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Alternate Pathways of Secretion of Simian Immunodeficiency Virus Envelope Glycoproteins

C. PETER SPIES[1] AND RICHARD W. COMPANS[2]*

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294-0005,[1] and Department of Microbiology and Immunology, Emory University School of Medicine, 3001 Rollins Research Center, Atlanta, Georgia 30322[2]

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A biotinylation assay was used to detect the envelope glycoprotein of the simian immunodeficiency virus (SIV) envelope glycoprotein expressed by a recombinant vaccinia virus on the surface of HeLa T4 cells. The relationship between the detection of the envelope glycoprotein on the cell surface and its secretion from the cell was examined. It was found that much more gp120 was released into the culture medium than could be accounted for by shedding of the biotinylated SIV envelope protein from the cell surface. Treatment with the ionophore monensin showed that this drug did not block the secretion of gp120 into the culture medium even though the expression of gp120 on the cell surface was strongly downregulated. Similar results were observed for the secretion of gp120 in HUT78 cells infected with $SIV_{mac}251$ virus. Brefeldin A, on the other hand, inhibited both the detection of gp120 on the cell surface and its secretion into the culture medium. On the basis of these results, we propose that gp120 can be secreted into the culture medium via at least two pathways. One pathway involves the dissociation of gp120 from membrane-associated gp41-gp120 complexes on the cell surface. However, the major pathway involves the secretion of gp120 without its transitory appearance on the cell surface as part of a gp41-gp120 complex.

The envelope glycoproteins of the human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) have important functions in the binding of the virus to the target cell and the induction of fusion between the viral and the target cell membranes (11, 16, 20). The envelope protein is synthesized as a precursor protein which is cleaved into two subunits by a host enzyme (10). For viruses with a full-length envelope protein, the precursor protein is referred to as gp160, and the cleavage products are the surface protein, gp120, and the transmembrane protein, gp41. gp120 has the function of binding to the CD4 receptor on target cells (20), whereas gp41 has a hydrophobic domain at its amino terminus which is thought to play an important role in the fusion between the viral and target cell membranes induced by the envelope glycoprotein (20). In addition, gp41 has a membrane-spanning domain which anchors the envelope glycoprotein complex to the viral or cell membranes. The gp120 protein is associated noncovalently with the gp41 transmembrane protein (12). The transport of the envelope glycoprotein precursor proceeds via the endoplasmic reticulum (ER) and Golgi complex. gp160 acquires high-mannose sugars in the ER which are processed in the Golgi complex to yield a highly glycosylated envelope glycoprotein with a mixture of high-mannose, hybrid, and complex carbohydrate structures (7, 13). The cellular site of precursor cleavage may be cell type dependent and has been reported to occur in a late Golgi compartment or in the trans-Golgi network (10, 32). The gp120 and gp41 cleavage products are transported as a complex to the cell surface, where they can be incorporated into budding virions.

It has been frequently observed that the gp120 subunit of the envelope glycoprotein is shed from the cell or virion surface into the culture medium. This shedding has been presumed to occur via the dissociation of a fraction of the gp120-gp41 complexes on the cell or virion surface (19, 27, 31). It has been reported that binding of gp120 to a soluble form of its cellular CD4 receptor induces conformational changes in the envelope complex and that CD4 binding can also induce shedding of gp120 (26). Some studies have also presented evidence which suggests that the gp120-gp41 complex dissociates before reaching the cell surface (12, 18). Such an event could lead to the direct secretion of gp120 from the cell without its prior expression on the cell surface. In this study, we examined possible pathways for the secretion of the SIV envelope protein. We directly analyzed the fate of the SIV glycoproteins detected on the cell surface and their relationship to the SIV glycoproteins secreted into the culture medium.

MATERIALS AND METHODS

Cells and viruses. HeLa T4, HEp-2, and TK⁻ 143 cells were maintained in Dulbecco's minimal essential medium (DMEM) supplemented with 10% newborn calf serum. HUT78 cells were maintained in RPMI 1640 medium supplemented with 15% fetal calf serum. A recombinant vaccinia virus expressing the $SIV_{mac}239$ envelope protein was made by standard methods (25). This virus was grown and titers were determined on TK-143 cells.

Vaccinia virus infection and ^{35}S radiolabelling. Eighty percent-confluent HeLa T4 cells in 35-mm-diameter dishes were infected with the recombinant vaccinia virus at a multiplicity of infection of 2. After a 1-h adsorption period, the inoculum was replaced with DMEM containing 10% newborn calf serum. After a 10-h incubation, the cells were starved in cysteine- and methionine-deficient DMEM for 30 min. The cells were then pulse-labelled with 80 μCi of [^{35}S]cysteine and [^{35}S]methionine per ml and chased for various periods with DMEM containing 10% newborn calf serum.

Cell surface biotinylation and SDS-PAGE. Cell surface proteins were identified by a procedure similar to that described by Le Bivic et al. (17). At the end of the pulse or the chase periods, the supernatants were collected. The cells were

* Corresponding author.

washed three times with ice-cold phosphate-buffered saline (PBS) containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ (PBS/CM) and incubated with 1 ml of a 0.5-mg/ml solution of sulfosuccinimidyl-2-(biotinamido) ethyl-1,3-dithiopropionate (NHS-SS-Biotin; Pierce) in PBS/CM at 4°C for 30 min. In preliminary experiments using various times and amounts of reagents, these conditions were found to allow optimal levels of biotinylation of accessible envelope proteins on the cell surface. Free biotin was blocked by being mixed with DMEM, and the cells were washed three times with ice-cold PBS/CM. The cells were then lysed in 150 mM NaCl–50 mM Tris-HCl (pH 7.5)–1% Triton X-100–1% sodium dodecyl sulfate (SDS)–1 mM EDTA plus protease inhibitors (0.3 μM aprotinin, 1 μM pepstatin, 1 μg of leupeptin per ml). A total of 250 μl of the supernatant and 500 μl of the cell lysate samples were immunoprecipitated for 16 h with SIV-specific antisera from an infected rhesus monkey and protein A-agarose beads (Pierce). The cell lysate beads were then washed three times in lysis buffer and divided into two equal aliquots. One of each of the aliquots was boiled in 20 μl of 10% SDS for 5 min and diluted with 1 ml of lysis buffer. The boiled beads were removed by centrifugation for 1 min in a Microfuge, and the supernatant was incubated with streptavidin-agarose beads (Pierce) for 5 h at 4°C. The beads were then washed three times with lysis buffer. Proteins were characterized by SDS–8.5% polyacrylamide gel electrophoresis (PAGE) and subsequent autoradiography.

Treatment with monensin or brefeldin A. HeLa T4 cells were infected with the recombinant vaccinia virus as described above. At 10 h postinfection, the cells were starved in cysteine- and methionine-deficient DMEM for 30 min in the presence of various concentrations of monensin or brefeldin A (BFA). The cells were then pulse-labelled with 80 μCi of [^{35}S]cysteine and [^{35}S]methionine for 15 min and chased for various periods with DMEM containing 10% newborn calf serum. Monensin (Sigma) or BFA (Epicenter Technologies) was present during both the pulse and the chase periods. The cells were then biotinylated and lysed, and SIV-specific proteins in the cell medium, in the cell lysate, or on the cell surface were recovered by immunoprecipitation and streptavidin treatment as described above. Proteins were characterized by SDS–8.5% PAGE and subsequent autoradiography.

Endo H treatment. Proteins secreted into the culture medium were collected and immunoprecipitated with SIV-specific antiserum and protein A-agarose beads. The beads were washed with lysis buffer and treated with or without 5 mU of endoglycosidase H (Endo H; Boehringer Mannheim) in 100 mM sodium acetate (pH 5.5) for 16 h at 37°C. Proteins were characterized by SDS–8.5% PAGE and subsequent autoradiography.

SIV_{mac}251 virus infection and radiolabelling. HUT78 cells were infected with SIV_{mac}251 (10^4 50% tissue culture infective doses) and passaged with the addition of fresh cells every 3 to 4 days. At 2 weeks postinfection, 2×10^6 infected cells were added to 35-mm-diameter dishes and starved with cysteine- and methionine-deficient RPMI 1640 medium for 25 min. The cells were then labelled with 200 μCi of [^{35}S]cysteine and [^{35}S]methionine per ml for 45 min. At the end of the labelling period, fetal calf serum was added to the cells to a final concentration of 15%; monensin was added to final concentrations of 0, 1, or 10 μM; and the cells were incubated for an additional 6.5 h. At the end of the incubations, the culture medium was removed and the cells were biotinylated with NHS-SS-Biotin as described above. Culture medium, cell lysate, and cell surface proteins were determined as described above.

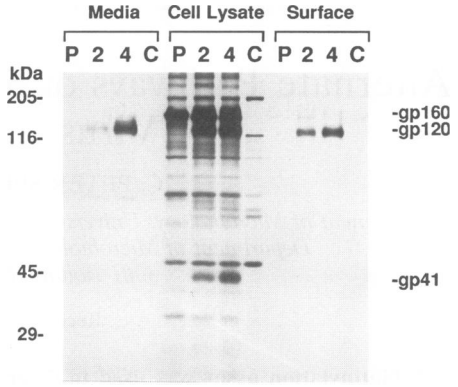

FIG. 1. Detection of SIV envelope glycoproteins on the cell surface. HeLa T4 cells infected with a recombinant vaccinia virus expressing the SIV envelope glycoprotein were pulse-labelled (lanes P) with [^{35}S]cysteine and [^{35}S]methionine for 15 min and chased with cold cysteine and methionine for 2 or 4 h (lanes 2 and 4, respectively). At the end of the chase period, the cell surface proteins were biotinylated. SIV-specific proteins secreted into the culture medium and in the cell lysate were immunoprecipitated with a polyclonal SIV antiserum. SIV-specific proteins on the cell surface were detected by precipitating half of the cell immunoprecipitate with streptavidin-agarose beads as described in Materials and Methods. Lanes C, vaccinia virus control samples.

RESULTS

Expression of the SIV envelope glycoprotein on the cell surface. In order to examine the relationship between the SIV envelope glycoprotein on the cell surface and its secretion into the culture medium, we utilized an assay which combines cell surface biotinylation and immunoprecipitation to differentiate SIV-specific proteins expressed in the whole cell and on the cell surface. Briefly, cells infected with a vaccinia virus recombinant expressing the SIV envelope protein were radiolabelled with [^{35}S]cysteine and [^{35}S]methionine and then reacted with the membrane-impermeable biotinylation reagent NHS-SS-Biotin. The cells were then lysed and immunoprecipitated with an SIV-specific polyclonal antibody. The biotinylated SIV envelope proteins from the cell surface were recovered by incubating half of the immunoprecipitated sample with streptavidin-agarose beads.

The results of a pulse-chase analysis of the appearance on the cell surface of the SIV envelope glycoprotein are shown in Fig. 1. HeLa T4 cells infected with rVV-SIV_{mac}239*env*, a vaccinia virus recombinant expressing the SIV envelope glycoprotein, were pulsed with [^{35}S]cysteine and [^{35}S]methionine for 15 min and chased with cold cysteine and methionine for 2 or 4 h. At the end of the pulse and each of the chases, the cells were biotinylated and subsequently treated as described above. The unprocessed gp160 precursor protein was present in the cell lysate of the pulse sample (lanes P). The processed gp120 and gp41 cleavage products appeared in the chase samples (lanes 2 and 4). Secretion of gp120 into the culture medium was observed in the 2-h chase sample (lanes 2) and increased significantly in the 4-h chase sample (lanes 4). gp120 was first detected on the cell surface in the 2-h chase sample, and it accumulated in the 4-h chase (lanes 2 and 4). A small amount of gp160 was also observed on the cell surface. We were not able to detect biotinylated gp41 on the cell surface in these experiments.

The secretion of gp120 can only partially be accounted for by dissociation from gp41 on the cell surface. To examine the

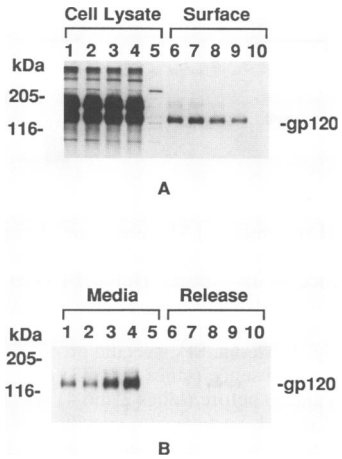

FIG. 2. Release of biotinylated gp120 into the culture medium. HeLa T4 cells infected with a recombinant vaccinia virus expressing the SIV envelope glycoprotein were pulse-labelled with [^{35}S]cysteine and [^{35}S]methionine for 15 min and chased with cold cysteine and methionine for 3 h. The cells were then biotinylated at 4°C for 30 min. Subsequently, fresh medium was added and the cells were incubated for an additional 1, 4, or 7 h. SIV-specific proteins present in the cell lysate and on the cell surface (A) and released into the cell medium (B, Media) were detected as outlined in Materials and Methods. Additionally, biotinylated SIV-specific proteins released into the culture medium from the cell surface were detected by precipitating half of the immunoprecipitated culture medium with streptavidin-agarose beads (B, Release). (A) Lanes 1 and 6, samples at end of initial 3-h chase; lanes 2 and 7, 1 h; lanes 3 and 8, 4 h; lanes 4 and 9, 7 h postbiotinylation; lanes 5 and 10, vaccinia virus control samples. (B) Lanes 1 to 5, total SIV-specific protein released into the medium; lanes 6 to 10, biotinylated SIV-specific proteins released into the medium. Individual lanes correspond to the same time points as in panel A.

relationship between the gp120 detected on the cell surface and the gp120 secreted into the culture medium, we decided to monitor the release of biotinylated gp120 from the cell surface into the culture medium. HeLa T4 cells infected with a recombinant vaccinia virus expressing the SIV envelope glycoprotein were pulsed and chased for 3 h as described before. The cells were then treated with NHS-SS-Biotin for 30 min at 4°C, after which fresh medium was added to the cells. The biotinylated cells were incubated for an additional 1, 4, or 7 h at 37°C. We ensured that the cells remained viable after the biotinylation by staining with trypan blue. SIV-specific proteins present in the cell lysate, surface, and culture medium fractions were recovered by immunoprecipitation and streptavidin treatment as described in Materials and Methods. In addition, half of the immunoprecipitated medium fractions were treated with streptavidin to recover biotinylated gp120 released into the culture medium from the cell surface at 1, 4, or 7 h after biotinylation. As shown in Fig. 2A, the level of biotinylated gp120 remaining on the cell surface decreased progressively in samples examined 1, 4, and 7 h after the cells had been biotinylated (Fig. 2A, lanes 7, 8, and 9). As shown in Fig. 2B, biotinylated gp120 was released from the cell surface and first appeared in the culture medium 1 h after biotinylation of the cells (Fig. 2B, lane 7). The level of biotinylated gp120 in the culture medium increased slightly in the 4- and 7-h postbiotinylation samples (Fig. 2B, lanes 8 and 9). As expected, none of the gp120 present in the medium at the end of the initial 3-h chase period (before biotinylation) was precipitated by streptavidin (Fig. 2B, compare lanes 1 and 6). We also examined the total amount of gp120 released into the culture medium 1, 4, and 7 h after the cells were biotinylated and the media were replaced. As shown in Fig. 2B, the total amount of radiolabelled gp120 released into the culture medium progressively increased at 1, 4, and 7 h after biotinylation of the cells (lanes 2, 3, and 4, respectively). Interestingly, the total levels of radiolabelled gp120 released into the medium were much greater than the levels of biotinylated gp120 released into the medium (Fig. 2B, compare lanes 2 and 7, 3 and 8, and 4 and 9). This finding indicates that the majority of the SIV envelope protein released into the culture medium had not been exposed on the cell surface and detected by biotinylation. This finding may be due to an unstable or transient expression of the envelope protein on the cell surface. However, the slow kinetics of shedding of biotinylated gp120 into the medium show that the gp120-gp41 association of the SIV envelope glycoprotein complex on the cell surface is relatively stable. These findings suggest that an alternative pathway exists for the release of gp120 into the culture medium, which does not require expression of an unstable gp41-gp120 complex on the cell surface.

Monensin downregulation of cell surface expression does not prevent secretion of gp120. The results we obtained by analyzing the release of biotinylated gp120 from the cell surface suggested that the SIV envelope protein is released from cells by different transport pathways. To further investigate the transport of the SIV glycoprotein to the cell surface and its release into the culture medium, we investigated the effect of monensin treatment. Monensin is a monovalent ionophore which has been shown to inhibit vesicular transport and to prevent terminal N-glycosylation and protein processing, which normally occur in the trans-Golgi complex (9). Treatment of infected cells with monensin has been reported to affect the transport and processing of the HIV envelope glycoprotein as well as that of other enveloped viral glycoproteins (1, 3, 5, 8, 14, 23, 29, 30). HeLa T4 cells infected with a recombinant vaccinia virus expressing the SIV envelope protein were radiolabelled with [^{35}S]cysteine and [^{35}S]methionine for 15 min and chased for 3 h in the presence of monensin concentrations from 0 to 50 μM. At the end of the chases, the cells were biotinylated and analyzed as described before. As shown in Fig. 3, at the end of the 3-h chase periods both the unprocessed gp160 precursor and the processed gp120 cleavage product were present in each of the cell lysate fractions. At all concentrations of monensin used, we observed an increase in electrophoretic mobility of gp120 compared with gp120 generated in the absence of monensin. This result has also been observed by other investigators and was shown to be because of differences in the glycosylation of the envelope protein in the presence of monensin (5, 23, 30). At the highest monensin concentration tested, a partial inhibition of precursor cleavage was also observed. Importantly, the cell surface expression of the processed SIV glycoprotein was markedly inhibited by monensin concentrations of 2 μM or greater. Virtually no gp120 was detected on the cell surface in the presence of 10 μM monensin, even though the rate of synthesis of gp120 was unaffected. We also observed no inhibition of release of gp120 into the medium by monensin concentrations up to 10 μM. Since monensin blocks protein transport to the plasma membrane, the continued secretion of gp120 into the culture medium in the presence of monensin provides further evidence for an alternate transport pathway available for the SIV glycoproteins which does not involve their transient expression on the cell surface. It has previously been reported that the HIV-1 envelope precursor protein can retard the transport of CD4 by forming a complex with CD4 in the ER (4). To determine whether the presence of CD4 has an effect

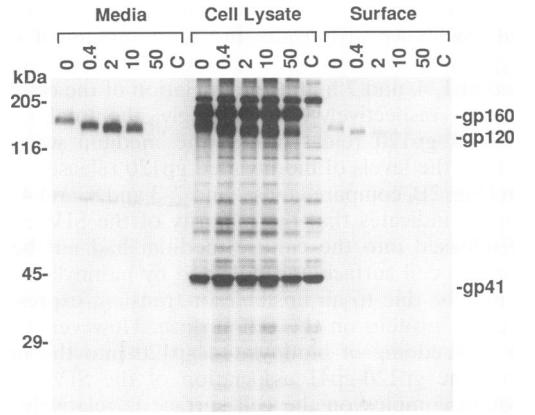

FIG. 3. Effects of monensin concentration on transport of the SIV envelope glycoprotein. HeLa T4 cells infected with a recombinant vaccinia virus expressing the SIV envelope glycoprotein were pulse-labelled with [^{35}S]cysteine and [^{35}S]methionine for 15 min and chased with cold cysteine and methionine for 3 h in the presence of monensin concentrations ranging from 0 to 50 μM. At the end of the chase period, the cell surface proteins were biotinylated. SIV-specific proteins secreted into the culture medium and in the cell lysate were immunoprecipitated with a polyclonal SIV antiserum. SIV-specific proteins expressed on the cell surface were detected by incubating half of the cell immunoprecipitate with streptavidin as described in Materials and Methods. Lanes C, vaccinia virus control samples.

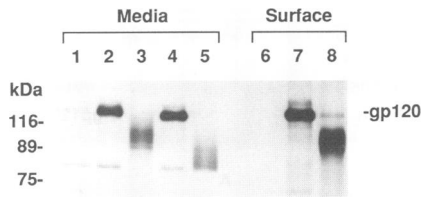

FIG. 4. Endo H treatment of SIV envelope glycoproteins secreted into the culture medium or expressed on the cell surface in the presence or absence of monensin. HeLa T4 cells infected with a recombinant vaccinia virus expressing the SIV envelope glycoprotein were pulse-chased with [^{35}S]cysteine and [^{35}S]methionine as outlined in the legend to Fig. 1. Media, SIV-specific proteins secreted into the culture medium in the absence (lanes 2 and 3) or presence of 10 μM monensin (lanes 4 and 5) before (lanes 2 and 4) and after (lanes 3 and 5) treatment with Endo H. Surface, SIV-specific proteins expressed on the cell surface (in the absence of monensin) before (lane 7) and after (lane 8) treatment with Endo H. Lanes 1 and 6, vaccinia virus control samples.

on the transport of the SIV glycoprotein in the presence of monensin, we have repeated the above experiment in the CD4 cell line HEp-2. Our results showed no major differences between the transport of the SIV envelope protein in HeLa T4 cells compared with HEp-2 cells, i.e., expression of gp120 on the cell surface was inhibited, whereas secretion into the medium was not (data not shown). This finding indicates that the presence of CD4 had no detectable effect on the transport pathways of the SIV glycoprotein.

SIV glycoproteins released into the culture medium have differing Endo H sensitivities in the presence of monensin. To directly test whether the SIV envelope glycoprotein had been exposed to a different set of sugar-modifying enzymes in the presence of monensin, we treated SIV glycoproteins secreted from cells in the presence or absence of 10 μM monensin with Endo H. Endo H removes N-linked oligosaccharide side chains from high-mannose glycoproteins, but it does not digest complex oligosaccharide side chains. As such, the resistance to detection with Endo H serves as a marker for transport to the Golgi complex. HeLa T4 cells infected with a recombinant vaccinia virus expressing the SIV glycoprotein were pulsed with [^{35}S]cysteine and [^{35}S]methionine for 20 min and then chased with cold cysteine and methionine for 3 h in the presence or absence of 10 μM monensin. The SIV glycoproteins that were released into the culture medium were then digested with Endo H. As shown in Fig. 4, there is a striking increase in Endo H sensitivity of gp120 secreted into the culture medium in the presence of monensin (Fig. 4, compare lanes 3 and 5). This result indicated that the gp120 released into the medium in the presence of monensin had not undergone normal oligosaccharide processing. It is interesting that the addition of these complex oligosaccharides is not required for the secretion of gp120 from the cell. In the absence of monensin, the susceptibility of the SIV envelope glycoproteins detected on the cell surface is similar to the Endo H susceptibility of the SIV envelope glycoprotein released into the medium (Fig. 4, compare lanes 3 and 8). When we treated gp120 secreted into the medium or expressed on the cell surface with neuraminidase (in the absence of monensin), we observed a slight increase in electrophoretic mobility for both secreted and cell surface-expressed gp120 (data not shown). Since the addition of sialic acid occurs in the trans-Golgi complex or the TGN, this finding suggests that secreted as well as membrane-associated envelope proteins are transported via the trans-Golgi complex.

BFA prevents release of SIV glycoproteins into the cell medium. BFA is a macrocyclic lactone which causes recycling of Golgi cisternae to the ER and completely blocks membrane traffic out of the ER (15). BFA has been shown to inhibit both the processing and the secretion of the HIV-1 envelope glycoprotein (24). In this study we used BFA to determine whether the observed secretion of the SIV envelope protein into the culture medium is prevented by this inhibitor. HeLa T4 cells infected with a vaccinia virus expressing the SIV envelope glycoprotein were pulse-labelled for 20 min with [^{35}S]cysteine and [^{35}S]methionine. The cells were then chased with cold cysteine and methionine for 3 h and 30 min in the presence of BFA concentrations ranging from 0 to 10 μg/ml. The cells were then biotinylated and treated as described above. As shown in Fig. 5, gp120 was detected on the cell surface only at the lowest BFA concentration (0.01 μg/ml). The inhibition of appearance of gp120 in the culture medium paralleled its detection on the cell surface. gp120 was not detected in the medium or on the cell surface at BFA concentrations higher than 0.01 μg/ml. At these higher BFA concentrations, the processing of the gp160 precursor was largely inhibited. This is not surprising, as the cellular protease responsible for the cleavage of the envelope precursor protein resides in a post-ER compartment (10). These results indicate that transport from the ER to the Golgi complex is a requirement for the release of the SIV glycoprotein into the culture medium.

An alternate pathway for the secretion of the SIV envelope protein also exists in cells infected with SIV. In order to investigate whether an alternate pathway for the secretion of the SIV envelope glycoprotein can also be demonstrated in cells infected with live SIV, we analyzed the appearance of gp120 on the cell surface and in the culture medium of $SIV_{mac}251$-infected HUT78 cells in the presence or absence of monensin. We radiolabelled $SIV_{mac}251$-infected HUT78 cells with [^{35}S]cysteine and [^{35}S]methionine for 7 h and 15 min in

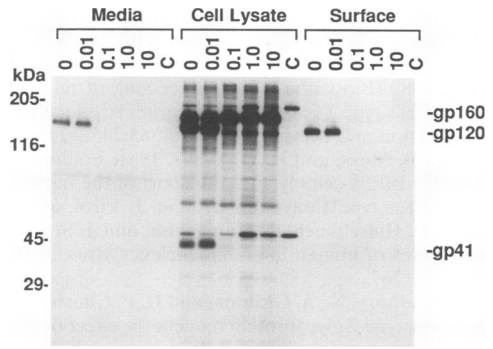

FIG. 5. Expression of the SIV envelope glycoprotein in the presence of BFA. HeLa T4 cells infected with a recombinant vaccinia virus expressing the SIV envelope glycoprotein were pulse-labelled with [^{35}S]cysteine and [^{35}S]methionine for 15 min and chased with cold cysteine and methionine for 3 h and 30 min in the presence of BFA at concentrations ranging from 0 to 10 μg/ml. At the end of the chase period, the cell surface proteins were biotinylated. SIV-specific proteins secreted into the media and in the cell lysate were immunoprecipitated with a polyclonal SIV antiserum. SIV-specific proteins expressed on the cell surface were detected by precipitating half of the cell immunoprecipitate with streptavidin-agarose beads as described in Materials and Methods. Lanes C, vaccinia virus control samples.

the presence of 0, 1, or 10 μM monensin. At the end of the incubations, the cells were biotinylated with NHS-SS-Biotin, and SIV-specific proteins present in the cell lysate, surface, and culture medium fractions were recovered by immunoprecipitation and streptavidin treatment as described above (Fig. 6). Treatment with monensin caused an increase in the electrophoretic mobility of gp120 and a partial inhibition of precursor cleavage, as found with the vaccinia virus-expressed glycoproteins. In the absence of monensin, gp120 and gp160 were readily apparent on the cell surface and gp120 was secreted into the culture medium. The expression of gp160 on the cell surface was not prevented by monensin. Importantly, at both monensin concentrations used, a strong downregulation of gp120 expressed on the cell surface was observed while appreciable amounts of gp120 were still released into the culture medium. This finding indicates that an alternative pathway for the secretion of the SIV envelope glycoprotein also occurs in SIV-infected T cells.

DISCUSSION

We have demonstrated that the SIV envelope protein can be detected on the cell surface by a biotinylation assay and that

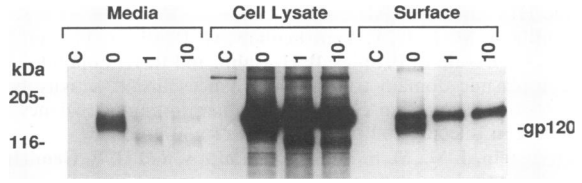

FIG. 6. Expression of the SIV envelope glycoprotein in HUT78 cells infected with $SIV_{mac}251$. HUT78 cells infected with $SIV_{mac}251$ were radiolabelled with [^{35}S]cysteine and [^{35}S]methionine for 7 h and 15 min in the presence of 0, 1, or 10 μM monensin as described in Materials and Methods. At the end of the chase period, the cell surface proteins were biotinylated. SIV-specific proteins present in the cell lysate, surface, and culture medium fractions were recovered by immunoprecipitation and streptavidin treatment as described above. Lanes C, uninfected cell controls.

the surface-expressed gp120 shows considerable stability with only a low level of shedding into the culture medium over time. In addition, we have found that a much larger fraction of gp120 is continuously secreted into the culture medium without being detected on the cell surface. Other investigators have also suggested that more than a single population of freely secreted or virion-associated HIV-1 gp120 exists. Earl et al. (6) have reported that radiolabelled HIV-1 envelope protein expressed in BSC-1 cells was efficiently shed for about 4 h postlabelling. After that, little additional shedding occurred over the next 20 h, even though the envelope protein continued to be present in the cell lysates. Helseth and coworkers (12) have examined HIV-1 envelope mutant proteins which exhibit a decreased association between the gp120 and gp41 subunits. They have reported that the levels of cell-associated gp41 of several mutants were decreased compared with those of the wild-type envelope, suggesting the possibility of a dissociation of the gp120-gp41 complex before reaching the cell surface. Unusually high levels of secretion of gp120 were reported with an HIV-1 envelope protein with a mutated glycosylation site in gp41, which suggests possible early dissociation of gp120 and gp41 (18). In a previous study, our laboratory has observed that virtually all of the envelope protein of an HIV-2 variant, ST#2, is secreted into the culture medium (21). McKeating and coworkers (19) have examined the shedding of gp120 from the virions of various HIV-1 strains and reported that the SF-2 virus isolate exhibited a high level of gp120 shedding but that the residual gp120 population left on virions was more resistant to shedding. These results point to the presence of a heterogeneous population of envelope proteins in cells and on the surface of virions.

The results we obtained by studying the release of biotinylated SIV glycoproteins suggested that the SIV envelope protein is exported from the cell by a mechanism which does not involve shedding from the cell surface. We further addressed this possibility by the use of the ionophore monensin. Monensin inhibits the transport of many proteins at the level of the medial- to trans-Golgi complex. It can block protein transport along a number of pathways depending on both the cell type and the protein involved (9). It has previously been observed that monensin has differential effects on distinct pathways of transport to the cell surface and secretion of glycoproteins. For instance, monensin blocked the secretion of acetylcholinesterase from chicken embryo skeletal muscle cells, even though the transport of the acetylcholine receptor to the cell surface was not inhibited (28). Monensin also blocked transport of the vesicular stomatitis virus G protein to the basolateral surface of MDCK cells without preventing the transport of the influenza virus HA glycoprotein to the apical surface of the same cell type (1). Bosch and Schwarz (3) have studied the effects of monensin on the transport of the Rous sarcoma virus glycoprotein in chicken embryo fibroblasts and have reported that treatment with monensin prevents the processing of high-mannose-type oligosaccharides to complex-type oligosaccharides, resulting in the formation of a 75-kDa envelope glycoprotein instead of the normal 85-kDa glycoprotein. Interestingly, this 75-kDa envelope protein was incorporated into released virions. Since the addition of complex-type oligosaccharides occurs in the Golgi complex, Bosch and Schwarz suggested that an alternative transport pathway to the cell surface exists for the Rous sarcoma virus glycoprotein. Several other studies have shown that monensin inhibits the transport to the cell surface of certain other viral glycoproteins (8, 9, 14, 29). Effects of monensin on the processing and transport of the HIV envelope protein have been studied by several investigators (5, 23, 30). Monensin appears to prevent

the cleavage of the HIV-1 envelope precursor protein and cause changes in glycosylation resulting in glycoproteins with faster electrophoretic mobilities and reduced or abolished incorporation of sialic acid, fucose, and galactose residues. Our results indicate that monensin exhibits differential effects on the cell surface expression and the secretion of the SIV envelope glycoprotein both in SIV-infected cells and in cells infected with a vaccinia virus recombinant expressing the SIV envelope protein. The inhibition of transport of the SIV envelope protein by monensin appears to be limited to those glycoproteins which are stably expressed on the cell surface. This finding suggests the presence of another, monensin-insensitive pathway for the secretion of SIV envelope glycoproteins. One likely explanation for this finding would be the dissociation of the gp120-gp41 complex inside the cell, before reaching the cell surface, and the secretion of the dissociated gp120 by a process which is resistant to monensin.

The present results also indicate that the alternate pathways for transport of the SIV envelope glycoprotein diverge after the cleavage of the gp160 precursor. This cleavage has been reported to be accomplished by furin, an enzyme which resides in the trans-Golgi network (10). In other studies it was concluded that envelope cleavage occurs in the medial Golgi cisternae (32). Our laboratory has recently shown that sulfation of the HIV and SIV glycoproteins, a modification which occurs in the trans-Golgi network, is not required for envelope glycoprotein secretion (2). The results obtained here with BFA, a compound which blocks transport of proteins from the ER to the Golgi complex (15), demonstrate that inhibition of transport of the SIV envelope glycoprotein at this level blocks both secretion of gp120 and its expression on the cell surface. Cell-type-specific factors can also affect the transport of lentiviral glycoproteins. An example of a cell which shows apparent differences in the secretion of the envelope glycoprotein is the monocyte. Orenstein and coworkers have reported that HIV-1-infected monocytes have very few virions budding from the cell surface and that most particles are observed budding into cytoplasmic vacuoles (22).

The results presented here further indicate that the levels of gp120 released in the culture medium should not be used as a measure of the expression of the envelope complex on the cell surface. To evaluate the expression of the envelope glycoprotein on the cell surface, direct detection methods, such as biotinylation or fluorescence-activated cell sorter analysis, should be employed.

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REFERENCES

1. **Alonso, F. V., and R. W. Compans.** 1981. Differential effect of monensin on enveloped viruses that form at distinct plasma membrane domains. J. Cell Biol. **89:**700–705.
2. **Bernstein, H. B., and R. W. Compans.** 1992. Sulfation of the human immunodefiency virus envelope glycoprotein. J. Virol. **66:**6953–6959.
3. **Bosch, J. V., and R. T. Schwarz.** 1984. Processing of $gPr92^{env}$, the precursor to the glycoproteins of Rous sarcoma virus: use of inhibitors of oligosaccharide trimming and glycoprotein transport. Virology **132:**95–109.
4. **Crise, B., L. Buonocore, and J. K. Rose.** 1990. CD4 is retained in the endoplasmic reticulum by the human immunodeficiency virus type 1 glycoprotein precursor. J. Virol. **64:**5585–5593.
5. **Dewar, R. L., M. B. Vasudevachari, V. Natarajan, and N. P. Salzman.** 1989. Biosynthesis and processing of human immunodeficiency virus type 1 envelope glycoproteins: effects of monensin on glycosylation and transport. J. Virol. **63:**2452–2456.
6. **Earl, P. L., B. Moss, and R. W. Doms.** 1991. Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. J. Virol. **65:**2047–2055.
7. **Geyer, H., C. Holschbach, G. Hunsmann, and J. Schneider.** 1988. Carbohydrates of human immunodeficiency virus. J. Biol. Chem. **263:**11760–11767.
8. **Ghosh-Choudhury, N., A. Graham, and H. P. Ghosh.** 1987. Herpes simplex virus type 2 glycoprotein biogenesis: effect of monensin on glycoprotein maturation, intracellular transport and virus infectivity. J. Gen. Virol. **68:**1939–1949.
9. **Griffiths, G., P. Quinn, and G. Warren.** 1983. Dissection of the Golgi complex. I. Monensin inhibits the transport of viral membrane proteins from *medial* to *trans* Golgi cisternae in baby hamster kidney cells infected with Semliki Forest virus. J. Cell Biol. **96:**835–850.
10. **Hallenberger, S., V. Bosch, H. Angliker, E. Shaw, H.-D. Klenk, and W. Garten.** 1992. Inhibition of furin-mediated cleavage activation of HIV-1 glycoprotein gp160. Nature (London) **360:**358–361.
11. **Haseltine, W.** 1991. Molecular biology of the human immunodeficiency virus type 1. FASEB J. **5:**2349–2360.
12. **Helseth, E., U. Olshevsky, C. Furman, and J. Sodroski.** 1991. Human immunodeficiency virus type 1 gp120 envelope glycoprotein regions important for association with the gp41 transmembrane glycoprotein. J. Virol. **65:**2119–2123.
13. **Holschbach, C., J. Schneider, and H. Geyer.** 1990. Glycosylation of the envelope glycoprotein gp130 of simian immunodeficiency virus from sooty mangabey (*Cercocebus atys*). Biochem. J. **267:**759–766.
14. **Johnson, D. C., and M. J. Schlesinger.** 1980. Vesicular stomatitis virus and Sindbis virus glycoprotein transport to the cell surface is inhibited by ionophores. Virology **103:**407–424.
15. **Klausner, R. D., J. G. Donaldson, and J. Lippincott-Schwartz.** 1992. Brefeldin A: insights into the control of membrane traffic and organelle structure. J. Cell Biol. **116:**1071–1080.
16. **Kowalski, M., J. Potz, L. Basiripour, T. Dorfman, W. C. Goh, E. Terwilliger, A. Dayton, C. Rosen, W. Haseltine, and J. Sodroski.** 1987. Functional regions of the envelope glycoprotein of human immunodeficiency virus type 1. Science **237:**1351–1355.
17. **Le Bivic, A., F. X. Real, and E. Rodriguez-Boulan.** 1989. Vectorial targeting of apical and basolateral plasma membrane proteins in a human adenocarcinoma epithelial cell line. Proc. Natl. Acad. Sci. USA **86:**9313–9317.
18. **Lee, W.-R., X.-F. Yu, W.-J. Syu, M. Essex, and T.-H. Lee.** 1992. Mutational analysis of conserved N-linked glycosylation sites of human immunodeficiency virus type 1 gp41. J. Virol. **66:**1799–1803.
19. **McKeating, J. A., A. McKnight, and J. P. Moore.** 1991. Differential loss of envelope glycoprotein gp120 from virions of human immunodeficiency virus type 1 isolates: effects on infectivity and neutralization. J. Virol. **65:**852–860.
20. **McKeating, J. A., and R. L. Willey.** 1989. Structure and function of the HIV envelope. AIDS **3:**S35–S41.
21. **Mulligan, M. J., G. V. Yamshchikov, G. D. Ritter, F. Gao, M. J. Jin, C. D. Nail, C. P. Spies, B. H. Hahn, and R. W. Compans.** 1992. Cytoplasmic domain truncation enhances fusion activity by the exterior glycoprotein complex of human immunodeficiency virus type 2 in selected cell types. J. Virol. **66:**3971–3975.
22. **Orenstein, J. M., M. S. Meltzer, T. Phipps, and H. E. Gendelman.** 1988. Cytoplasmic assembly and accumulation of human immunodeficiency virus types 1 and 2 in recombinant human colony-stimulating factor 1-treated human monocytes: an ultrastructural study. J. Virol. **62:**2578–2586.
23. **Pal, R., R. C. Gallo, and M. G. Sarngadharan.** 1988. Processing of the structural proteins of human immunodefiency virus type 1 in the presence of monensin and cerulenin. Proc. Natl. Acad. Sci. USA **85:**9283–9286.
24. **Pal, R., S. Mumbauer, G. M. Hoke, A. Takatsuki, and M. G. Sarngadharan.** 1991. Brefeldin A inhibits the processing and

secretion of envelope glycoproteins of human immunodeficiency virus type 1. AIDS Res. Human Retroviruses **7:**707–712.
25. **Ritter, G. D., M. J. Mulligan, S. L. Lydy, and R. W. Compans.** Cell fusion activity of the simian immunodeficiency virus envelope protein is modulated by the intracytoplasmic domain. Virology, in press.
26. **Sattentau, Q., and J. P. Moore.** 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J. Exp. Med. **174:**407–415.
27. **Schneider, J., O. Kaaden, T. D. Copeland, S. Oroszlan, and G. Hunsmann.** 1986. Shedding and interspecies type sero-reactivity of the envelope glycopolypeptide gp120 of the human immunodeficiency virus. J. Gen. Virol. **67:**2533–2538.
28. **Smilowitz, H.** 1980. Routes of intracellular transport of acetylcholine receptor and esterase are distinct. Cell **19:**237–244.
29. **Srinivas, R. V., L. R. Melsen, and R. W. Compans.** 1982. Effects of monensin on morphogenesis and infectivity of Friend murine leukemia virus. J. Virol. **42:**1067–1075.
30. **Stein, B. S., and E. G. Engleman.** 1990. Intracellular processing of the gp160 HIV-1 envelope precursor. J. Biol. Chem. **265:**2640–2649.
31. **Willey, R. L., J. S. Bonifacino, B. J. Potts, M. A. Martin, and R. D. Klausner.** 1988. Biosynthesis, cleavage and degradation of the human immunodeficiency virus 1 envelope glycoprotein gp160. Proc. Natl. Acad. Sci. USA **85:**9580–9584.
32. **Willey, R. L., T. Klimkait, D. M. Frucht, J. S. Bonifacino, and M. A. Martin.** 1991. Mutations within the human immunodeficiency virus type 1 gp160 envelope glycoprotein alter its intracellular transport and processing. Virology **184:**319–329.