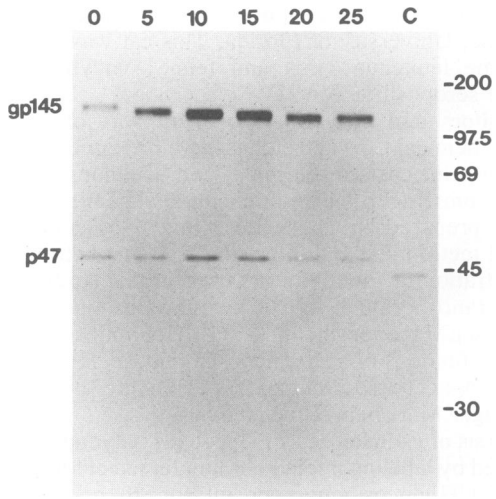

FIG. 2. Pulse-chase analyses of FIV proteins from the FIV-infected CRFK cell line. FIV-infected CRFK cells were incubated for 2 h in methionine-deficient medium and were then radiolabeled with 100 μCi of [^{35}S]methionine per ml for 5 min. Following the labeling period, the medium was removed, washed with medium containing excess cold methionine (150 mg/ml), and incubated in this medium containing excess methionine for various periods (0, 5, 10, 20, or 25 min), at which time FIV-specific proteins were immunoprecipitated from cells by using an FIV-positive, feline leukemia virus-negative cat antiserum as described in Materials and Methods. Uninfected CRFK cells that were pulsed-labeled for 5 min and chased for 25 min served as a negative control (C). All precipitated samples were analyzed under reducing conditions by SDS-PAGE (10% gel) and visualized by standard fluorographic techniques.

of gp130 was determined by pulse-labeling infected CRFK cells for 30 min in the presence or absence of 1 μg of tunicamycin per ml. As expected, a 30-min labeling of infected cells resulted in the biosynthesis of the gp130 precursor and the two minor proteins as described above (Fig. 4). In contrast, infected CRFK cells treated with tunicamycin prior to immunoprecipitation resulted in a protein product with an apparent M_r of 75,000 (Fig. 4). FIV proteins immunoprecipitated from untreated cells were also digested with endo H to determine the extent of glycosylation. Digestion of the immunoprecipitated gp130 with endo F also resulted in reduction of the precursor band from M_r 130,000 to 75,000 (Fig. 4). Both pieces of data suggest that at least 55,000 Da of N-linked glycans is initially added to the gp130 precursor. The gp95 released into culture medium was also treated with endo H or endo F to determine whether the added N-linked oligosaccharide chains were all processed to the complex form. Digestion of gp95 with endo H produced a protein with an M_r of 79,000, whereas digestion of gp95 with endo F resulted in a protein with an M_r of 49,000 (Fig. 5). The results indicate that the FIV gp95 released from cells contains both complex and high-mannose oligosaccharide side chains (Fig. 5).

Inhibitor studies. To determine whether the processing of the FIV glycoprotein was altered in the presence of inhibitors of N-linked glycosylation or glycoprotein transport, FIV-infected CRFK cells were treated with either monensin, castanospermine, or swainsonine. The results of pulse-chase analyses of FIV-infected CRFK cells in the presence of various concentrations of monensin are shown in Fig. 6. Monensin is a sodium ionophore that is known to alter the transport of many membrane proteins. In the presence of 10 μM monensin, gp130 was observed at the 0-h chase period and appeared to be reduced in size with time (Fig. 6A). However, gp95 and gp40 were never observed during any of the chase periods. No glycoprotein was found to be released from the cell (data not shown). In the presence of 1.0 μM monensin, gp130 was the major species labeled throughout the 8-h chase period. However, unlike the situation for treatment with 10 μM monensin, two minor proteins with apparent M_rs of 95,000 and 84,000 were labeled throughout the chase period (Fig. 6B). It appeared that the levels of both gp130 and the two minor species remained constant throughout the chase period. As with infected CRFK cells treated with 10 μM monensin, no significant amounts of FIV glycoproteins were released into the culture medium (data not shown). In the presence of 0.01 μM monensin, cleavage of the glycoprotein precursor was evident although the sizes of the two components were reduced (apparent M_rs of 84,000 and 36,000) (Fig. 6C). However, the kinetics of transport and release from the cell did not appear to be altered since the glycoprotein with M_r 84,000 was released at the 2-h chase period. As with the untreated cultures, the glycoprotein with an M_r of 36,000 was not transported from the cell (Fig. 6D).

The results of treatment of FIV-infected CRFK cells with

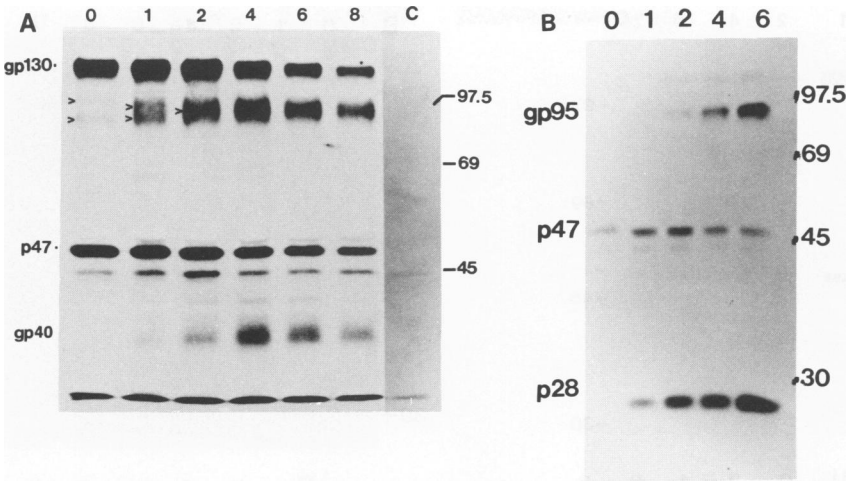

FIG. 3. Pulse-chase analyses of FIV proteins from the FIV-infected CRFK cell line with extended chase periods. FIV-infected CRFK cells were incubated for 2 h in methionine-deficient medium and were then radiolabeled with 100 μCi of [^{35}S]methionine per ml for 30 min. Following the labeling period, the medium was removed, washed with medium containing excess cold methionine (150 mg/ml), and incubated in this medium containing excess methionine for various periods (0, 1, 2, 4, 6, or 8 h), at which time FIV-specific proteins were immunoprecipitated by using an FIV-positive, feline leukemia virus-negative antiserum. Uninfected CRFK cells that were pulse-labeled for 30 min and chased for 8 h served as a negative control (C). (A) FIV-specific proteins immunoprecipitated from cell lysates. (B) FIV-specific proteins immunoprecipitated from cell culture medium. Samples were analyzed under reducing conditions by SDS-PAGE (10% gel) and visualized by standard fluorographic techniques.

two specific inhibitors of the enzyme of the N-linked glycosylation pathway, castanospermine and swainsonine, followed by immunoprecipitation with an antiserum specific for FIV are summarized in Fig. 7. Castanospermine is an inhibitor of α-glucosidase I of the rough endoplasmic reticulum. In the presence of castanospermine, the precursor glycoprotein had an increased apparent M_r, of 145,000. The processed molecule was also larger than the unmodified gp95 (M_r 100,000), whereas the transmembrane component had a lower apparent M_r, of 38,000. Pulse-chase analyses revealed that the FIV glycoprotein was processed slowly relative to untreated cultures since the gp95 component did not appear in significant amounts until the 4-h chase period (Fig. 8A). This slow processing of gp130 was also reflected in the amount of gp95 that was released from infected cells. gp95 was not detected in the supernatant until the 6-h chase period (Fig. 8B). In contrast to treatment with castanospermine, treatment of infected CRFK cells with swainsonine, an inhibitor of α-mannosidase II of the Golgi complex,

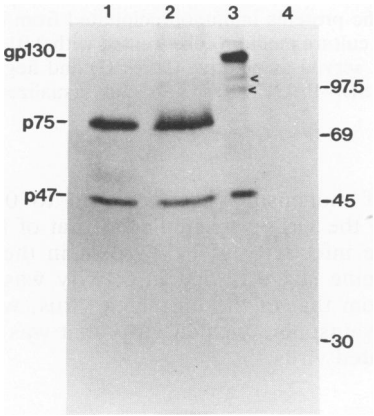

FIG. 4. Glycosylation of gp130. FIV-infected CRFK cells were incubated for 2 h in methionine-deficient medium in the presence or absence of 1 μg of tunicamycin per ml. Cells were then labeled with 100 μCi of [^{35}S]methionine for 30 min, and FIV proteins were immunoprecipitated with an FIV-positive antiserum. Lanes: 1, FIV proteins synthesized in the presence of tunicamycin; 2, FIV proteins synthesized in the absence of tunicamycin but treated with endo H for 15 h; 3, FIV proteins synthesized in the absence of tunicamycin; 4, FIV proteins immunoprecipitated from uninfected CRFK cells (negative control). All samples were analyzed under reducing conditions by SDS-PAGE (10% gel) and visualized by standard fluorographic techniques.

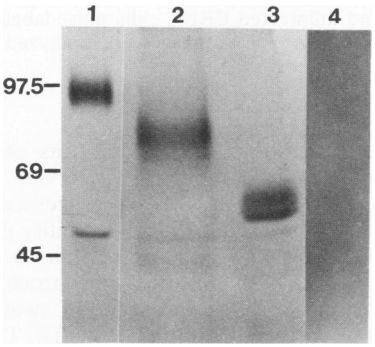

FIG. 5. Glycosylation of gp95. FIV-infected CRFK cells were incubated for 2 h in methionine-deficient medium and then labeled with 100 μCi of [^{35}S]methionine for 20 h. The culture medium was collected, and FIV proteins were immunoprecipitated by using FIV-positive antiserum. Lanes: 1, FIV proteins isolated from the culture medium of FIV-infected CRFK cells; 2, FIV proteins isolated from the culture medium of FIV-infected CRFK cells and treated with endo H for 15 h; 3, FIV proteins isolated from the culture medium of FIV-infected CRFK cells and treated with endo F for 4 h; 4, FIV proteins isolated from the culture medium of uninfected CRFK cells. All samples were analyzed under reducing conditions by SDS-PAGE (10% gel) and visualized by standard fluorographic techniques.

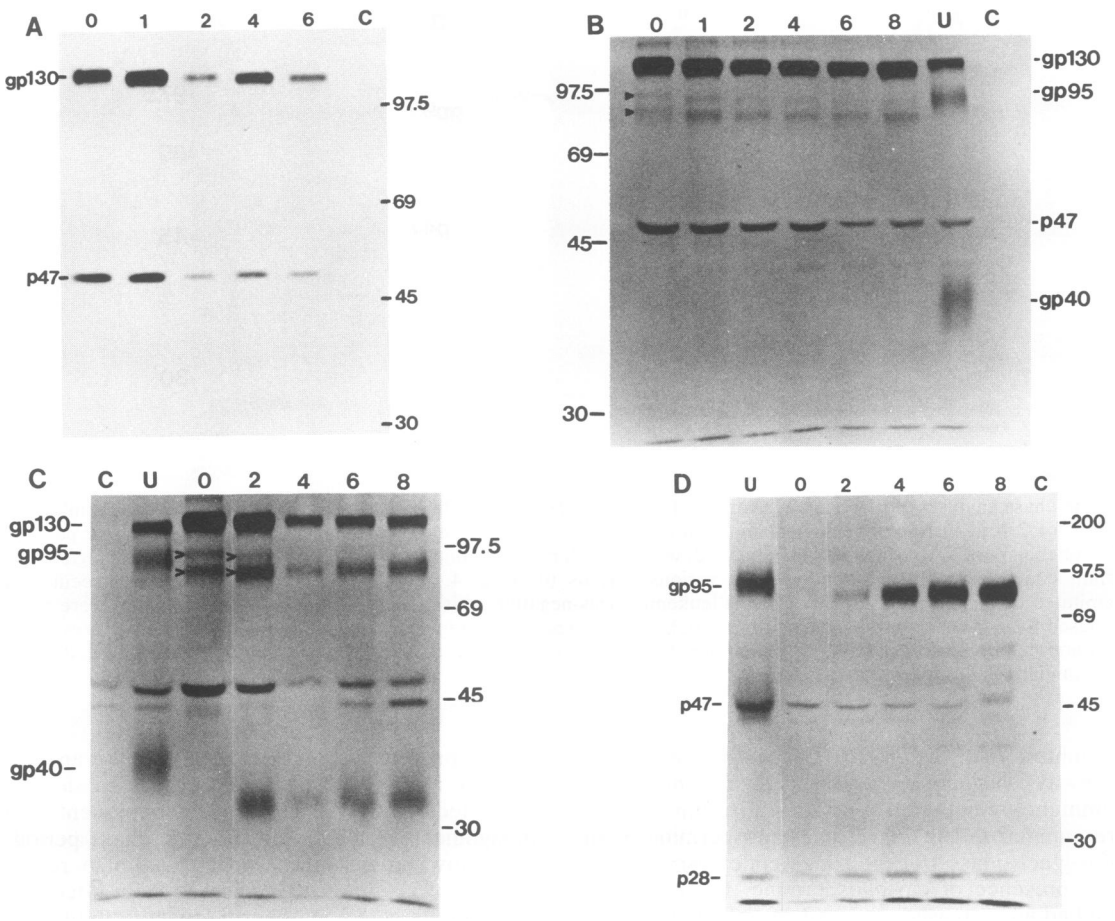

FIG. 6. Pulse-chase analyses of FIV proteins from FIV-infected CRFK cells in the presence of various concentrations of monensin. Analyses were identical to those for Fig. 3, except that cells were treated with 10, 1, or 0.01 μM monensin for 2 h prior to labeling and were maintained at these concentrations of monensin during the various chase periods (0, 1, 2, 4, and 6 h for 10 μM monensin; 0, 1, 2, 4, 6, and 8 h for 1 and 0.01 μM monensin). FIV-specific proteins were immunoprecipitated from cell lysates by using an antiserum from an FIV-positive cat. (A) FIV-specific proteins immunoprecipitated from lysates of cells treated with 10 μM monensin. (B) FIV-specific proteins immunoprecipitated from lysates of cells treated with 1 μM monensin. (C) FIV-specific proteins immunoprecipitated from lysates of cells treated with 0.01 μM monensin. (D) FIV-specific proteins immunoprecipitated from the culture medium cells treated with 0.01 μM monensin. FIV-infected and uninfected CRFK cells pulse-labeled for 30 min and chased for 8 h served as positive (lanes U) and negative (lanes C) controls, respectively. All samples were analyzed under reducing conditions by SDS-PAGE (10% gel) and visualized by standard fluorographic techniques.

resulted in a precursor molecule that was essentially the same size as the gp130 molecule observed in untreated cultures (Fig. 8C). However, the two processed forms were smaller, as evidenced by their faster mobility on polyacrylamide gels (M_r 85,000 and 36,000). Relative to castanospermine-treated cultures, the intracellular processing of the glycoprotein precursor in the presence of swainsonine was similar to that of untreated cultures (Fig. 8D). The precursor glycoprotein was cleaved in detectable amounts as early as the 1-h chase period; the gp95 was released into the culture medium by the 2-h chase period.

Infectivity studies with glycosylation inhibitors. The effect of monensin, castanospermine, and swainsonine on the infectivity of FIV produced from persistently infected CRFK cells is presented in Fig. 9. The ability of the virus to infect and replicate was determined by the level of reverse transcriptase activity with respect to time. Growth of FIV in the presence of 10 μM monensin resulted in the complete loss of virus infectivity. Similar results were obtained with tunicamycin (data not shown). In contrast, when the concentration of monensin was decreased to 0.01 μM, the infectivity of the virus was similar to that of the untreated samples. The infectivity of FIV grown in the presence of castanospermine suggests that infectivity was significantly decreased from that of the untreated virus, whereas treatment with swainsonine yielded virus that was as infectious as the untreated virus.

DISCUSSION

Previous studies have reported the nucleotide sequence of FIV, but no studies have used various inhibitors of glycosylation and membrane protein transport to examine the processing and transport of the FIV-encoded glycoprotein (21, 22, 39). The nucleotide sequence of FIV predicts a single glycoprotein precursor of 856 amino acids with several domains or sequences typical of many glycoproteins encoded by replication-competent retroviruses (21, 22). These include (i) two hydrophobic domains located at the amino-

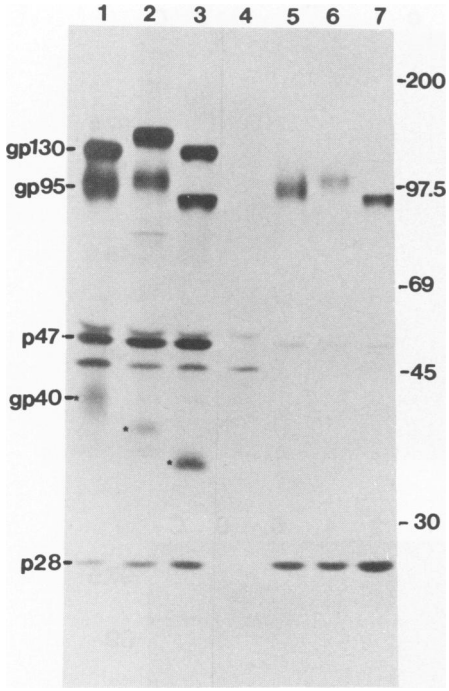

FIG. 7. Synthesis of FIV proteins in the presence of castanospermine and swainsonine. FIV-infected CRFK cells were incubated for 2 h in methionine-deficient medium and in the presence of 3 mM castanospermine or 300 ng of swainsonine per ml or were left untreated. Cells were then labeled with 50 μCi of [^{35}S]methionine for 18 h, and FIV proteins were immunoprecipitated from cell lysates and cell culture medium from cells with an FIV-positive antiserum. FIV-specific proteins were immunoprecipitated from FIV-infected CRFK cells without inhibitor (lane 1), with castanospermine (lane 2), or with swainsonine (lane 3). FIV-specific proteins immunoprecipitated from CRFK cells that were labeled under the same conditions served as a negative control (lane 4). FIV-specific proteins immunoprecipitated from the cell culture medium are shown from untreated (lane 5), castanospermine-treated (lane 6), and swainsonine-treated (lane 7) FIV-infected CRFK cell cultures. Samples were analyzed under reducing conditions by SDS-PAGE (10% gel) and visualized by standard fluorographic techniques.

terminal region that could serve as a potential signal sequence for translocation across the rough endoplasmic reticulum; (ii) a putative cleavage site, Arg-Arg-Lys-Arg, at the carboxyl terminus of the putative receptor-binding protein; and (iii) a putative transmembrane protein with two hydrophobic domains, one at the amino terminus which could be a potential fusion peptide for entry into susceptible cells and one toward the carboxyl terminus which could serve to anchor the membrane glycoprotein. The predicted precursor glycoprotein has 16 or 17 N-linked glycosylation sites within the putative receptor-binding protein and 4 sites within the putative transmembrane protein.

Radiolabeling CRFK cells persistently infected with the Petaluma strain of FIV revealed the presence of three glycoproteins with apparent molecular weights of 130,000, 95,000, and 40,000 (Fig. 1). These results are similar to what has been previously reported (22). Radiolabeling of infected cells for short periods (5 min) followed by short chase periods (up to 25 min) resulted in the synthesis of a glycoprotein with an apparent M_r of 145,000 that was gradually transformed over the course of the short chase period into a smaller protein with an M_r of 130,000 by 20 min (Fig. 2). This decrease was most probably due to the trimming of the terminal glucose and mannose residues from the N-linked oligosaccharide glycans added to the nascent polypeptide chain during translocation across and transport through the rough endoplasmic reticulum. Additional data demonstrating that gp130 was the precursor to the mature FIV glycoprotein came from experiments in which infected cells were treated with tunicamycin. This antibiotic prevents the addition of the dolichol-phosphate saccharide intermediate to the nascent polypeptide chain as it is translocated across the rough endoplasmic reticulum (33). Treatment of infected cells with tunicamycin for 2 h followed by radiolabeling resulted in the biosynthesis of a precursor in which the apparent molecular weight shifted from 130,000 to approximately 75,000, suggesting the addition of at least 55,000 Da of N-linked oligosaccharides to the nascent polypeptide chain (Fig. 4). The size of the glycoprotein precursor following treatment with tunicamycin (M_r of approximately 75,000) is similar to the predicted size of the precursor (M_r of approximately 78,000) if the signal peptidase cleavage site occurs within the first area of significant hydrophobicity (amino acids 150 to 180), as has been predicted (39). Taken together, these results indicate that the gp130 molecule is the FIV glycoprotein precursor. Extension of the pulse-chase period through 8 h revealed what appears to be at least one intermediate during the processing of the FIV glycoprotein in CRFK cells. Radiolabeling of infected cells for 30 min resulted in the appearance of the major precursor (gp130) and two minor components with apparent M_rs of approximately 100,000 and 90,000. With time, these two proteins were chased into two major cleavage products with M_rs of 95,000 and 40,000. Approximately 50% of the glycoprotein precursor was processed by the 2- to 4-h chase period (Fig. 3). The results obtained with FIV differ from those reported for HIV, in which the gp160 glycoprotein precursor was demonstrated to be inefficiently processed (45). Only a small percentage (5 to 15%) of the gp160 glycoprotein precursor of HIV was processed into mature gp120, which was subsequently transported to the cell surface and released from the cell (45). Because degradation of gp160 was inhibited by the lysosomotropic agent NH_4Cl, it was suggested that the majority of the gp160 precursor was being shunted and degraded in the lysosomes of lymphocytes (45). Additional evidence for the existence of an intermediate during the processing of this glycoprotein comes from the experiments with monensin, a sodium ionophore known to inhibit the intracellular transport of membrane and secretory proteins through the rough endoplasmic reticulum-Golgi complex (20, 40, 41). In addition, monensin has been shown to disrupt the transport and processing of several retrovirus glycoproteins such as the HIV gp120/gp41, the murine leukemia virus gp70/p15E, and the Rous sarcoma virus gp85/gp37 (4, 34, 38). Pulse-chase analyses of infected cells in the presence of 1 μM monensin clearly reveal that two proteins are present in addition to the precursor (gp130) after the 30-min labeling and that the levels of these two proteins persist throughout the chase periods examined (up to 8 h). When the concentration of monensin is reduced to 0.01 μM to allow more efficient processing, the intermediate is initially present but is gradually chased into two major components with apparent M_rs of approximately 84,000 and 34,000. Monensin has been used to study the processing and transport of the HIV glycoproteins synthesized in cells chronically infected with HIV or in adenovirus-infected cells expressing the HIV glycoprotein (4, 23). Monensin was shown to inhibit the processing of the gp160 precursor into gp120 and gp41 in lymphocyte cultures chron-

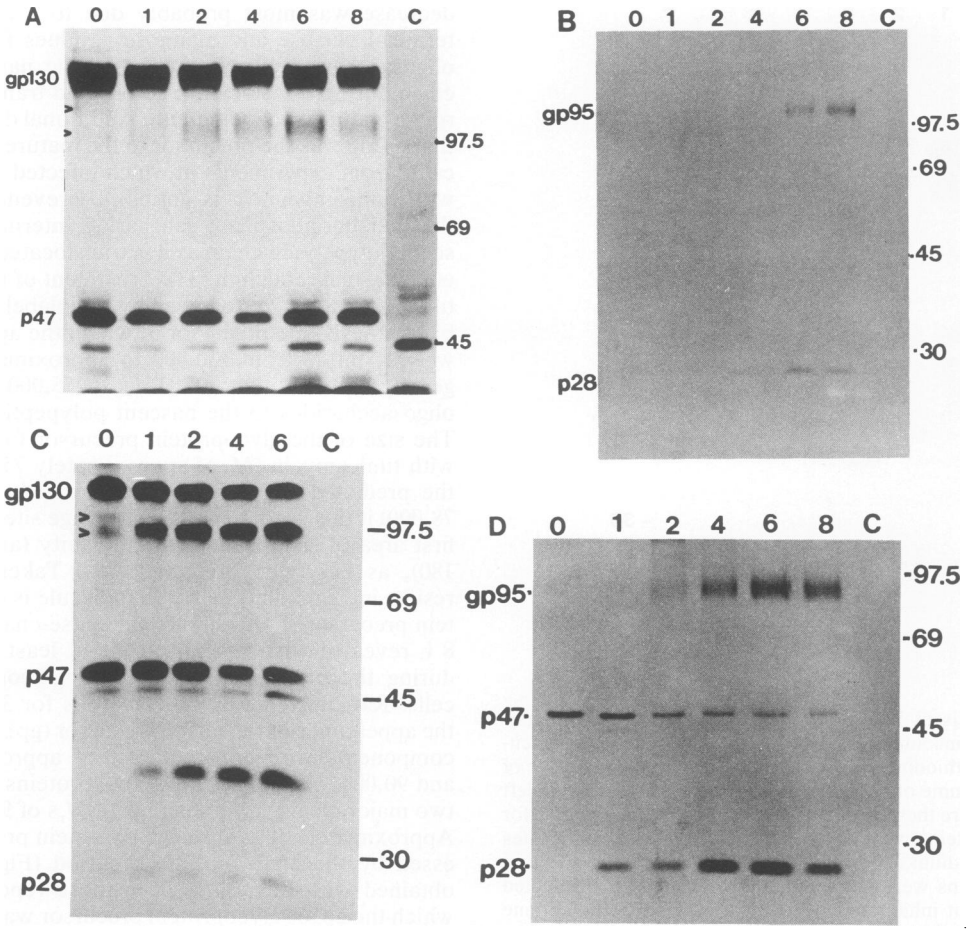

FIG. 8. Pulse-chase analyses of FIV proteins in the presence of castanospermine or swainsonine. Pulse-chase analyses were identical to the procedures described for Fig. 3, except that cells were treated with 3 mM castanospermine (A and B) or 300 ng of swainsonine per ml (C and D) prior to the pulse-labeling period and were maintained at 3 mM during the various chase periods (0, 1, 2, 4, 6, and 8 h for castanospermine; 0, 1, 2, 4, and 6 h for swainsonine). (A) FIV-specific proteins from cell lysates of castanospermine-treated cells. (B) FIV-specific proteins immunoprecipitated from the cell culture medium of castanospermine-treated cells. (C) FIV-specific proteins immunoprecipitated from cell lysates of swainsonine-treated cells. (D) FIV-specific proteins immunoprecipitated from the cell culture medium of swainsonine-treated cells. CRFK cells that were pulse-labeled for 30 min and chased for 8 h served as a negative control (lanes C). Samples were analyzed by SDS-PAGE (10% gel) and visualized by standard fluorographic techniques.

ically infected with HIV (23). In addition to a gp120 cleavage product, these investigators observed a protein with an apparent M_r of 100,000 in monensin-treated cultures (23). The effect of monensin on the synthesis and processing of the HIV glycoprotein expressed from a recombinant adenovirus vector has also been investigated (4). In the presence of monensin, the viral glycoprotein was reduced in size in both HIV-infected lymphocytes and human 293 cells infected with an adenovirus vector expressing the HIV glycoprotein (4). However, unlike in the previous study, these investigators did not demonstrate additional proteins other than the gp120 cleavage product, but unlike our results obtained with FIV-infected cells, these investigators were unable to find an intermediate during the processing of this glycoprotein.

Pulse-chase analyses revealed that gp95 was efficiently transported from cells and released into the culture medium, whereas gp40 was inefficiently released from cells. The reason for the inefficient release of gp40 from infected cells even after a 24-h labeling with [^{35}S]methionine is unclear. Possible explanations for these findings may be (i) the possibility that the gp40 is solidly embedded in the membrane and is not efficiently incorporated into maturing virions and/or (ii) the weak interaction between the gp95 and gp40 proteins that permits the FIV glycoprotein to dissociate at the cell surface. With respect to the second possibility, the two glycoprotein components are clearly separated on SDS-polyacrylamide gels under reducing and nonreducing conditions, suggesting the lack of disulfide bonds between this heterodimer (data not shown). The nucleic acid sequence of the FIV glycoprotein gene predicts the presence of 22 cysteine residues following the proposed hydrophobic signal sequence in the gp95, whereas only 2 cysteine residues exist within the ectodomain of the transmembrane gp40 molecule (38). If the two cysteine residues within the gp40 molecule (they are only six residues apart) formed an intrachain disulfide bond, the glycoprotein heterodimer would not be disulfide linked. Also, analysis of the gp95 sequence has failed to detect any regions of significant hydrophobicity at the carboxyl terminus that might allow the interaction with the hydrophobic amino acid terminus of the gp40 molecule. Such hydrophobic interactions have been postulated to exist

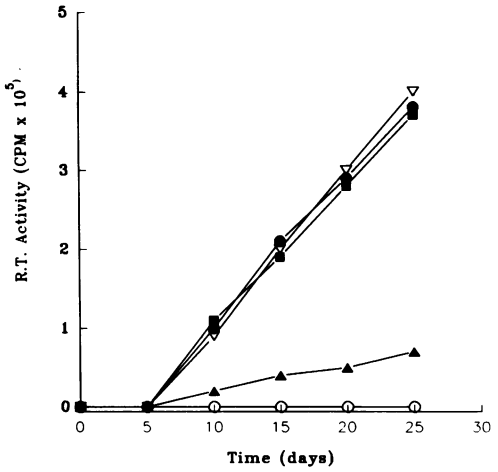

FIG. 9. Infectivity of virus grown in the presence of various inhibitors of glycosylation. FIV-infected CRFK cells were grown in the presence of each inhibitor for 4 h, and then the medium was replaced with fresh medium containing the same concentration of inhibitor. Cells were further incubated for 24 h, the cell supernatants were collected, and the virus was used to infect CRFK cells. The reverse transcriptase (R.T.) activity was then determined at 5, 10, 15, 20, and 25 days. Symbols: ▲, 3 mM castanospermine; ●, 300 ng of swainsonine per ml; ○, 10^{-6} M monensin; ■, 10^{-8} M monensin; ▽, untreated.

for other retrovirus glycoproteins such as gp70/p15E of murine leukemia virus (28). It is possible that gp95 and gp40 are being transported as a heterodimer complex to the cell surface, at which time the majority of the gp95 dissociates from the membrane-bound gp40. Following the release of the gp95, the gp40 may undergo a conformational change that would signal its endocytosis by the cell and ultimately result in its accelerated breakdown.

Recently, several inhibitors of specific enzymatic steps of the N-linked glycan processing pathway have been isolated and used to study the roles of N-linked glycan structures on the glycoproteins targeted to the cell surface and viral glycoprotein functions such as infectivity (1, 7, 9, 10, 24). Castanospermine is an inhibitor of the enzyme α-glucosidase I, which has been localized within the rough endoplasmic reticulum (30, 31). Growth of FIV in the presence of castanospermine resulted in a glycoprotein precursor with an apparent M_r of 145,000. This probably represents the size of the true glycoprotein precursor molecule with untrimmed oligosaccharide chains, since it inhibits α-glucosidase I of the rough endoplasmic reticulum. It is also in agreement with the results obtained when infected cells were pulse-labeled for short periods. This intermediate was probably not observed in the absence of the drug because trimming of the glucose residues on the nascent oligosaccharide chain probably occurs rapidly after addition to the polypeptide chain in the rough endoplasmic reticulum. The putative receptor-binding protein (gp95) also had a higher molecular weight in the presence of castanospermine. However, the transmembrane protein (gp40) had a decreased molecular weight (gp34), suggesting that this inhibitor had differential effect on processing of the FIV glycoproteins. The reasons for this effect are still unclear. Swainsonine is an inhibitor that has been shown to be active against α-mannosidase II, which has been localized to the Golgi complex (8, 42). Swainsonine did not alter the size of the FIV precursor but did reduce the size of the two major cleavage products, gp95 and gp40. This reduction in size is probably due to the inability of the cells to add terminal sugars to the oligosaccharide in the presence of this inhibitor.

We examined the infectivity of virus grown in the presence of glucosidase and mannosidase inhibitors. Virus grown in the presence of castanospermine but not swainsonine had a reduced infectivity for a feline T-lymphoid cell line. At present it is unknown whether the reduced infectivity of virus grown in the presence of castanospermine is due to altered N-linked glycan side chains on the putative receptor-binding glycoprotein or to slowed processing and transport of the gp95/gp40 complex to the cell surface. Previous studies have examined the infectivity of virus grown in the presence of glucosidase and mannosidase inhibitors. Both castanospermine and swainsonine were found to prevent the modification of the high-mannose side chains to those of the complex carbohydrate type of glycoproteins of influenza virus (9, 24). Growth of influenza virus in the presence of either swainsonine or castanospermine did not result in a decrease in virus infectivity compared with that of virus grown in the absence of the drug (9, 24). These results indicated that addition of complex carbohydrates to the glycoprotein is not required for virus infectivity. The effects of glucosidase and mannosidase inhibitors on the synthesis and infectivity of HIV have been examined (12, 44). These studies indicate that inhibitors of glucosidase activity (castanospermine and deoxynojirimycin) but not of mannosidase activity (1-deoxymannojirimycin) were able to alter the size of the HIV gp120/gp41 glycoprotein and were shown to prevent syncytium formation by cell-to-cell fusion (12, 44). However, the effects of these inhibitors on the kinetics of gp120/gp41 processing have not been examined. The results presented here indicate that FIV displays a similar infectivity in the presence of glucosidase and mannosidase inhibitors. These results suggest that the FIV-cat system may be a useful model for testing new antiviral drugs targeted against the glycoproteins of lentiviruses.

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