Human Immunodeficiency Virus Type 1 Envelope Glycoprotein Is Modified by O-linked Oligosaccharides

HELENE B. BERNSTEIN,¹ SIMON P. TUCKER,² ERIC HUNTER,¹ JOHN S. SCHUTZBACH,¹
AND RICHARD W. COMPANS^{3*}

Department of Microbiology, University of Alabama at Birmingham, Birmingham, Alabama 35294¹; Infectious Disease Section, Searle Research and Development, St. Louis, Missouri 63198²; and Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, Georgia 30322³

Received 10 June 1993/Accepted 28 September 1993

The human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein has been shown to be extensively modified by N-linked glycosylation; however, the presence of O-linked carbohydrates on the glycoprotein has not been firmly established. We have found that enzymatic deglycosylation of the HIV-1 envelope glycoprotein with neuraminidase and O-glycosidase results in a decrease in the apparent molecular weight of the envelope glycoprotein. This result was observed in both vaccinia virus recombinant-derived envelope glycoproteins and glycoproteins derived from the IIIB, SG3, and HXB2, strains of HIV-1. The decrease in molecular weight was also observed when the envelope glycoprotein had been deglycosylated with N-glycanase F after treatment with neuraminidase and O-glycosidase, indicating that the decrease in apparent molecular weight was not attributable to the removal of N-linked carbohydrate. Treatment with neuraminidase, O-glycosidase, and N-glycanase F was found to be necessary to remove all radiolabel from [³H]glucosamine-labelled envelope glycoprotein, a result seen for both recombinant and HIV-1-derived envelope glycoprotein. [³H]glucosamine-labelled carbohydrates liberated by O-glycosidase treatment were separated by paper chromatography and were found to be of a size consistent with O-linked oligosaccharides. We, therefore, conclude that the HIV-1 envelope glycoprotein is modified by the addition of O-linked carbohydrates.

The envelope glycoprotein plays a critical role in the life cycle of the human immunodeficiency virus (HIV) as it mediates the attachment and entry of the virus into susceptible cells via the cellular receptor, CD4 (17, 20, 21, 33). The envelope glycoprotein is first synthesized as a polyprotein precursor (gp160), the majority of which is retained in an intracellular compartment and undergoes subsequent degradation (1, 34, 38, 39). A fraction of gp160 is proteolytically cleaved in the Golgi complex to yield the transmembrane (gp41) and surface subunits (gp120) of the mature envelope glycoprotein (4, 5, 9, 29, 36). Following proteolytic cleavage, gp120 and gp41 remain associated via noncovalent interactions (35) and are expressed on the surface of infected cells and incorporated into budding virions (37). The surface unit is responsible for the interaction of the virus with CD4 (22), and the transmembrane subunit of the envelope glycoprotein is responsible for CD4-dependent fusion leading to infectious entry and the formation of multinucleated syncytia (7).

The HIV type 1 (HIV-1) envelope glycoprotein precursor, gp160, is heavily glycosylated, with a peptide core of approximately 90 kDa (1). gp120 is also extensively glycosylated, with over 20 utilized N-linked glycosylation sites (9). The presence of O-linked oligosaccharides on the HIV-1 envelope glycoprotein, however, has not been conclusively established. O-linked glycosylation has been demonstrated, however, for other retroviral glycoproteins, including Friend spleen focus-forming virus (SFFV), Friend mink cell focus-forming virus (FrMCF), Rauscher murine leukemia virus (R-MuLV), and feline leukemia virus (10, 31, 32). The O-linked carbohydrates were localized to the surface subunits of the FrMCF glycoproteins. In the present

study, we have examined the HIV-1 envelope glycoprotein for the presence of O-linked carbohydrates by using enzymatic deglycosylation and chromatographic analysis of the released glycans.

MATERIALS AND METHODS

Cells and viruses. BHK-21 cells were obtained from the American Type Culture Collection (ATCC) and grown in Dulbecco's modified Eagle's medium containing 10% newborn calf serum and 10% tryptic peptide broth. H9 cells were also obtained from ATCC and were grown in RPMI 1640 medium supplemented with 15% fetal calf serum. The IIIB isolate of HIV-1 was used to persistently infect H9 cells as described previously (8). Sup-T1 cells infected with the HXB2 strain of HIV-1 were obtained from John Wakefield. Peripheral blood mononuclear cells (PBMCs) and CEMx174 cells infected with the SG3 strain of HIV-1 were a gift from Sajal Ghosh (10a). Vaccinia virus recombinants VV-env-1 and VV-SC11 (28) were grown as previously described (14).

Recombinant protein expression. Dishes (100 mm) of confluent BHK-21 cells were infected with recombinant vaccinia viruses at a multiplicity of infection of 5. The cells were starved at 10 h postinfection for 30 min in methionine- and cysteine-deficient or in glucose-deficient Eagle's medium and then metabolically labelled with 100 μ Ci of [³⁵S]methionine and [³⁵S]cysteine (New England Nuclear) per ml or 100 μ Ci of [³H]glucosamine (ICN) per ml in deficient medium for the indicated times. Cells were lysed in a buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, and 1% Nonidet P-40. Lysates were precleared with *Staphylococcus aureus* preloaded with normal human antisera, and HIV-specific polypeptides were immunoprecipitated by using pooled HIV-positive patient sera and protein A-agarose beads (Pierce) as described previously (2).

HIV-1 protein expression. HIV-1-infected cells and mock-

* Corresponding author. Mailing address: Department of Microbiology and Immunology, Emory University School of Medicine, 3001 Rollins Research Center, Atlanta, GA 30322. Phone: (404) 727-5947. Fax: (404) 727-3659.

infected cells were starved for 30 min in methionine- and cysteine-deficient or in glucose-deficient Eagle's medium and then metabolically labelled with 100 μ Ci of [35 S]methionine and [35 S]cysteine (New England Nuclear) per ml or 100 μ Ci of [3 H]glucosamine (ICN) per ml in deficient medium for 3 h. Cells were lysed, and proteins were immunoprecipitated as described above.

Neuraminidase and *O*-glycosidase digestion. Immunoprecipitated proteins bound to agarose beads were suspended in buffer containing 0.5% Nonidet P-40, 10 mM Tris (pH 7.4), and 0.5 M NaCl. Neuraminidase (Boehringer Mannheim) was added to a final concentration of 150 mU/ml, and the samples were incubated for 2 h at 37°C. *O*-Glycosidase digestions were performed by suspending immunoprecipitated, neuraminidase-digested proteins bound to agarose beads in 20 mM Tris-maleate (pH 6.8) and treating with 2 mU of *O*-glycosidase (Boehringer Mannheim) for 17 h at 37°C, as recommended by Boehringer Mannheim. In the case of multiply digested proteins, samples were treated first with neuraminidase, followed by *O*-glycosidase, followed by *N*-glycanase F (PNGase F). Control samples were incubated under identical conditions, but in the absence of enzyme.

PNGase F and endo F digestion. Immunoprecipitated proteins were dissociated from the agarose beads by boiling the samples for 5 min in 10 μ l of sample loading buffer containing 1% sodium dodecyl sulfate (SDS), 10% glycerol, 5% mercaptoethanol, and 62 mM Tris (pH 6.8). Aliquots of eluted protein samples were deglycosylated with 0.4 U of PNGase F (Boehringer Mannheim) for 17 h at 37°C in 100 μ l of buffer containing 1% *N*-octylglucoside, 50 mM Tris (pH 7.4), and 10 mM EDTA. Endoglycosidase F (Endo F) digestions were carried out on identical aliquots of eluted protein by using 0.05 U of enzyme in 100 μ l of buffer containing 0.1 M sodium phosphate (pH 6.1), 50 mM EDTA, 1% Nonidet P-40, and 1% *N*-octylglucoside for 17 h at 37°C. In the case of double digestions, samples were treated with Endo F and then with PNGase F. Proteins were then precipitated with 10 volumes of acetone for 2 h at -20°C, and the pellets were redissolved in sample loading buffer and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE).

Paper chromatography. Radiolabelled glycans and glycan standards were spotted on Whatman no. 1 filter paper and subjected to ascending paper chromatography with *n*-propanol-ethyl acetate-water (7:1:2). The lanes containing radiolabelled glycans were cut into 1-cm sections, and the counts were measured by using a scintillation counter. Glycan standards were detected colorimetrically by using *p*-anisidine phthalate.

RESULTS

***O*-Glycosidase causes a decrease in the apparent molecular weight of the envelope glycoprotein.** Enzymatic deglycosylation has been used as a tool to demonstrate the presence of O-linked carbohydrates on viral glycoproteins (6, 31, 32). We found that neuraminidase treatment caused a slight decrease in the electrophoretic mobility of [35 S]methionine- and [35 S]cysteine-labelled, recombinant HIV-1 envelope glycoprotein immunoprecipitated from BHK-21 cells infected with VV-env-1, a vaccinia virus which expresses the HIV-1 envelope glycoprotein (Fig. 1, lane 2). A larger decrease in the apparent molecular weight of the HIV-1 envelope glycoprotein was observed after additional digestion with *O*-glycosidase (Fig. 1, lane 3). To provide further evidence that the size decrease seen in envelope glycoprotein following *O*-glycosidase treatment is attributable to the removal of O-linked glycans and not to the removal of N-linked oligosaccharides, we deglycosylated enve-

FIG. 1. Deglycosylation of recombinant envelope glycoprotein. BHK-21 cells infected with VV-env-1 were labelled with 100 μ Ci of [35 S]Met and [35 S]Cys per ml for 5 h, and cells were lysed and then immunoprecipitated. Aliquots of protein were untreated (lane 1), treated with neuraminidase (lane 2), or treated with neuraminidase and then with *O*-glycosidase (lane 3). Following enzymatic deglycosylation, samples were analyzed via SDS-8% PAGE and then fluorography.

lope glycoprotein with both *O*-glycosidase and PNGase F. We observed a greater decrease in the size of gp160 after deglycosylation with both PNGase F and *O*-glycosidase than with gp160 deglycosylated with PNGase F alone (Fig. 2, lane 5).

Neuraminidase and *O*-glycosidase treatment of envelope glycoproteins immunoprecipitated from persistently infected H9/IIIB cells also resulted in an increase in the electrophoretic mobility of gp160 and gp120 (Fig. 3A). In this case, an additional band was observed above gp160 following neuraminidase treatment (Fig. 3A, lane 2). Subsequent *O*-glycanase digestion resulted in a decrease in mobility for this band and an increase in mobility of gp120 and gp160. The identity of this high-molecular-weight band was not established. Other strains of HIV-1 were also examined for the presence of O-linked glycans by this method. On the basis of the observed decrease in molecular weight after treatment with *O*-glycosidase, the HXB2 (Fig. 3B) and SG3 (Fig. 3C and D) strains of HIV-1 also appear to contain O-linked glycans. These experiments were performed on glycoproteins isolated from several different cell types, including PBMCs, demonstrating that O-linked glycosylation of HIV-1 occurs in several cloned cell lines as well as in normal blood cells.

FIG. 2. Removal of N- and O-linked glycans from recombinant envelope glycoprotein. BHK-21 cells infected with VV-env-1 were labelled with 100 μ Ci of [35 S]Met and [35 S]Cys per ml for 5 h, and cells were lysed and then immunoprecipitated. Aliquots of glycoprotein were deglycosylated with PNGase F (lane 2); Endo F (lane 3); Endo F followed by PNGase F (lane 4); or neuraminidase, *O*-glycosidase, and PNGase F (lane 5) or were untreated (lane 1). Following enzymatic deglycosylation, samples were analyzed via SDS-6% PAGE and then fluorography.

FIG. 3. Deglycosylation of viral envelope glycoprotein. HIV-1 infected cells were metabolically labelled with 100 μ Ci of [35 S]Met and [35 S]Cys per ml for 6 h. Immunoprecipitated cell lysates were untreated (lane 1), treated with neuraminidase (lane 2), or treated with neuraminidase and then with *O*-glycosidase (lane 3), and proteins were analyzed by SDS-10% PAGE and fluorography. (A) Persistently infected H9-IIIB cells; (B) HXB2-infected Sup-T1 cells; (C) SG3-infected CEMx174 cells; (D) SG3-infected PBMCs.

***O*-Glycosidase and PNGase F digestion of [3 H]glucosamine-labelled envelope glycoprotein.** To increase the sensitivity of the assay and to provide a more precise means of establishing glycosylation states, the HIV-1 envelope glycoprotein was labelled with [3 H]glucosamine prior to enzymatic deglycosylation of the immunoprecipitated envelope glycoproteins. BHK-21 cells were infected with VV-env-1 and labelled with 100 μ Ci of [3 H]glucosamine per ml for 2 h at 10 h postinfection prior to cell lysis. The envelope glycoprotein was then deglycosylated by using enzymes which remove N-linked glycans, including PNGase F, Endo F, or a combination of both enzymes to ensure that all N-linked glycans had been removed. One aliquot of glycoprotein was additionally subjected to *O*-glycosidase treatment (Fig. 4, lane 5). On overnight exposure of the fluorograph, [3 H]glucosamine appeared to have been completely removed from the glycoproteins which were treated with N-linked carbohydrate-specific endoglycosidases (Fig. 4A). However, upon prolonged exposure, [3 H]glucosamine-labelled proteins at the expected molecular weights for deglycosylated gp160 (90 kDa) and gp120 (60 kDa) were seen in these samples, indicating that deglycosylation was incomplete (Fig. 4B). In contrast, the sample which had been treated with neuraminidase and *O*-glycosidase prior to treatment with PNGase F (Fig. 4B) contained no residual labelled proteins. In addition, the sample which had been deglycosylated with Endo F (Fig. 4B, lane 3) appeared to retain more radiolabelled glycan than the other samples. This is consistent with the fact that Endo F cleaves glycans distal to the GlcNAc-Asn linkage, which would leave a residual GlcNAc on deglycosylated peptides.

These studies were repeated by using [3 H]glucosamine-labelled envelope glycoprotein immunoprecipitated from lysates of H9/IIIB cells and yielded essentially identical results. Neuraminidase followed by *O*-glycosidase treatment prior to PNGase F digestion was found to be required to remove all [3 H]glucosamine label from the viral glycoprotein. It is of note that the glycoprotein obtained from H9/IIIB cells appears to have most of the residual [3 H]glucosamine-labelled glycan attached to gp160 (Fig. 5B, lane 2), whereas an almost equal amount of [3 H]glucosamine-labelled gp120 and gp160 remained following N-linked deglycosylation of glycoproteins

FIG. 4. Deglycosylation of [3 H]glucosamine-labelled recombinant envelope glycoprotein. BHK-21 cells infected with VV-env-1 were labelled with 100 μ Ci of [3 H]glucosamine per ml for 2 h, and cells were lysed. Lysates were immunoprecipitated, and the aliquots were deglycosylated with PNGase F (lane 2); Endo F (lane 3); Endo F followed by PNGase F (lane 4); or neuraminidase, *O*-glycosidase, and PNGase F (lane 5) or were untreated (lane 1). Proteins were analyzed by SDS-10% PAGE and fluorography. (A) Overnight exposure of film; (B) 3-week exposure of film. In panel B, lane 3, gp160 appears to retain an increased amount of radiolabel compared with that in lanes 2 and 4. This is likely because Endo F hydrolyzes glycans distal to the terminal GlcNAc. However, it is uncertain why additional radiolabel was not also retained by gp120.

derived from vaccinia virus-infected BHK-21 cells. These results are probably attributable to differences in glycosylation between the two cell lines used in these experiments. These results provide further evidence for *O*-linked glycosylation of the HIV-1 envelope glycoprotein and, in combination with the increase in electrophoretic mobility of amino acid-labelled glycoprotein seen after *O*-glycosidase treatment (Fig. 1, 2, and 3), suggest that both gp120 and gp160 contain *O*-linked glycans. To estimate the approximate number of *O*-linked oligosaccharide chains on the HIV-IIIB envelope glycoprotein, we compared the amount of [3 H]glucosamine (and [3 H]galactosamine) released from desialated H9/IIIB-derived glycoprotein after removal of *O*- or N-linked glycans. Based on the assumption that there are 24 utilized N-linked glycosylation sites on gp120 (19) and 4 utilized N-linked glycosylation sites on gp41 (18) and that each N-linked glycan contains an average of 2.88 GlcNAcs per molecule (24), we estimate that

FIG. 5. Deglycosylation of [^3H]glucosamine-labelled viral envelope glycoprotein. Persistently infected H9/IIIB cells were labelled with 100 μCi of [^3H]glucosamine for 6 h, and cells were lysed. Lysates were immunoprecipitated, and the aliquots were untreated (lanes 1), deglycosylated with PNGase F (lanes 2), or deglycosylated with neuraminidase, *O*-glycosidase, and PNGase F (lanes 3). Proteins were analyzed by SDS-10% PAGE and fluorography. (A) Overnight exposure of film; (B) 3-week exposure of film.

there are approximately eight O-linked carbohydrate chains per molecule of gp160.

Paper chromatography of released glycans. O-linked glycans typically have a lower molecular weight than their N-linked counterparts. To confirm that the glycans released by *O*-glycosidase treatment of HIV-1 envelope glycoprotein are of a size consistent with O-linked glycans, ascending paper chromatography of released, [^3H]glucosamine-labelled glycans was conducted in a polar solvent consisting of propanol-ethyl acetate-water (7:1:2). This solvent will separate short glycans of one to four saccharides, and longer glycans (including N-linked glycans) will be retained at the origin. Immunoprecipitated glycoprotein bound to agarose beads was digested with neuraminidase and was then extensively washed with 20 mM Tris-maleate (pH 6.8) to remove the radiolabel released during neuraminidase treatment. Aliquots were then treated with *O*-glycanase or mock digested with no enzyme. The resulting supernatants, which contained the released glycans, were then subjected to paper chromatography. [^3H]glucosamine-labelled glycan released by *O*-glycosidase from recombinant HIV-1 envelope glycoprotein yielded a single peak following paper chromatography which migrated 10 cm from the origin (Fig. 6). The mock-digested sample contained few counts and yielded no peak on the chromatogram. As a control, [^3H]glucosamine-labelled, immunoprecipitated proteins from lysates of cells infected with VV-SC11, a recombinant vaccinia virus expressing β -galactosidase, were deglycosylated by using *O*-glycosidase, and the reaction products were subjected to paper chromatography. There were no resulting peaks on the paper chromatogram, indicating that the peak derived from vaccinia virus-expressed gp160 is HIV-1 specific (data not shown). To confirm that N-linked glycans are retained at the origin by using our chromatography system, N-linked [^3H]glucosamine-labelled glycans were liberated from immunoprecipitated HIV-1 envelope glycoproteins by using PNGase F and subjected to paper chromatography. As expected, all of the released glycans remained at the origin, demonstrating that N-linked glycans are too large to be resolved by paper chromatography with this solvent system (data not shown).

O-Glycosidase treatment of the H9/IIIB-derived HIV-1 envelope glycoprotein released carbohydrates which were found to segregate into two peaks at 9 and 18 cm (Fig. 7). These peaks were not evident in the mock-digested controls (Fig. 7).

FIG. 6. Paper chromatography of [^3H]glucosamine-labelled oligosaccharides liberated from recombinant HIV-1 envelope glycoprotein by *O*-glycosidase. Immunoprecipitated glycoprotein bound to agarose beads was treated with 150 mU of neuraminidase per ml for 2 h at 37°C. Aliquots of glycoprotein were treated with 2 mU of *O*-glycosidase or mock digested for 16 h at 37°C. Reaction products were separated via paper chromatography. Strips (1 cm) were analyzed for radioactivity by using a scintillation counter. $\circ$, *O*-glycosidase-digested glycoprotein; $\square$, mock-digested glycoprotein. Glycan standards: 1, Gal-Gal-GlcNAc; 2, Gal-GalNAc; 3, Glu.

As an additional control, [^3H]glucosamine-labelled lysates of mock-infected H9 cells were immunoprecipitated with HIV-1-specific antisera, and the *O*-glycosidase digestion products were analyzed by paper chromatography. No peaks were evident on the chromatogram (data not shown). The differences in the carbohydrates released from recombinant and H9/IIIB-derived glycoproteins are likely to be attributable to the different cell lines utilized in these experiments. The increased expression of HIV-1 envelope glycoprotein seen in

FIG. 7. Paper chromatography of [^3H]glucosamine-labelled oligosaccharides liberated from H9/IIIB-derived envelope glycoprotein by *O*-glycosidase. Immunoprecipitated glycoproteins bound to agarose beads were treated with 150 mU of neuraminidase per ml for 2 h at 37°C. Aliquots of glycoprotein were treated with 2 mU of *O*-glycosidase or mock digested for 16 h at 37°C. Reaction products were separated via paper chromatography. Strips (1 cm) were analyzed for radioactivity by using a scintillation counter. $\circ$, *O*-glycosidase-digested glycoprotein; $\square$, mock-digested glycoprotein. Glycan standards: 1, mannotriose; 2, *O*-mannose.

recombinant vaccinia virus-infected cells may also contribute to the differences in glycosylation observed between H9/IIIB cells and BHK-21 cells. These results indicate that the carbohydrates released from the HIV-1 glycoprotein by *O*-glycosidase are of a size consistent with their being *O*-linked glycans.

DISCUSSION

O-linked glycosylation is a commonly occurring form of posttranslational modification. However, considerably less is known about the synthesis and function of *O*-linked carbohydrates on glycoproteins than about their *N*-linked counterparts, and there are no currently accepted consensus sequences for *O*-linked glycosylation (16, 25). *O*-linked glycosylation begins with the addition of the core sugar GalNAc in an α -glycosidic linkage to a serine or threonine residue. The basic structure may then be enhanced by the subsequent addition of galactose, *N*-acetylglucosamine, fucose, and sialic acid by glycosyltransferases found in the Golgi apparatus (30). The structures of *O*-linked carbohydrates are varied, and there is no common carbohydrate core or processing pathway similar to that involved in *N*-linked glycosylation.

There are several well-established cases of *O*-linked glycosylation of viral glycoproteins. The spike protein (GP) of Marburg virus has been shown to contain *O*-linked carbohydrate (6), and two distinct *O*-linked oligosaccharides have been isolated from the E1 glycoprotein of mouse hepatitis virus (26). Pinter and Honnen have provided evidence for the *O*-linked glycosylation of several retroviral envelope glycoproteins, including Friend SFFV, FrMCF, R-MuLV, and feline leukemia virus (31, 32), and the *O*-linked glycans of Friend MuLV were localized by Geyer et al. (10). However, there has been uncertainty about the presence of *O*-linked oligosaccharides on the HIV-1 envelope glycoprotein. Indirect evidence supporting the presence of *O*-linked oligosaccharides on the HIV-1 envelope glycoprotein has previously been obtained (11, 23, 36); however, our data provide the first biochemical identification of these *O*-linked glycans. Stein and Engleman observed a difference in the pI between envelope glycoprotein that had been deglycosylated with PNGase F and envelope glycoprotein that had been treated with neuraminidase prior to deglycosylation with PNGase F (36), suggesting that sialated *O*-linked carbohydrates may be present on the HIV-1 envelope glycoprotein. Our observation that treatment with neuraminidase followed by *O*-glycosidase causes a decrease in the apparent molecular weight of gp120 and gp160 correlates with their data and suggests that both the precursor and surface unit forms of the glycoprotein contain *O*-linked glycans. Additional evidence for the presence of *O*-linked carbohydrates has been provided by studies involving anticarbohydrate monoclonal antibodies which recognize structures restricted to *O*-linked carbohydrates. These antibodies were found to immunoprecipitate gp120 and inhibit infection and syncytium formation in CD4⁺ lymphocytic cell lines (12). However, Kozarsky et al. (15) were unable to demonstrate *O*-linked glycosylation of recombinant HIV-1 envelope glycoprotein expressed by CHO *IdD* cells, a cell line which is reversibly defective in *O*-linked glycosylation because of its inability to synthesize UDP-Gal and UDP-GalNAc. Glycosylation is host cell specific and is dependent on the presence of glycosyltransferases, which vary among different cell types (25). In addition, variation in glycosylation may even be seen within a single cell line, as observed by Mizuochi et al., who analyzed the *N*-linked carbohydrate structures present on the envelope glycoprotein of HIV-IIIB isolated from H9 cells (24).

When cells are labelled with [³H]glucosamine, there is

metabolism of the radiolabelled carbohydrate which is restricted to the amino sugars GlcNAc, GalNAc, and NeuNAc (3). Radiolabel is therefore incorporated into *O*-linked carbohydrates via the core GalNAc residue as well as any GalNAc, GlcNAc, or NeuNAc that is added to the core. Using [³H]glucosamine labelling of glycoproteins and then enzymatic deglycosylation, we have demonstrated the release of radiolabel by *O*-glycosidase treatment of the HIV-1 envelope glycoprotein. This result was seen for envelope glycoprotein obtained from BHK-21 cells infected with vaccinia virus expressing the HIV-1 envelope glycoprotein and by persistently infected H9/IIIB cells. Ascending paper chromatography analysis of the carbohydrate released by *O*-glycosidase yielded results consistent with those expected for *O*-linked glycans.

The function of *O*-linked glycosylation is not well understood. Jentoft has proposed that *O*-linked glycosylation may cause steric interactions between the carbohydrates and the peptide core of glycoproteins, resulting in a stiff extended conformation of the protein in the region of *O*-glycosylation (13). Other results indicate that *O*-linked glycosylation may play a role in the stability of cell surface glycoproteins, including Epstein-Barr virus glycoprotein, and prevent their proteolysis (16). Studies which have analyzed envelope glycoproteins from two phenotypically different strains of SFFV suggest another role for *O*-linked glycosylation. SFFV_p envelope glycoprotein contains *O*-linked carbohydrate, and the virus causes polycythemia, while SFFV_A envelope glycoprotein is not *O*-glycosylated, and the virus causes anemia. It has been suggested that this difference in glycosylation may be related to the difference in leukemogenicity seen between these two viruses (31, 32). A recent report by Overbaugh and Rudensky demonstrates that simian immunodeficiency virus variants isolated late in the progression to simian AIDS contain envelope sequences rich in serine and threonine, and they suggest that these changes may be accompanied by increased glycosylation, including potential sites for the addition of *O*-linked glycans (27). Such addition of oligosaccharides may play a role in escape of viruses from immune surveillance mechanisms by masking antigenic epitopes on the glycoprotein. Glycosylation has also been reported to play a functional role in the attachment of viruses to the cell surface (30), an observation which is consistent with the notion that certain envelope glycans may serve as targets for viral neutralization (12). Therefore, several possibilities exist for the function of *O*-linked carbohydrates on the HIV-1 envelope glycoprotein, and it is anticipated that further research will define a role for such structures.

ACKNOWLEDGMENTS

We thank Mark J. Mulligan and the AIDS Center Clinical Core Repository for pooled HIV-positive antisera. We are indebted to John Wakefield and Sajal Ghosh for kindly providing HXB2-infected Sup-T1 cells and SG3-infected PBMCs and CEMx174 cells. We thank Patrick B. Johnston for helpful scientific discussions.

HIV culture was carried out in the UAB Center for AIDS Research Central Virus Culture Core Facility under program grant P30-AI-27767 from the National Institutes of Health. This work was supported by grants AI-33319, AI-28147, AI-34242, and AI-27290 from the National Institutes of Health.

REFERENCES

1. Allan, J. S., J. E. Coligan, F. Barin, M. F. McLane, J. G. Sodroski, C. A. Rosen, W. A. Haseltine, T. H. Lee, and M. Essex. 1985. Major glycoprotein antigens that induce antibodies in AIDS patients are encoded by HTLV-III. *Science* **228**:1091-1094.
2. Bernstein, H. B., and R. W. Compans. 1992. Sulfation of the human immunodeficiency virus envelope glycoprotein. *J. Virol.* **66**:6953-6959.

3. Cummings, R. D., R. K. Merkle, and N. L. Stults. 1989. Separation and analysis of glycoprotein oligosaccharides. *Methods Cell Biol.* **32**:141–183.
4. 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.
5. 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.
6. Feldmann, H., C. Will, M. Schikore, W. Slenczka, and H. Klenk. 1991. Glycosylation and oligomerization of the spike protein of marburg virus. *Virology* **182**:353–356.
7. Gallaher, W. R. 1987. Detection of a fusion peptide sequence in the transmembrane protein of the human immunodeficiency virus. *Cell* **50**:327–328.
8. Gartner, S., and M. Popovic. 1990. Virus isolation and purification, p. 53–69. In A. Aldovini and B. D. Walker (ed.), *Techniques in HIV research*. Stockton Press, New York.
9. Geyer, H., C. Holschbach, G. Hunsmann, and J. Schneider. 1988. Carbohydrates of human immunodeficiency virus. *J. Biol. Chem.* **263**:11760–11767.
10. Geyer, R., J. Dabrowski, U. Dabrowski, D. Linder, M. Schluter, H. H. Schott, and S. Stirm. 1990. Oligosaccharides at individual glycosylation sites in glycoprotein 71 of Friend murine leukemia virus. *Eur. J. Biochem.* **187**:95–110.
- 10a. Ghosh, S. K., P. N. Fultz, E. Keddie, M. S. Saag, P. M. Sharp, B. H. Hahn, and G. M. Shaw. 1993. A molecular clone of HIV-1 tropic and cytopathic for human and chimpanzee lymphocytes. *Virology* **194**:858–864.
11. Hammar, L., S. Eriksson, and B. Morein. 1989. Human immunodeficiency virus glycoproteins: lectin binding properties. *AIDS Res. Hum. Retroviruses* **5**:495–506.
12. Hansen, J.-E. S., H. Clausen, C. Nielsen, L. S. Teglbjaerg, L. L. Hansen, C. M. Nielsen, E. Dabelsteen, L. Mathiesen, S.-I. Hakomori, and J. O. Nielsen. 1990. Inhibition of human immunodeficiency virus (HIV) infection in vitro by anticarbohydrate monoclonal antibodies: peripheral glycosylation of HIV envelope glycoprotein gp120 may be a target for virus neutralization. *J. Virol.* **64**:2833–2840.
13. Jentoft, N. 1990. Why are proteins O-glycosylated? *Trends Biochem. Sci.* **15**:291–294.
14. Kilpatrick, D. R., R. V. Srinivas, E. G. Stephens, and R. W. Compans. 1987. Effects of deletion of the cytoplasmic domain upon surface expression and membrane stability of a viral envelope glycoprotein. *J. Biol. Chem.* **262**:16116–16121.
15. Kozarsky, K., M. Penman, L. Basiripour, W. Haseltine, J. Sodroski, and M. Krieger. 1989. Glycosylation and processing of the human immunodeficiency virus type 1 envelope protein. *J. Acquired Immune Defic. Syndr.* **2**:163–169.
16. Krieger, M., P. Reddy, K. Kozarsky, D. Kingsley, L. Hobbie, and M. Penman. 1989. Analysis of the synthesis, intracellular sorting, and function of glycoproteins using a mammalian cell mutant with reversible glycosylation defects. *Methods Cell Biol.* **32**:57–84.
17. Lasky, L. A., J. E. Groopman, C. W. Fennie, P. M. Benz, D. J. Capon, D. J. Dowbenko, G. R. Nakamura, W. M. Nunes, M. E. Renz, and P. W. Berman. 1986. Neutralization of the AIDS retrovirus by antibodies to a recombinant envelope glycoprotein. *Science* **233**:209–212.
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. Leonard, C. K., M. W. Spellman, L. Riddle, R. J. Harris, J. N. Thomas, and T. J. Gregory. 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. *J. Biol. Chem.* **265**:10373–10382.
20. Matthews, T. M., K. Winhold, H. Lyerly, A. Langlois, H. Wigzell, and D. Bolognesi. 1987. Interaction between the human T-cell lymphotropic virus type IIIB envelope glycoprotein gp120 and the surface antigen CD4: role of carbohydrate in binding and cell fusion. *Proc. Natl. Acad. Sci. USA* **84**:5424–5428.
21. McDougal, J., J. Nicholson, G. Cross, S. Cort, M. Kennedy, and A. Mawle. 1986. Binding of the human retrovirus HTLV/LAV/ARV/HIV to the CD4 (T4) molecule: conformation dependence, epitope mapping, antibody inhibition and potential for idiotype mimicry. *J. Immunol.* **137**:2937–2944.
22. McDougal, J. S., M. S. Kennedy, J. M. Sligh, S. P. Cort, A. Mawle, and J. K. A. Nicholson. 1986. Binding of HTLV-III/LAV to T4+ cells by a complex of the 110K viral protein and the T4 molecule. *Science* **231**:382–385.
23. Merkle, R. K., D. E. Helland, J. L. Welles, A. Shilatfard, W. A. Haseltine, and R. D. Cummings. 1991. gp160 of HIV-1 synthesized by persistently infected molt-3 cells is terminally glycosylated: evidence that cleavage of gp160 occurs subsequent to oligosaccharide processing. *Arch. Biochem. Biophys.* **290**:248–257.
24. Mizuuchi, T., T. J. Matthews, M. Kato, J. Hamako, K. Titani, J. Solomon, and T. Feizi. 1990. Diversity of oligosaccharide structures on the envelope glycoprotein gp120 of human immunodeficiency virus 1 from the lymphoblastoid cell line H9. *J. Biol. Chem.* **265**:8519–8524.
25. Moore, B. R., and S. J. Free. 1985. Protein modification and its biological role. *Eur. J. Biochem.* **17**:283–289.
26. Niemann, H., R. Geyer, H. D. Klenk, D. Linder, S. Stirm, and M. Wirth. 1984. The carbohydrates of mouse hepatitis virus (MHV) A59: structures of the O-glycosidically linked oligosaccharides of glycoprotein E1. *EMBO J.* **3**:665–670.
27. Overbaugh, J., and L. M. Rudensky. 1992. Alterations in potential sites for glycosylation predominate during evolution of the simian immunodeficiency virus in macaques. *J. Virol.* **66**:5937–5948.
28. Owens, R. J., and R. W. Compans. 1989. Expression of the human immunodeficiency virus envelope glycoprotein is restricted to the basolateral surfaces of polarized epithelial cells. *J. Virol.* **179**:827–833.
29. 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.
30. Paulson, J. C. 1989. Glycoproteins: what are the sugar chains for? *Trends Biochem. Sci.* **14**:272–275.
31. Pinter, A., and W. J. Honnen. 1988. O-linked glycosylation of retroviral envelope gene products. *J. Virol.* **62**:1016–1021.
32. Pinter, A., and W. J. Honnen. 1989. Biochemical characterization of cell-associated and extracellular products of the Friend spleen focus-forming virus *env* gene. *Virology* **173**:136–143.
33. Putney, S. D., T. J. Matthews, W. G. Robey, D. L. Lynn, M. Robert-Guroff, W. T. Mueller, A. J. Langlois, J. Ghayeb, J. S. R. Petteway, K. J. Weinhold, P. J. Fischinger, F. Wong-Staal, R. C. Gallo, and D. P. Bolognesi. 1986. HTLV-III/LAV-neutralizing antibodies to an *E. coli*-produced fragment of the virus envelope. *Science* **234**:1392–1395.
34. Robey, W. G., B. Safai, S. Oroszlan, L. O. Arthur, M. A. Gonda, R. C. Gallo, and P. J. Fischinger. 1985. Characterization of envelope and core structural gene products of HTLV-III with sera from AIDS patients. *Science* **228**:593–595.
35. Schneider, J., O. Kaaden, T. D. Copeland, S. Oroszlan, and G. Hunsmann. 1986. Shedding and interspecies type sero-reactivity of the envelope glycopeptide gp120 of the human immunodeficiency virus. *J. Gen. Virol.* **67**:2533–2538.
36. Stein, B. S., and E. G. Engleman. 1990. Intracellular processing of the gp160 HIV-1 envelope precursor. *J. Biol. Chem.* **265**:2640–2649.
37. Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Benach, and E. G. Engleman. 1987. pH-independent HIV-1 entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. *Cell* **49**:659–668.
38. Veronese, F. D., A. L. DeVico, T. D. Copeland, S. Oroszlan, R. C. Gallo, and M. G. Sarngadharan. 1985. Characterization of gp41 as the transmembrane protein coded by the HTLV-III/LAV envelope gene. *Science* **229**:1402–1405.
39. Willey, R. L., J. S. Bonifacio, 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.