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Role of oligosaccharides in the processing and maturation of envelope glycoproteins of human immunodeficiency virus type 1*

(syncytium formation/virus infectivity/carbohydrate trimming enzymes/immunoprecipitations)

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ABSTRACT **The processing and maturation of envelope glycoproteins of human immunodeficiency virus type 1 (HIV-1) were studied in infected cells treated with inhibitors of oligosaccharide processing. In MOLT-3 cells chronically infected with HIV-1 (strain HTLV-III$_B$), tunicamycin severely inhibited the glycosylation of envelope proteins. Deoxynojirimycin, an inhibitor of glucosidase I in the rough endoplasmic reticulum, inhibited the proteolytic processing of gp160, whereas no such effect was noted with either deoxymannojirimycin or swainsonine, inhibitors of mannosidase I and II, respectively, in the Golgi complex. The processed gp120 and gp41 synthesized in the presence of deoxymannojirimycin were found to contain mannose-rich oligosaccharide cores as evidenced by their susceptibility to endoglycosidase H digestion. The formation of syncytia normally observed when CEM cells are cocultured with HIV-1-infected cells was markedly inhibited in the presence of deoxynojirimycin, but such inhibition was not observed in cells treated with deoxymannojirimycin or swainsonine. The infectivity of virions released from MOLT-3/HTLV-III$_B$ cells treated with deoxynojirimycin or deoxymannojirimycin was significantly lower than the infectivity of virions released from untreated cells. On the other hand, treatment with swainsonine did not affect the infectivity of the progeny virus. These results suggest that the proteolytic processing of gp160 takes place in infected cells when the glycoprotein has mannose-rich oligosaccharide structures. Trimming of glucose residues and the primary trimming of mannose residues are necessary for the release of infectious virus.**

The major glycosylation pathway utilized by the viral and cell membrane glycoproteins begins with the transfer of the precursor oligosaccharide $Glc_3Man_9(GlcNAc)_2$ from the carrier lipid, dolichol phosphate, to the nascent polypeptide chain of the glycoprotein (1). The complex-type oligosaccharides are generated via the trimming pathway. The first step of such processing is initiated in the endoplasmic reticulum by the removal of three glucose residues by glucosidases I and II. Subsequently the action of mannosidase I in the Golgi complex completes the removal of four mannose residues. The addition of *N*-acetylglucosamine, the removal of two more mannose residues by mannosidase II, and the addition of peripheral sugar residues such as galactose, *N*-acetylglucosamine, and sialic acid by specific glycosyltransferases result in the synthesis of complex-type oligosaccharide moieties in the glycoprotein molecule (1).

Identification of drugs capable of inhibiting either the synthesis or the processing of oligosaccharides has made it possible to investigate the role of glycosylation in the synthesis and maturation of glycoproteins. One of the first inhibitors of this group to be described is tunicamycin, an antibiotic that inhibits the addition of any carbohydrate molecules to asparagine residues of potential glycoproteins (2). Several inhibitors of the trimming enzymes are now available (2). Thus 1-deoxynojirimycin (DNM) and castanospermine have been shown to inhibit glucosidase I activity in the endoplasmic reticulum, whereas 1-deoxymannojirimycin (MNM) and swainsonine inhibit mannosidases I and II, respectively, in the Golgi complex (2–5). Some of these inhibitors have been shown to affect the secretion and maturation of glycoproteins in some enveloped viruses (6–11).

One of the unique features of human immunodeficiency virus type 1 (HIV-1) is its tropism for CD4-bearing human T lymphocytes (12). The *env* gene of HIV-1 encodes information for two glycosylated structural proteins that are initially synthesized in the form of a precursor polyprotein, gp160. This protein is subsequently processed to an amino-terminal exterior component, gp120, and a carboxyl-terminal transmembrane protein, gp41 (13, 14). A key interaction between gp120 and the CD4 molecule initiates the infection process. One of the consequences of HIV-1 infection is the formation of multinucleated giant cells resulting from cell–cell fusion (15, 16). It has been demonstrated that free gp120 can temporarily interfere with the viral infection and block the process of cell fusion (17). However, if the carbohydrate chains were removed from gp120 by enzymatic treatment, CD4 binding and blockage of cell fusion were reduced significantly (17). Mannosyl residues in the oligosaccharide chain of gp120 were found to be involved in HIV-1-induced pathogenesis, as mannose-specific lectins completely inactivated the virus (18). It is thus apparent that the understanding of oligosaccharide processing of viral glycoproteins may help to study the mode of infection of CD4-positive cells by HIV-1. In this communication we present a comparative study of the processing of envelope glycoproteins of HIV-1 in the presence of some known inhibitors of glycoprotein biogenesis. The effects of these inhibitors on the virus-induced cell fusion and viral infectivity were also studied.

MATERIALS AND METHODS

Virus and Cells. Chronically infected MOLT-3/HTLV-III$_B$ cells and uninfected CEM cells were maintained in RPMI-1640 medium (Advanced Biotechnology, Columbia, MD) supplemented with 10% fetal bovine serum, 1 mM glutamine, and 100 units of penicillin and 100 μg of streptomycin per ml.

Radiolabeling of Cells With [^{35}S]Methionine and Immunoprecipitation of HIV-1 Proteins by Antibodies. MOLT-3/HTLV-III$_B$ cells were incubated at 37°C for 7 hr in

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Abbreviations: HIV-1, human immunodeficiency virus type 1; MNM, 1-deoxymannojirimycin; DNM, 1-deoxynojirimycin; endo H, endoglycosidase H.

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methionine-free medium supplemented with [^{35}S]methionine (100 μCi/ml; 1 μCi = 37 kBq) (14). The labeled cells and the cell-free medium were collected and lysed at 4°C in phosphate-buffered saline (PBS: 0.5% NaCl/10 mM sodium phosphate, pH 7.2) containing 0.1% $NaDodSO_4$, 1% Triton X-100, and 0.5% sodium deoxycholate (PBS/TDS). The lysates were absorbed for 3 hr at room temperature with protein A-Sepharose and normal human serum and centrifuged. Immunoprecipitation reactions were performed by the addition, to 1 ml of the labeled clarified cell extract, of 10 μl of a HIV-1 antibody-positive human serum and 0.2 ml of a 10% suspension of protein A-Sepharose. The samples were incubated at 4°C overnight and the immunoprecipitates were collected by centrifugation at 2000 × *g* for 10 min. The immunoprecipitates were washed extensively in PBS/TDS, resuspended in 75 μl of 65 mM Tris·HCl, pH 6.7/1% $NaDodSO_4$/10% glycerol/0.25% 2-mercaptoethanol/0.1% bromophenol blue, heated for 3 min at 90°C, and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis. For endoglycosidase H (endo H) treatment, the immunoprecipitates were washed with 0.05 M sodium acetate buffer (pH 5.5) containing 0.025% $NaDodSO_4$ and then were digested with 6 milliunits of endo H (Boehringer Mannheim) at 37°C for 6 hr. The digested samples were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis.

Syncytium Assay. The syncytium assay was performed in 96-well plates by cocultivating 10^5 CEM cells with 10^4 MOLT-3/HTLV-III$_B$ cells in 0.2 ml of medium. The plates were incubated in a CO_2 incubator at 37°C for 40 hr, and the number of multinucleated giant cells in each well was determined by microscopic examination.

RESULTS

Tunicamycin, But Not MNM or Swainsonine, Inhibit the Synthesis and Processing of gp160. The effect of specific inhibitors of oligosaccharide processing on the synthesis of viral proteins in HIV-1-infected cells was studied in MOLT-3/HTLV-III$_B$ cells. The cells were labeled for 7 hr with [^{35}S]methionine in the presence of the inhibitors, and the immunoreactive proteins in the lysates were precipitated with a HIV-1 antibody-positive human serum. Tunicamycin (1.5 μg/ml; Calbiochem) severely inhibited the glycosylation of the envelope protein, resulting in the accumulation of an 80-kDa molecule (Fig. 1*A*, lane 4). Treatment of cells with MNM (5 mM; Boehringer Mannheim) or swainsonine (10 μg/ml; Boehringer Mannheim) had no effect on the processing of gp160, although a major 100-kDa protein was observed in each case in addition to the usual gp120 (lanes 2 and 3). As described in our earlier study (9), DNM (5 mM) inhibited the proteolytic processing of gp160. Treatment of MOLT-3/HTLV-III$_B$ cultures with MNM or swainsonine did not change the overall protein profile or affect the yield of the virus obtained from the cell-free medium (Fig. 1*B*) with the exception that the gp120 equivalent was smaller in size in the treated cultures. None of the inhibitors studied affected the processing of the *gag*-encoded precursor Pr53^{gag} to p24.

Cleavage of gp160 Occurs Before Trimming of Mannose Residues. As described in the Introduction, the biosynthesis of an N-glycosylated protein starts with the addition of an oligosaccharide to an asparagine residue and is followed by stepwise trimming of glucose and mannose residues and the addition of peripheral sugars. At what stage of sugar-trimming does HIV gp160 undergo cleavage to gp120 and gp41? This question was analyzed by examining the sensitivity of the three HIV-1 glycoproteins to endo H treatment. Endo H specifically cleaves N-linked oligosaccharides containing cores with high mannose content, such as is present before the mannose trimming. In contrast, the complex oligosaccharides formed in the Golgi are not affected by endo H (1).

MOLT-3/HTLV-III$_B$ cultures were labeled with [^{35}S]methionine in the presence or absence of MNM, and the labeled cells and free virus in the medium were separately extracted with detergents. The HIV-1 proteins in the lysates were immunoprecipitated with a broadly reactive serum from a HIV-1-infected subject. Portions of the immunoprecipitates were digested with endo H and then analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis along with their undigested controls. Irrespective of whether the culture was treated with MNM or not, gp160 was always totally sensitive to endo H treatment (Fig. 2*A*), indicating that gp160 exists always in the high-mannose state. On the other hand, gp120 from untreated cells was only partially sensitive to endo H treatment. However, the 100-kDa gp120 species of the MNM-treated culture contained mannose-rich cores, as it was totally digested by endo H. It is clear that the reduced size of this protein is the result of a block in the normal maturation of the sugar moieties to the complex carbohydrate type. Both gp120 (Fig. 2*A*, lane 2) and the 100-kDa variant

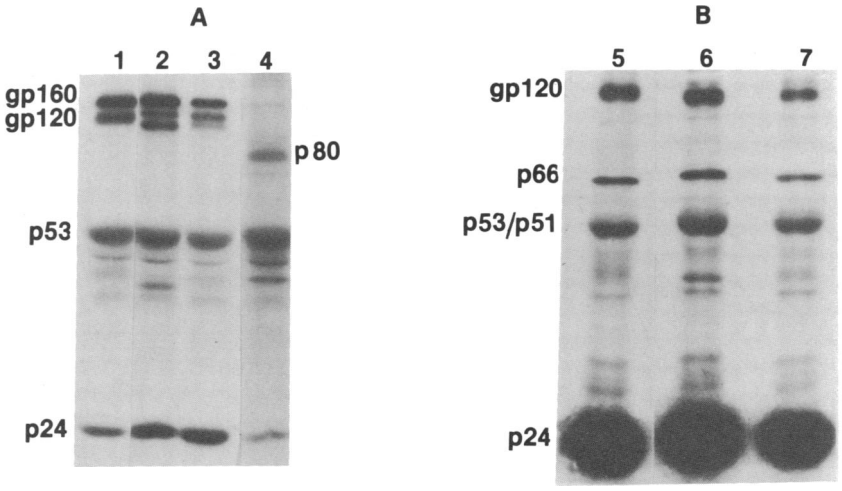

FIG. 1. Profile of viral proteins from HIV-1-infected cells in the presence of MNM, swainsonine, or tunicamycin. MOLT-3/HTLV-III$_B$ cells were labeled with [^{35}S]methionine for 7 hr in the absence or presence of MNM (5 mM), swainsonine (10 μg/ml), or tunicamycin (1.5 μg/ml). The cells and media were separated, lysed, and immunoprecipitated with a HIV-1 antibody-positive human serum. (*A*) Cell-associated viral proteins from untreated cells (lane 1) or cells treated with MNM (lane 2), swainsonine (lane 3), or tunicamycin (lane 4). (*B*) Viral proteins from culture supernatant of untreated cells (lane 5) or of cells treated with MNM (lane 6) or swainsonine (lane 7).

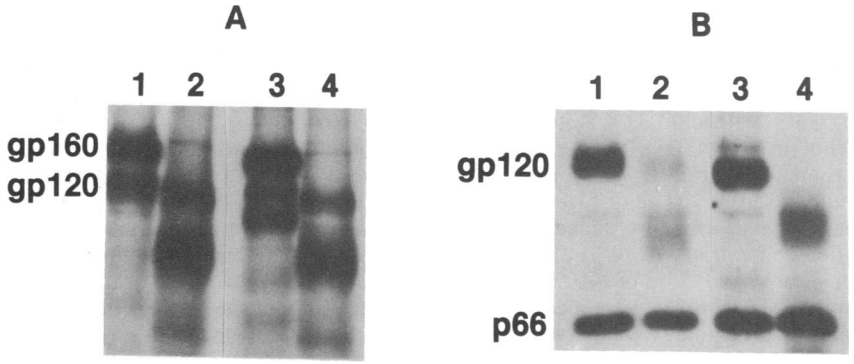

FIG. 2. Susceptibility of glycoproteins from HIV-1-infected cells and cell-free media to endo H digestion. MOLT-3/HTLV-III$_B$ cells were labeled with [^{35}S]methionine for 7 hr in the presence or absence of 5 mM MNM. The cells and media were separated, lysed, and immunoprecipitated with a HIV-1 antibody-positive human serum, and portions of the immunoprecipitates were digested with endo H. Proteins were then analyzed by gel electrophoresis. (*A*) Cell extracts. (*B*) Viral lysates. Lanes (both panels): 1 and 2, untreated cultures; 3 and 4, cultures treated with MNM. Proteins in lanes 2 and 4 were digested with endo H, and those in lanes 1 and 3 were untreated.

(lane 4) gave rise to the same-size product after endo H treatment. The properties of secreted gp120 and the corresponding 100-kDa protein were similar to those of the cell-associated proteins. Total endo H sensitivity was seen only with the protein secreted from MNM-treated cells (Fig. 2*B*).

The carbohydrate residues of gp41 exist mostly in the complex state and therefore were largely resistant to endo H digestion (Fig. 3, lanes 1 and 2). However, as expected, gp41 from cells treated with MNM contained mannose-rich sugar residues, as evidenced by the total sensitivity to endo H digestion (Fig. 3, lanes 3 and 4).

MNM and Swainsonine Do Not Affect HIV-1-Induced Syncytium Formation. The carbohydrate residues of gp120 play a significant role in the interaction with the CD4 receptor on the target cells (17). The proper oligosaccharide processing of gp120 may thus be needed for the cell fusion process resulting in syncytium formation. The effect of inhibitors of oligosaccharide processing on syncytium formation was determined by cocultivation of CEM cells with MOLT-3/HTLV-III$_B$ cells in the presence of various concentrations of DNM, MNM, or swainsonine (Table 1). Syncytium formation was not affected by either MNM or swainsonine even at high concentrations. In sharp contrast, as shown in our earlier study, a significant inhibition of syncytium formation was noted in the presence of DNM (9). Experiments with MNM and swainsonine have shown that gp120 containing mannose-rich oligosaccharides can be expressed on the plasma membrane and interact with the CD4 antigen of the target cell to induce cell–cell fusion.

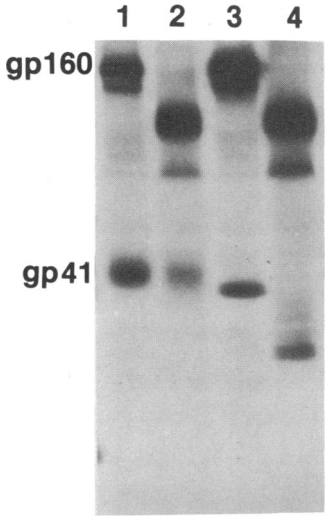

FIG. 3. Susceptibility of gp41 from HIV-1-infected cells to endo H. MOLT-3/HTLV-III$_B$ cells were labeled with [^{35}S]methionine for 7 hr in the presence or absence of 5 mM MNM and immunoprecipitated with a rabbit anti-gp41. Lanes: 1 and 2, untreated cells; 3 and 4, cells treated with MNM. Immunoprecipitates in lanes 2 and 4 were digested with endo H.

Effect of Inhibitors of Oligosaccharide Trimming Enzymes on the Infectivity of Progeny Virus. The infectivity of HIV-1 released from MOLT-3/HTLV-III$_B$ cells treated with the inhibitors of trimming enzymes was compared to that from untreated cells. MOLT-3/HTLV-III$_B$ cells were treated with DNM or MNM for 5 days. The cell-free supernatant was then collected and the reverse transcriptase activity was determined. There was no significant difference in reverse transcriptase activity between the drug-treated and untreated cell culture supernatants (Fig. 4*A*), indicating that the amount of virus in each culture medium was similar. The results were also similar when cells were treated with swainsonine (data not shown). These cell-free virions were then used to infect CEM cells at three different dilutions and the cultures were monitored for reverse transcriptase activity. The infectivity of the virions released from DNM-treated cells was about half that of the virus from untreated cells, but MNM treatment resulted in a drastic reduction in the infectivity of the progeny virus (Fig. 4*B*). In sharp contrast, the infectivity of HIV-1 from swainsonine-treated cells was comparable to that of virions from untreated cells (Fig. 4*C*). This suggests that the primary mannose-trimming of HIV-1 glycoproteins by mannosidase I is required for the expressed virus to be infectious. The secondary trimming by mannosidase II does not seem to be necessary for virus infectivity.

Table 1. Effect of inhibitors of oligosaccharide processing on syncytium formation by MOLT-3/HTLV-III$_B$ cells

Drug	Concentration	Syncytia, no. per field
None (control)	—	32
DNM	1 mM	1
	2 mM	0
	3 mM	1
MNM	1 mM	29
	2 mM	36
	3 mM	30
Swainsonine	5 μg/ml	40
	7.5 μg/ml	36
	10 μg/ml	40

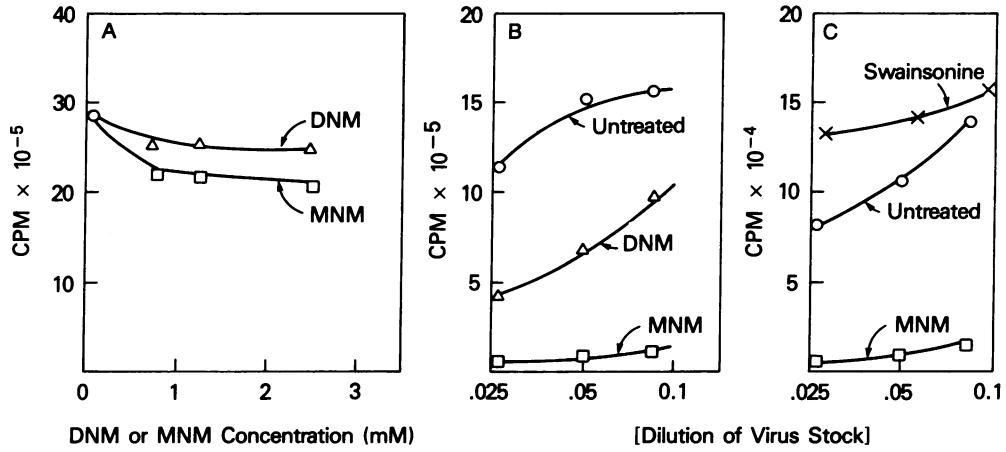

FIG. 4. Yield and infectivity of HIV-1 released from MOLT-3/HTLV-III$_B$ cells treated with DNM, MNM, or swainsonine. (*A*) MOLT-3/HTLV-III$_B$ cells (10^6) were cultured in 3 ml of medium containing DNM or MNM as indicated. After 2 days, cells were centrifuged and resuspended in fresh medium containing the drugs, and culture continued for 3 more days. Particle-associated reverse transcriptase activity in the medium was measured as described (12). (*B* and *C*) Supernatants from cultures treated with DNM (4 mM), MNM (4 mM), or swainsonine (10 μg/ml) were used to infect CEM cells. For this, the medium was added at three different dilutions to 10^5 CEM cells in 0.1 ml of RPMI-1640 medium. After a 1-hr incubation at 37°C the medium was removed and the cells were cultured in fresh medium. Reverse transcriptase activity in the supernatant was determined after 10 days (*B*) or after 7 days (*C*).

DISCUSSION

The results demonstrate that in HIV-1-infected cells the cleavage of gp160 to gp120 takes place in proteins containing unprocessed oligosaccharide chains. Thus the uncleaved gp160 is totally sensitive to endo H digestion and is in a high-mannose state irrespective of the presence of mannosidase inhibitors in the culture. Such a total endo H sensitivity of gp120 and gp41 was detected only in cells treated with MNM. Treatment of MOLT-3/HTLV-III$_B$ cells with MNM or swainsonine leads to a protein of 100 kDa containing mannose-rich cores. It is possible that a different cleavage site in gp160 may be used to generate this smaller protein, but this seems unlikely because endo H digestion of gp120 and of the 100-kDa molecule gave products of similar size (Fig. 2). The oligosaccharide structures linked to gp120 have recently been shown to carry oligomannosidic glycans with eight or more mannose residues (19). The cleavage of a precursor glycoprotein containing mannose-rich oligosaccharide structures was also noted in other enveloped viruses. Thus the proteolytic cleavage of PE_2 to E_2 in Sindbis virus took place at a stage when the oligosaccharide in the viral glycoproteins had the composition $Man_9(GlcNAc)_2$ (11). MNM treatment did not affect this cleavage.

We recently demonstrated that in MOLT-3 cells chronically infected with HTLV-III$_B$, monensin inhibited the proteolytic processing of gp160 to gp120 (20). However, the limited processing that occurs in these cells led to a 100-kDa product in addition to gp120, as was seen in the present study with MNM-treated cells. This suggests that the cleavage of gp160 takes place at an intracellular site, probably within the Golgi complex. Intracellular cleavage of precursor proteins has been observed in Newcastle disease virus and mumps virus (21, 22). Thus the processing of F_0 protein was shown to occur either in trans-Golgi cisternae or in a cell compartment occupied by the glycoprotein soon after its transit through the Golgi complex. Among retroviruses, studies with murine leukemia virus and Rous sarcoma virus have shown that the precursor envelope proteins are devoid of fucose or sialic acid, although the mature glycoproteins contain complex-type carbohydrates (23–25). However, a recent study with avian reticuloendotheliosis virus (REV) has demonstrated that the proteolytic processing of envelope glycoproteins takes place via an endo H-resistant polyprotein, suggesting that the intracellular cleavage of REV envelope precursor protein occurs after the final stages of glycosylation (26).

A role of carbohydrate residues of gp120 in viral infectivity has been demonstrated by lectin binding to HIV-1. It has been shown that mannose-specific lectins such as concanavalin A can inactivate HIV-1 and block virus-induced syncytium formation (18, 27). As shown in the present study, virus released from MNM- or DNM-treated cells had lower infectivity than that from swainsonine-treated or untreated cells. A similar finding was recently reported in another study (28). Unlike the DNM-treated cells, the MNM-treated cells behaved like untreated cells in syncytium induction with CEM cells. This suggests that although cell–cell fusion is an important and necessary step for the transmission of HIV-1 from an infected cell, we still do not understand the detailed mechanism of infection by the cell-free virus. It is quite likely that the glucose and mannose residues of gp120 have to be partially trimmed before the virus becomes fully infectious. These requirements may not be stringent for cell–cell fusion. The inability of swainsonine to affect the infectivity of the virus suggests that the carbohydrate residues such as sialic acid and fucose in HIV-1 glycoproteins that are added subsequent to the final mannose-trimming may not be essential for viral infectivity. It is possible that some of the oligosaccharide residues are in close proximity to regions important for infectivity. Thus alteration of oligosaccharide structures may induce a local perturbation of the protein conformation, resulting in attenuation of the viral infectivity.

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