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Biosynthesis and Processing of Human Immunodeficiency Virus Type 1 Envelope Glycoproteins: Effects of Monensin on Glycosylation and Transport

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When human immunodeficiency virus type 1 envelope glycoproteins were expressed in 293 cells by using a recombinant adenovirus expression vector, the envelope precursor (gp160) was initially glycosylated by cotranslational addition of N-linked high-mannose oligosaccharide units to the protein backbone and then cleaved to gp120 and gp41. The subunits gp120 and gp41 were then further modified by the addition of fucose, galactose, and sialic acid, resulting in glycoproteins containing a mixture of hybrid and complex oligosaccharide side chains. A fraction of glycosylated gp160 that escaped cleavage was further modified by the terminal addition of fucose and galactose, but the addition of sialic acid did not occur, consistent with the notion that it is compartmentalized separately from the gp120 envelope protein. Processing and transport of gp160 were blocked by the monovalent ionophore monensin, which at high concentrations (25 μM and above) was a potent inhibitor of the endoproteolytic cleavage of gp160; at lower concentrations (1 to 10 μM), it selectively blocked the secondary glycosylation steps so that smaller products were produced. Monensin (1 μM) treatment also resulted in a reduction in syncytium formation, which was observed when recombinant infected cells were cocultivated with CD4-bearing HeLa cells. The infectivity of human immunodeficiency virus type 1 was also reduced by monensin treatment, a decrease that may be due to incompletely glycosylated forms of gp120 that have a lower affinity for the CD4 receptor.

Human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome and related disorders (2, 6, 15, 22, 24), displays a selective tropism for CD4-bearing cells, and the CD4 molecule has been shown to be the receptor for the virus (3, 11, 16, 19). The recognition of this receptor by the virus and the interaction between the viral envelope protein gp120 and the CD4 antigen are critical steps in the infectious cycle, and recent studies have indicated that endoproteolytic cleavage of gp160 and glycosylation of gp120 are required for infectivity of HIV-1 and for binding of virions to CD4 (8, 17, 18, 33). The demonstrated importance of the viral envelope proteins prompted us to examine more closely the intracellular events leading to the formation of biologically functional gp120 and gp41.

We previously reported the construction of a recombinant human adenovirus type 5 (Ad5env) containing the entire HIV-1 *env* gene (4). Cells infected with this recombinant were shown to produce the HIV-1 envelope proteins gp160, gp120, and gp41 and to form syncytia when mixed with CD4-bearing cells. The Ad5env recombinant was used in this study to define the cellular processes involved in the biosynthesis and subsequent posttranslational processing of gp160. The monovalent ionophore monensin, which inhibits intracellular transport of proteins through the rough endoplasmic recticulum-Golgi complex (20, 27–30), was used to localize various steps in the processing pathway. As has been reported for gp160 encoded by HIV-1 (1), we observed that gp160 encoded by the recombinant adenovirus was cotranslationally glycosylated via the addition of N-linked high-mannose oligosaccharide side chains, a process that was inhibited by the addition of tunicamycin, resulting in the accumulation of a 95-kilodalton apoprotein. Cleavage of gp160 precedes the secondary glycosylation steps, which include the addition of fuctose, galactose, and sialic acid to gp120 and gp41.

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MATERIALS AND METHODS

Reagents. Monensin was obtained from Sigma Chemical Co. Radioactive isotopes were purchased from ICN Pharmaceuticals Inc. Endo-β-*N*-acetylglucosaminidase H (endo H) and neuraminidase were obtained from Boehringer Mannheim Biochemicals.

Cells and viruses. Monolayers of 293 cells were maintained in Eagle minimal essential medium containing 10% fetal bovine serum. HeLa $CD4^+$ cells were the kind gift of Richard Axel of the Howard Hughes Medical Institute, Columbia University, and were maintained in Dulbecco modified Eagle medium containing 10% fetal bovine serum and geneticin. HIV-1-infected human peripheral blood lymphocytes (PBLs) were maintained in RPMI 1640 medium supplemented with interleukin-2 and 5% fetal bovine serum. The construction of Ad5env has been previously described (4). The virus was plaque purified twice, and titers were determined on 293 cells.

Cell labeling. Ad5env-infected cells were labeled with either [^{35}S]methionine and [^{35}S]cysteine ([^{35}S]Met-[^{35}S]Cys) in serum-free, methionine-free Dulbecco modified medium or with [^{14}C]glucosamine, [^{3}H]mannose, [^{3}H]fucose, or [^{3}H]galactose in Eagle modified essential medium 24 h postinfection for the incubation periods noted below. Cell lysates, prepared as previously described (23) with RIPA buffer (20 mM Tris hydrochloride [pH 7.5], 2 mM EDTA, 150 mM NaCl, 1% Desoxycholate [BBL Microbiology Systems], 1% Triton X-100, and 0.25% sodium dodecyl sulfate), were used for radioimmunoprecipitation with pooled sera from HIV-1-seropositive persons and protein A-Sepharose. Immunoprecipitated proteins were analyzed by sodium do-

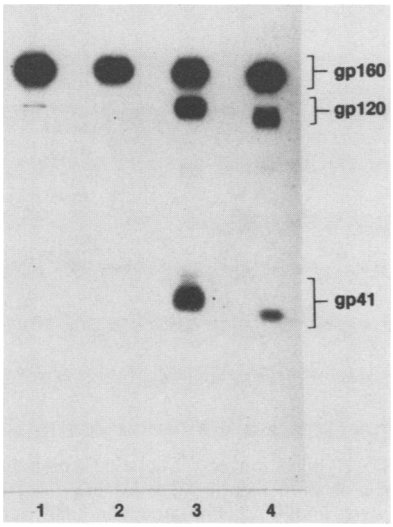

FIG. 1. Synthesis and processing of Ad5env-encoded HIV-1 glycoproteins: At 24 h after infection with Ad5env, 293 monolayer cells were pulse-labeled for 30 min with [^{35}S]Met-[^{35}S]Cys and then chased for 4 h in medium containing an excess of unlabeled methionine. Cell lysates were prepared and proteins were immunoprecipitated as described in Materials and Methods. Precipitated proteins were subjected to electrophoresis on a 10% sodium dodecyl sulfate–polyacrylamide gel (13). The fluorogram depicts immunoprecipitated polypeptides after a 30-min pulse in the absence (lane 1) and the presence (lane 2) of 1 μM monensin and a 4-h chase in the absence (lane 3) and the presence (lane 4) of 1 μM monensin. The positions of gp160, gp120, and gp41 are indicated.

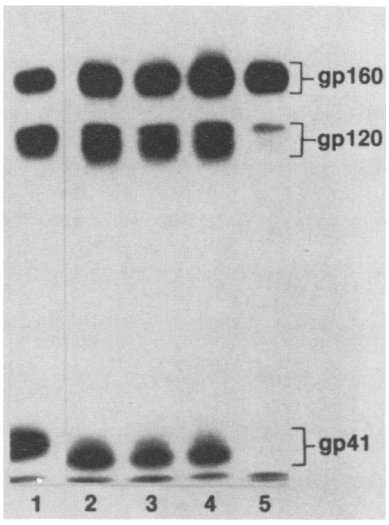

FIG. 2. Effects of high and low monensin concentrations on the cleavage and processing of gp160: ^{35}S-labeled proteins from Ad5env-infected 293 cells grown in the presence or absence of monensin were immunoprecipitated as described in Materials and Methods. Immunoprecipitated polypeptides were analyzed as indicated in the legend to Fig. 1. The fluorogram shows envelope glycoproteins produced in the absence of monensin (lane 1) and in the presence of monensin at 1 μM (lane 2), 5 μM (lane 3), 10 μM (lane 4), and 25 μM (lane 5).

decyl sulfate-polyacrylamide gel electrophoresis (13) and fluorography. HIV-1-infected PBLs were metabolically labeled for 4 h with [^{35}S]Met-[^{35}S]Cys in RPMI 1640 medium lacking methionine and cysteine and containing 5% fetal bovine serum. Cell lysates were prepared and analyzed as described above.

Syncytium-formation assay. At 24 h postinfection, Ad5env-infected 293 cells were incubated for 4 h in the presence or absence of 1 μM monensin. HeLa CD4$^+$ cells obtained from monolayer cultures were mixed with either the monensin-treated or control cells and incubated at 37°C in four-chamber tissue culture slides (Titertek). After a 24-h incubation, cultures were scored for syncytia.

Reverse transcriptase assay. Virion-associated reverse transcriptase activity was measured as previously described (34) with [^{3}H]TTP as the substrate.

Deglycosylation of envelope proteins. Proteins were immunoprecipitated and then digested with 5 mU of endo H in buffer containing 50 mM sodium acetate (pH 5.5), 0.1% sodium dodecyl sulfate, and 0.1% bovine serum albumin at 37°C for 12 h. Neuraminidase digestion was performed in buffer containing 100 mM sodium acetate (pH 5.5), 0.5% Nonidet P-40, and 500 mM NaCl at 37°C for 12 h.

RESULTS

Effects of high and low monensin concentration on the cleavage and processing of gp160, gp120, and gp41. The first newly synthesized HIV-1 protein that was detected by immunoprecipitation, when Ad5env-infected 293 cells were pulse-labeled for 30 min with [^{35}S]Met-[^{35}S]Cys, had a molecular weight of 160,000 (gp160) on sodium dodecyl sulfate-polyacrylamide gels (Fig. 1, lane 1). After a 4-h chase period in meidum supplemented with an excess of unlabeled methionine, its cleavage products gp120 and gp41 were observed (Fig. 1, lane 3), but a significant fraction of the gp160 still remained. Previous results (4) showed that the cleavage products can be detected within cells as early as 2 h after pulse-labeling and demonstrated a precursor-product relationship between gp160 and the subunits of gp120 and gp41. Allan et al. (1) reported that cotranslational glycosylation of a protein backbone was involved in the synthesis of HIV-1-encoded gp160, since the appearance of gp160 could be inhibited by the addition of the inhibitor tunicamycin (9). Glycosylation of the Ad5env-encoded precursor could also be inhibited by tunicamycin, and the incompletely glycosylated molecules were not subjected to proteolytic processing (data not shown). To examine the role of posttranslational processing events on the production of gp160, gp120, and gp41, Ad5env-infected 293 cells were pulse-labeled for 30 min with [^{35}S]Met-[^{35}Cys] and chased for 4 h in the presence or absence of monensin at concentrations from 1 to 25 μM. Monensin impedes glycoprotein processing in the Golgi apparatus by altering the pathway or rate of protein translocation through this organelle (10, 14, 20, 21, 25, 27–30). In control cells, gp120 and gp41 constituted most of the immunoprecipitated envelope products (Fig. 2, lane 1), although some residual gp160 remained at the end of the chase period. At low monensin concentrations (1 to 10 μM), the gp120 and gp41 that were formed were of lower apparent molecular weight than the cleavage products in control cells (Fig. 2, lanes 2 through 4; see also Fig. 5, lanes 1 through 4), and the amount of uncleaved gp160 in these cells was greater than that in control cells (Fig. 2, compare lane 1 with lanes 2 through 4). At a monensin concentration of 25 μM, proteolytic cleavage of gp160 was strongly inhibited, as evidenced by greatly reduced amounts of the cleavage products (Fig. 2, lane 5), whereas concentrations greater than 25 μM completely inhibited cleavage of gp160 (data not shown). High concentrations of monensin have been shown to interfere

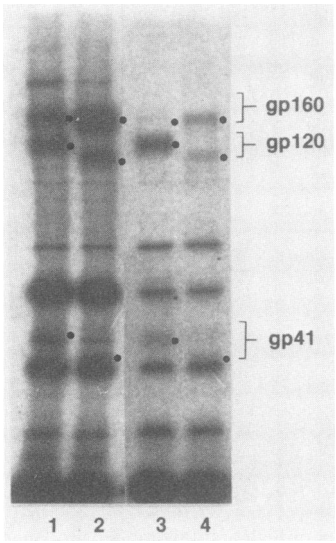

FIG. 3. Analysis of HIV-1 proteins synthesized in monensin-treated PBLs. Cells were pretreated with monensin for 1 h and then labeled with [^{35}S]Met-[^{35}S]Cys for 4 h in the continued presence of monensin. ^{35}S-labeled proteins from HIV-1-infected PBLs grown in the presence or absence of 1 μM monensin were immunoprecipitated. Shown are polypeptides untreated (lane 1) monensin-treated (lane 2) cell lysates; and from untreated (lane 3) and monensin-treated (lane 4) culture supernatants. The positions of the envelope proteins are indicated.

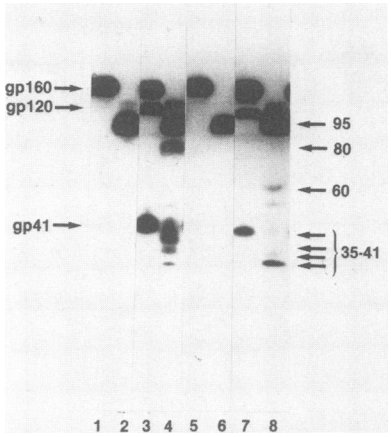

FIG. 4. Endo H digestion of ^{35}S-labeled polypeptides synthesized in control or monensin-treated Ad5env-infected 293 cells. Polypeptides were immunoprecipitated and analyzed before and after complete digestion with endo H. Shown are pulse-labeled (30 min) samples before (lane 1) and after (lane 2) endo H digestion of immunoprecipitates from control cells, samples from a 4-h chase before (lane 3) and after (lane 4) digestion of precipitated proteins from control cells, Pulse-labeled (30 min) samples before (lane 5) and after (lane 6) endo H digestion of immunoprecipitates from monensin-treated cells, and samples from a 4-h chase before (lane 7) and after (lane 8) endo H digestion of proteins immunoprecipitated from monensin-treated cells. The positions of gp160, gp120, gp41, and the digestion products are indicated.

with multiple steps in the pathway of glycoprotein processing (10, 21, 25, 28, 30). Although its effect on the protease(s) responsible for cleavage of gp160 was not seen at lower monensin concentrations, other processing steps still remained susceptible, resulting in the formation of lower-molecular-weight species of gp120 and gp41. Monensin at a concentration of 1 μM was therefore used in subsequent experiments to examine the maturation process of gp120 and gp41.

Viral protein synthesis in monensin-treated cells. To first determine that a monensin concentration of 1 μM did not inhibit protein synthesis, Ad5env-infected cells were pulse-labeled for 30 min with [^{35}S]Met-[^{35}S]Cys and then chased for 4 h in the absence or presence of 1 μM monensin. Cell lysates were prepared in RIPA buffer, and equal volumes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Since adenoviruses inhibit synthesis of host protein (7, 31), most of the newly synthesized proteins are virally encoded. The addition of 1 μM monensin to Ad5env-infected cells did not alter the overall pattern or levels of proteins either during the 30-min pulse or after the 4-h chase period (data not shown). There was no change seen in the rates of migration of adenovirus proteins that resulted from the monensin treatment. These observations were extended to HIV-1 proteins synthesized in PBLs. HIV-1-infected PBLs were metabolically labeled for 4 h with [^{35}S]Met-[^{35}S]Cys in the presence or absence of 1 μM monensin. HIV-1-specific proteins were immunoprecipitated from cell lysates and culture supernatants to determine the effects of monensin on cell-associated and virion-associated envelope proteins. The envelope gene products gp120 and gp41 synthesized in monensin-treated, HIV-1-infected PBLs were smaller than those in control cells, and there was partial inhibition of gp160 cleavage. Immunoprecipitation of culture supernatants revealed the full complement of virion-associated proteins (Fig. 3, compare lanes 3 and 4) and indicated that the cleavage products of lower apparent molecular weights were incorporated into virions produced in the presence of monensin. In addition, there was more gp160 associated with these virions than with those released from control cells.

Monensin inhibits terminal glycosylation of envelope proteins. The increase in electrophoretic mobility of envelope proteins in monensin-treated cells seemed most likely attributable to alterations in oligosaccharide processing. These proteins were examined with endo H, which catalyzes the removal of N-linked oligosaccharide side chains from high-mannose and some hybrid glycoproteins but will not digest complex oligosaccharide units (12, 26, 32). The Ad5env-encoded gp160 synthesized in either the absence or presence of 1 μM monensin during a 30-min pulse with [^{35}S]Met-[^{35}S]Cys was completely digested by endo H, yielding the apoprotein with an apparent molecular weight of 95,000 (Fig. 4, lanes 2 and 6), in agreement with earlier studies (1). Therefore, newly synthesized gp160 contains only high-mannose or hybrid oligosaccharide units. Most of the residual gp160 remaining after a 4-h chase period in control cells was completely deglycosylated by endoH, indicating that little subsequent modification of the high-mannose side chains to the complex variety had occurred. The subunits gp120 and gp41 produced in untreated cells have complex carbohydrate side chains, since these products are only partially susceptible to endo H digestion. The molecular weight of gp120 was reduced to 80,000, whereas digestion of gp41 resulted in a series of products ranging in molecular weight from 35,000 to 41,000 (Fig. 4, lane 4). In contrast, the complete endoH susceptibility of gp120 and gp41 from monensin-treated cells (Fig. 4, lane 8) indicated that the carbohydrate residues on these molecules were still of the high-mannose or hybrid variety. Thus, mature gp120 and

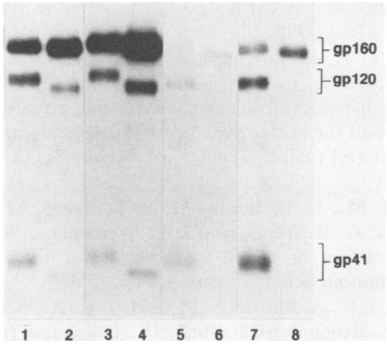

FIG. 5. Glycosylation of envelope proteins in control or monensin-treated, Ad5env-infected 293 cells. Infected cells were labeled for 4 h at 24 h postinfection with ^{14}C- or ^{3}H-labeled sugars in the absence or presence of 1 μM monensin. Cell lysates were prepared, and proteins were immunoprecipitated and analyzed on a 10% sodium dodecyl sulfate–polyacrylamide gel. Shown are [^{14}C]glucosamine-labeled proteins in the absence (lane 1) or presence (lane 2) of monensin, [^{3}H]mannose-labeled proteins in the absence (lane 3) and presence (lane 4) of monensin, [^{3}H]fucose-labeled proteins in the absence (lane 5) and presence (lane 6) of monensin, and [^{3}H]galactose-labeled proteins in the absence (lane 7) and presence (lane 8) of monensin.

gp41 synthesized in control cells contained a mixture of complex and high-mannose or hybrid oligosaccharide side chains, whereas the products formed in the presence of monensin contained only high-mannose or hybrid side chains. To investigate the specific changes in oligosaccharide composition, Ad5env-infected 293 cells were radiolabeled with various sugar precursors in the absence or presence of 1 μM monensin. The addition of the terminal sugars fucose and galactose to gp120 and gp41 was significantly reduced in the presence of monensin (Fig. 5). A densitometric scan of the fluorogram (not shown) indicated that there was about a 90% reduction in labeling of gp120 and gp41 with fucose or galactose in the presence of monensin. Digestion of envelope products from monensin-treated and control cells with neuraminidase indicated the presence of sialic acid residues on gp120 and gp41 from control cells but not on gp120 and gp41 from ionophore-treated cells or on the precursor gp160 in either control or monensin-treated cells (data not shown). Based on studies in other systems (12), it is likely that *N*-acetylglucosamine is also added as a terminal sugar.

Effects of monensin on the functional properties of the envelope glycoproteins. The ability of gp120 synthesized in the presence of monensin to form syncytia was assayed by mixing Ad5env-infected 293 cells maintained in the presence or absence of 1 μM monensin with untreated CD4-bearing HeLa monolayer cells. Monensin treatment markedly reduced the rate of syncytium formation; at 24 h after mixing, the numbers and sizes of syncytia were significantly reduced compared with those of the controls (data not shown). During Ad5env infection of 293 cells, some of the gp120 expressed on the cell surface was shed into the culture medium. Monensin treatment of these cells caused an almost complete inhibition of the appearance of gp120 in the cell culture supernatant, suggesting that less gp120 was present on the surface of monensin-treated cells than on controls. It seems likely that monensin inhibits envelope-induced syncytium formation by altering both gp120 glycosylation and expression on the cell surface. Since monensin inhibited gp120-induced syncytium formation and caused the appearance of HIV-1 containing modified envelope proteins, it was possible that virions produced by monensin-treated PBLs might display a reduced ability to infect susceptible cells. To investigate this possibility, virions released from monensin-treated and control HIV-1-infected cells were used to infect fresh PBLs. Each inoculum was normalized by using equivalent amounts of reverse transcriptase activity. Supernatants were harvested from cultures, and reverse transcriptase activity was measured. There was a 50% reduction in reverse transcriptase activity after monensin treatment.

DISCUSSION

The synthesis and processing of HIV-1 envelope glycoproteins expressed by a recombinant adenovirus vector were examined in human cells infected with the recombinant Ad5env, which codes for the envelope protein precursor gp160 (Fig. 1). The core-glycosylated precursor undergoes proteolytic cleavage to yield gp120 and gp41, which is apparent 2 to 4 h after synthesis of gp160. Cleavage of gp160 probably occurs in *cis* or medial regions of the Golgi apparatus, since cleavage can be inhibited by high concentrations of monensin (Fig. 2), a drug known to perturb events in these locations (10, 20, 21, 25, 27–30). Recently (18) it has been shown that endoproteolytic cleavage of gp160 is required for activation of HIV-1. Further localization of the subcellular site of cleavage and identification of the protease(s) involved in the cleavage process may provide insights about inhibition of HIV-1 replication.

At low monensin concentrations, the cleavage event did occur; however, the products gp120 and gp41 were of a lower apparent molecular weight than the normal products (Fig. 2 and 5). Since the size of the precursor gp160 is identical in control cells and in cells exposed to monensin (Fig. 1, compare lanes 1 and 2), it is apparent that late processing events necessary to generate mature gp120 and gp41 occur after cleavage of gp160 and that these events are not required for the endoproteolytic step.

The late processing steps include the formation of complex and hybrid oligosaccharide side chains through the addition of fucose, galactose, and sialic acid to the trimmed high-mannose units on gp120 and gp41. Additionally, some gp160 apparently escapes cleavage in control cells, and these molecules contain fucose and galactose residues but lack sialic acid. An examination of HIV-1 virion envelope proteins demonstrated some uncleaved gp160 in association with virions, and this amount was increased in the presence of monensin (Fig. 3). This implies that gp160 can follow an alternate pathway that allows secondary glycosylation before cleavage. Studies with another retroviral envelope protein, the mouse mammary tumor virus glycoprotein precursor Pr73env (5), demonstrated that some uncleaved precursor molecules appeared on the cell surface and contained fucose and galactose.

We have previously reported that gp120 synthesized in Ad5env-infected cells could bind to CD4-bearing cells and form syncytia (4). It is shown here that the extent of syncytium formation and the infectivity of HIV-1 produced from monensin-treated cells are reduced a consequence of the effects of monensin on gp120.

Other studies that have examined the role of glycosylation on gp120-induced syncytium formation and viral infectivity (8, 18, 33) have also been emphasized the importance of properly processed oligosaccharide moieties for the biological activity of gp120 and infectivity of HIV-1.

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