Glycopeptides of Murine Leukemia Viruses

I. Comparison of Two Ecotropic Viruses

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The glycopeptides obtained by pronase digestion of two ecotropic strains of murine leukemia virus (MuLV) were compared by gel filtration. Four different glycopeptide size classes, designated G₁, G₂, G₃, and G₄, with molecular weights of approximately 5,100, 2,900, 2,200, and 1,500, respectively, were shown to be associated with Rauscher MuLV virions grown in JLS-V9 cells. Various sugar precursors, including glucosamine, galactose, fucose, and mannose were incorporated into G₁ and G₂, suggesting that these are complex (type I) glycopeptides. The two smaller glycopeptide size classes, G₃ and G₄, were shown to be mannose-rich (type II) glycopeptides. G₄ was more sensitive to digestion with endo- β -N-acetylglucosaminidase H than G₃, suggesting that the core of G₃ may contain fewer mannose residues. Glycopeptides of the same size class as G₁ and G₂ were associated with both Rauscher MuLV and AKR-MuLV grown in III6A (mouse embryo) cells. Previous studies have shown that gp52, a proteolytic cleavage product of gp70, possessed primarily G₁ glycopeptides and that gp52 was more highly sulfated than gp70. We observed that G₁ is approximately twofold more highly sulfated than G₂, explaining the observed difference in sulfation of gp52. The unusually large size of G₁ suggested that infection with MuLV may alter the host cell glycosylation pattern. To test this possibility, glycopeptides from Sindbis virions grown in uninfected and Rauscher MuLV-infected JLS-V9 cells were compared, and no differences were observed. G₁ was not detected in Sindbis virions, indicating that acquisition of G₁ depends on properties of the virus-coded polypeptide backbone of the gp70 molecule.

Previous studies have shown that glycoproteins of enveloped viruses grown in cells of various species origin acquire glycosyl side chains of the same approximate size class, although some host cell-dependent differences have been observed (1, 2, 4, 14, 19, 21-23a, 30). Evidence has been obtained that asparagine-linked oligosaccharides are synthesized by the transfer of a preformed oligosaccharide core from a lipid-linked intermediate to the polypeptide backbone; subsequently, a portion of the protein-bound oligosaccharides may undergo processing and further elongation by stepwise addition of monosaccharides (13, 27, 32, 33). Whether a complex (type I) or mannose-rich (type II) glycopeptide is linked to a particular glycosylation site may be a function of the amino acid sequence or the conformation surrounding an asparagine residue or both (2, 9), suggesting that although glycosylation is a function of cellular enzymes, the glycosylation pattern may be determined by the viral genome.

The glycoproteins of murine leukemia viruses (MuLV's) are located on the external surface of

the viral envelope. The larger of these glycoproteins, which has a molecular weight of approximately 70,000, is designated gp70 and is involved in a number of functions, including viral attachment and definition of viral host range. gp70 may be cleaved to yield minor glycoproteins which remain virion associated; the largest and most abundant of these has a molecular weight of 45,000 to 52,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and has been designated gp45 or gp52 in various laboratories (16, 17, 20, 26). Some differences in the immunological properties and amino acid composition between gp70 and gp45 have been reported, and the major difference reported (20) was an approximately fourfold higher carbohydrate content in gp70. We have previously reported that gp70 possessed two glycopeptide size classes, designated G₁ and G₂, with molecular weights of approximately 5,100 and 2,900, respectively, whereas gp52 possessed only the G₁ glycopeptide size class (16). In the present study we have further analyzed the carbohydrate components of two ecotropic

MuLV's. The results indicate that these viruses possess complex glycopeptides (containing glucosamine, fucose, mannose, and galactose), as well as mannose-rich glycopeptides lacking galactose or fucose. Glycosylation of the viral glycoproteins appears to be controlled by features of the viral polypeptide sequence as well as the host cell of origin.

MATERIALS AND METHODS

Virus and cells. Uninfected JLS-V9 cells and Rauscher MuLV (R-MuLV)-infected JLS-V9 cells were obtained from Pfizer Inc., Maywood, N.J. Both cell lines were cultured in RPMI 1640 supplemented with 10% fetal calf serum, and dexamethasone (4 to 6 μ g/ml; Sigma Chemical Co., St. Louis, Mo.) was added to the medium of R-MuLV-infected JLS-V9 cells to stimulate virus production (36). III6A cells chronically infected with R-MuLV (CL-1) or AKR-MuLV (L2CL-G12) were kindly provided by P. V. O'Donnell, Memorial Sloan-Kettering Cancer Center, and cultured in reinforced Eagle medium supplemented with 10% fetal calf serum. Dexamethasone was added to the culture medium of infected III6A cells before labeling, as described for R-MuLV-infected JLS-V9 cells. Sindbis virus stocks were grown and assayed in chicken embryo fibroblast (CEF) cells (26). Briefly, primary CEF cells were prepared from 10-day-old embryos and cultured in reinforced Eagle medium supplemented with 10% fetal calf serum. Secondary monolayers of CEF cells were inoculated at a multiplicity of infection of 1 PFU/cell. After a 2-h adsorption period, reinforced Eagle medium was added, and the infection was allowed to proceed for 20 h. Sindbis virus was grown in JLS-V9 or R-MuLV-infected JLS-V9 cells in the same manner. Stocks of the A/WSN(H₂N₁) strain of influenza virus were grown in MDBK cells (3) and assayed by plaqueing on MDCK cells (35).

Radioactive labeling and purification of viruses. MuLV virions were radiolabeled in reinforced Eagle medium supplemented with fetal calf serum and dexamethasone; the amounts of the labeled precursors used were 10 to 15 μ Ci of [³H]glucosamine, [³H]fucose, [³H]galactose, or [³H]mannose per ml or 50 to 100 μ Ci of ³⁵SO₄⁻² per ml. Radiolabeled virions were harvested at 24- to 36-h intervals; first and second viral harvests were pooled, and virus was purified as described previously (16).

Sindbis virus grown in uninfected or R-MuLV-infected JLS-V9 cells was radiolabeled with 5 μ Ci of [³H]glucosamine per ml. Sindbis and influenza marker viruses, grown in CEF and MDBK cells, respectively, were labeled with 0.5 μ Ci of [¹⁴C]glucosamine per ml. Medium containing Sindbis or influenza virus was clarified by centrifugation at 5,000 $\times$ g, and the virus was concentrated by centrifugation at 30,000 rpm for 1 h in a Beckman T35 rotor. Virions were purified by equilibrium zonal centrifugation on a 5 to 40% (wt/wt) potassium tartrate gradient at 30,000 rpm for 1 h at 4°C in an SW50.1 rotor. After centrifugation the visible band of virus was collected, diluted, and pelleted at 30,000 rpm for 1 h in an SW50.1 rotor.

Polyacrylamide gel electrophoresis. Samples

were prepared and analyzed on high-pH discontinuous sodium dodecyl sulfate-polyacrylamide slab gels as described previously (15) (modified from Laemmli [18]). Glycoproteins were eluted from the slab gel as described previously (16).

Pronase digestion. Radioactively labeled samples were exhaustively digested by the procedure of Sefton and Keegstra (32). After concentration by ethanol precipitation, the glycopeptides were treated with *Vibrio cholerae* neuraminidase (Calbiochem-Behring Diagnostic, Somerville, N.J.) as described by Moyer et al. (22), unless otherwise indicated.

Analysis of viral glycopeptides. Samples were dissolved in 0.1 M Tris-hydrochloride, pH 8.0, applied to a column (1.0 by 115 cm) of Bio-Gel P-10 or P-6 (200 to 400 mesh; Bio-Rad Laboratories, Richmond, Calif.) that was equilibrated with the same buffer, and eluted in 0.7-ml fractions with the same buffer. For radioactivity determinations, 0.3 ml of each fraction was mixed with 4 ml of Handifluor (Mallinckrodt Inc., St. Louis, Mo.) for counting.

endo H treatment of viral glycopeptides. After pronase digestion the viral glycopeptides were desalted on a Bio-Gel P-2 column and lyophilized. They were dissolved in 0.1 M sodium citrate buffer, and 0.01 U of endo- β -N-acetylglucosaminidase H (endo H; Miles Research Products, Elkhart, Ind.) was added. The mixture was digested for 24 h at 37°C and analyzed by chromatography on a Bio-Gel P-6 column. Previous studies of influenza viral glycopeptides have shown that only the mannose-rich glycopeptides are sensitive to this enzyme (23a).

Isotopes. D-[6-³H]glucosamine (6.3 Ci/mmol), D-[1-¹⁴C]glucosamine (58.2 mCi/mmol), D-[1-³H]galactose (2.8 Ci/mmol), and carrier-free ³⁵SO₄⁻² were obtained from ICN Pharmaceuticals, Irvine, Calif. D-[2-³H]mannose (2 Ci/mmol) and L-[6-³H]fucose (24 Ci/mmol) were obtained from Amersham/Searle, Arlington Heights, Ill.

RESULTS

Characterization of glycopeptides of R-MuLV. The elution profile of [³H]glucosamine-labeled glycopeptides obtained from virions grown in JLS-V9 cells was very heterogeneous (data not shown). As observed previously with glycopeptides of other viruses (7, 14, 19), treatment with neuraminidase eliminated the heterogeneity (Fig. 1A), and most of the resulting glycopeptides were found in a large peak (designated G₂) that is similar in size to the type I glycopeptides of influenza virus (16). A large glycopeptide (designated G₁) was also observed, eluting 10 to 14 fractions behind the void volume. The residual ³H label in the void volume contains host-derived glycosaminoglycans (M. C. Kemp, B. Caterson, and R. W. Compans, manuscript in preparation). To further characterize the oligosaccharides of MuLV glycoproteins, virions were radioactively labeled with [³H]galactose or [³H]fucose. The elution profiles indicate that both the G₁ and G₂ size classes

FIG. 1. Analysis of the glycopeptides of R-MuLV grown in JLS-V9 cells by gel filtration on Bio-Gel P-10. (A) [^3H]glucosamine-labeled glycopeptides. (B) [^3H]galactose-labeled glycopeptides. (C) [^3H]fucose-labeled glycopeptides. (D) Glycopeptides of R-MuLV virions doubly labeled with [^3H]glucosamine and $^{35}\text{S}_4^{-2}$. The arrows labeled G_1 and G_2 included here and in subsequent figures indicate the elution positions of R-MuLV glycopeptides grown in JLS-V9 cells, which have molecular weights of 5,100 and 2,900, respectively. —, ^3H disintegrations per minute; ---, ^{35}S disintegrations per minute.

contain galactose (Fig. 1B) as well as fucose (Fig. 1C), suggesting that they are complex glycopeptides. A comparison of Fig. 1B and C suggests that G_1 is more efficiently labeled with galactose than fucose. The [^3H]galactose-labeled glycopeptides corresponding to G_1 and G_2 were pooled and rechromatographed on a Bio-Gel P-6 column, but no additional glycopeptides could be resolved (data not shown).

Glycoproteins of R-MuLV have been reported to be sulfated (26). Moreover, the minor MuLV glycoprotein (gp52), which was shown to be a cleavage product of gp70, possesses primarily G_1 glycopeptides (16), and it is two- to threefold more highly sulfated than gp70 (26). Based on these observations, we wished to determine whether G_1 is more highly sulfated than the other viral glycopeptides. Virions were doubly labeled with [^3H]glucosamine and $^{35}\text{S}_4^{-2}$, and their glycopeptides were analyzed on Bio-Gel P-10 (Fig. 1D). The ratio of ^{35}S to ^3H was calculated to be 1.17 for G_1 and 0.63 for G_2 . Thus, G_1 is approximately twofold more highly sulfated than G_2 . The slight difference in elution between ^3H - and ^{35}S -labeled glycopeptides may be due to

charge exclusion, resulting from the negative charge imparted by the sulfate groups, and has also been observed with sulfated glycopeptides of influenza A/WSN virions (23).

When R-MuLV glycopeptides labeled with [^3H]mannose were analyzed by chromatography on Bio-Gel P-6 (Fig. 2A), three distinct peaks, designated G_2 , G_3 , and G_4 , were detected. They were estimated to have molecular weights of 2,900, 2,200, and 1,500 respectively, based on the [^{14}C]glucosamine-labeled influenza marker glycopeptides. In addition, a leading shoulder on G_2 was observed, which may correspond to G_1 glycopeptides. Neither G_3 nor G_4 was resolved as a

FIG. 2. Glycopeptides of R-MuLV labeled with mannose. (A) Glycopeptides of R-MuLV virions labeled with [^3H]mannose were analyzed on a Bio-Gel P-6 column. (B) [^3H]mannose-labeled glycopeptides were treated with endo H as described in the text and analyzed on Bio-Gel P-6. [^{14}C]glucosamine-labeled glycopeptides of influenza virions were included as internal markers, and the positions of the type I and II size classes are indicated by arrows.

distinct peak when other sugar precursors were used, indicating that these are mannose-rich glycopeptides. The profile of [^3H]mannose-labeled glycopeptides associated with purified gp70 closely resembled Fig. 2A (data not shown).

Susceptibility of R-MuLV glycopeptides to endo H. From the results described above it seemed likely that R-MuLV gp70 possessed both complex and mannose-rich glycopeptides. To further characterize the structure of the glycopeptides, we examined their sensitivity to endo H, which acts effectively on asparagine-linked oligosaccharides containing a core of more than three mannose residues, but is ineffective when fewer mannose residues are present (34). As Fig. 2B shows, G_4 appeared to be totally susceptible to endo H, and G_3 was at least partially digested. Isolation and further digestion of the residual G_3 peak resulted in further degradation of G_3 , but it was not totally cleaved (data not shown). Based on these results, it may be concluded that R-MuLV contains two complex glycopeptides (G_1 and G_2) and two glycopeptide size classes with large oligomannosyl cores, which appear to lack galactose and fucose (G_3 and G_4). The partial resistance of G_3 to endo H suggests that this peak is heterogeneous and may contain components which differ in the number of mannose-linked sugars.

Glycopeptides of R-MuLV grown in III6A cells. Because of the unusual size of G_1 , we were interested in determining whether this glycopeptide was unique to virions grown in JLS-V9 cells. For comparison, [^3H]glucosamine-labeled R-MuLV was grown in III6A cells, a clone of cells derived from the feral mouse embryo line WM1511 (11, 25), which was persistently infected with R-MuLV. The elution profile of the glycopeptides of III6A-grown R-MuLV analyzed on Bio-Gel P-10 (Fig. 3A) appears similar in most respects to that of the R-MuLV glycopeptides grown in JLS-V9 cells (Fig. 1A). In both cases the G_1 and G_2 glycopeptide size classes are observed. The III6A-grown R-MuLV glycopeptides appear to be slightly more heterogeneous in size than glycopeptides of R-MuLV grown in JLS-V9 cells. Since G_1 is present on R-MuLV virions grown in different cell types, the presence of this glycopeptide type may be a function of the primary or secondary structure of the polypeptide backbone.

Glycopeptide composition of AKR-MuLV. We also wished to examine whether the presence of the large glycopeptide G_1 may be characteristic of other MuLV gp70 molecules. Therefore, we analyzed the glycopeptides of AKR-MuLV, another ecotropic virus which shares group and interspecies determinants with

FIG. 3. Glycopeptides of R-MuLV (A) and AKR-MuLV (B) virions grown in III6A cells. The glycopeptides of virions labeled with [^3H]glucosamine were analyzed on Bio-Gel P-6.

R-MuLV. AKR-MuLV was grown in III6A cells labeled with [^3H]glucosamine, and the glycopeptides obtained by pronase digestion were analyzed on Bio-Gel P-10. The elution profile (Fig. 3B) closely resembles the glycopeptide pattern of R-MuLV virions grown in JLS-V9 (Fig. 1A) or III6A cells (Fig. 3A). Both G_1 and G_2 glycopeptides are detectable, and the major difference among the three profiles is the increased amount of glycosaminoglycans associated with virions grown in III6A cells.

Glycopeptides of Sindbis virus grown in uninfected or R-MuLV-infected JLS-V9 cells. Since ecotropic MuLV's acquire an unusually large glycopeptide when grown in cells of different origins, it seemed possible that MuLV infection may alter the cellular glycosylation process. Alternatively, as suggested above, the primary or secondary structure of the gp70 molecule may specify the attachment of these large glycopeptides. To determine whether R-MuLV infection alters the cellular glycosylation proc-

ess, [^3H]glucosamine-labeled Sindbis virus was grown in uninfected and R-MuLV-infected JLS-V9 cells. The glycopeptides of purified virions were prepared by pronase digestion and cochromatographed on Bio-Gel P-6 with [^{14}C]glucosamine-labeled Sindbis virus glycopeptides grown in CEF cells. No differences in the elution profiles of the Sindbis glycopeptides prepared in uninfected and R-MuLV-infected JLS-V9 cells were detected (Fig. 4). It may be noted that incorporation of glucosamine into S_4 , the mannose-rich type II oligosaccharide associated with Sindbis virus glycoproteins (32), is decreased in either infected or uninfected JLS-V9 cells relative to that in CEF cells. Glycopeptides of the same size class as G_1 were not observed, indicating that Sindbis viral glycoproteins do not acquire such glycopeptides in R-MuLV-infected cells (Fig. 4B).

DISCUSSION

The present results indicate that at least four different glycopeptide size classes are associated with glycoproteins of R-MuLV grown in JLS-V9 cells. The two largest size classes (G_1 and G_2) are complex glycopeptides, whereas the two smaller ones (G_3 and G_4), are mannose-rich glycopeptides. These designations are supported by the selective labeling of G_1 and G_2 with galactose or fucose, whereas G_3 and G_4 lack these sugars and

are highly labeled with [^3H]mannose. Furthermore, the sensitivity of G_3 and G_4 to cleavage by endo H supports the conclusion that these are high-mannose glycopeptides. The available information suggests that both complex and mannose-rich oligosaccharides are linked to polypeptide chains by an *N*-glycosidic bond between asparagine and *N*-acetylglucosamine. Initial glycosylation appears to involve an en bloc transfer of a mannose-rich oligosaccharide via a dolichol intermediate to an asparagine residue. This structure is then trimmed and becomes the precursor of a type I or II oligosaccharide (13, 24, 29, 31, 33). Whether G_1 is synthesized by this mechanism is uncertain, because mannose was incorporated poorly into G_1 , whereas glucosamine and galactose are readily incorporated. These results, as well as the unusually large size of G_1 , suggest that it may differ in structure from the well-characterized glycopeptides of vesicular stomatitis virus (6, 7, 13, 18, 28), influenza virus (23, 23a, 30), and Sindbis virus (1, 2, 14, 24, 27, 28, 32, 33). Further studies of the structure and synthetic pathway of G_1 are therefore of interest. Previous studies showed that the minor glycoprotein gp52, which is a cleavage product of gp70, was two- to threefold more highly sulfated than gp70 (16, 26), and the present results reveal that G_1 is highly sulfated compared with G_2 . Since G_1 was the only glycopeptide detected in gp52 (16), this result seems to explain the increased level of sulfation. The present results, as well as previous information on influenza virus (23), suggest that only complex (type I) glycopeptides are sulfated.

Our results suggest that G_1 may be a distinct oligosaccharide structure found in association with the glycoproteins of ecotropic MuLV's. A similar glycopeptide was found in R-MuLV grown in JLSV-9 or III6A cells, as well as in AKR-MuLV grown in III6A cells. It seems unlikely that G_1 represents two oligosaccharides which are situated on adjacent parts of the gp70 polypeptide backbone, although the possibility cannot be excluded. Recent studies in our laboratory have shown that a glycopeptide of similar size is associated with influenza C virions grown in chick kidney cells, whereas influenza A viruses grown in these cells lack such glycopeptides (24). This observation, as well as the association of G_1 with R-MuLV grown in two different cells and with AKR-MuLV, suggests that addition of such oligosaccharides to the gp70 polypeptide backbone appears to be controlled by specific amino acid sequences. Sindbis virus apparently lacks such sequences since it does not contain glycopeptides of the G_1 size class when grown in R-MuLV-infected cells. Moreover, G_1 is primarily

FIG. 4. Comparison of the glycopeptides of Sindbis virions grown in uninfected and R-MuLV-infected JLS-V9 cells with the glycopeptides of Sindbis virions grown in CEF cells. Sindbis virions grown in JLS-V9 cells were labeled with [^3H]glucosamine, and virions grown in CEF cells were labeled with [^{14}C]glucosamine. ^3H - and ^{14}C -labeled samples were mixed, digested with pronase, and analyzed on Bio-Gel P-6. (A) Elution profiles of the glycopeptides of Sindbis virions grown in uninfected JLS-V9 and CEF cells. (B) Elution profiles of the glycopeptides of Sindbis virions grown in R-MuLV-infected JLS-V9 and CEF cells. —, ^3H disintegrations per minute; ---, ^{14}C disintegrations per minute.

associated with the portion of gp70 which is retained after proteolytic cleavage to yield the minor glycoprotein gp52 (16). Hence, the appropriate amino acid sequence must reside in this region, and the limited studies to date suggest that the sequence may be conserved among different MuLV's.

Large glycopeptides have also been reported to be associated with avian RNA tumor virus glycoproteins (10, 19). In particular, the glycopeptides associated with avian sarcoma virus ranged in molecular weight from approximately 2,500 to 5,000 (10). However, these glycopeptides had not been treated with neuraminidase before analysis, and previous studies had indicated a significant reduction in size of glycopeptides by neuraminidase treatment (19). Lai and Duesberg (19) suggested that transformation of chick cells by avian RNA tumor viruses may lead to increased apparent size of the viral glycopeptides because of higher levels of sialyl transferase activity in such cells. Similarly, Forchhammer and Turnock (9) suggested that gp70 from Moloney murine sarcoma virus (MuLV)-transformed cultures exhibits a higher isoelectric point and increased molecular weight because of increased sialyl transferase activity. To determine whether infection by R-MuLV alters the cellular glycosylation pattern, we compared the oligosaccharides of Sindbis virus grown in uninfected and R-MuLV-infected JLS-V9 cells, but no detectable differences in the glycopeptide profiles were noted. If the sialyl transferase activity in the infected cell had been increased, an increased proportion of the [³H]glucosamine label would have been expected in the S₁ and S₂ glycopeptides of Sindbis virus, as had been observed when Sindbis virus was grown in Rous sarcoma virus-transformed chicken cells (14).

Further studies of the oligosaccharides of other murine leukemia viruses may reveal whether viruses exhibiting different host cell specificities show recognizable differences in their glycosylation patterns. In addition, certain glycopeptides, such as G₁, may prove to be useful markers for analysis of possible recombinants between MuLV envelope proteins, such as have been reported to occur (4, 5, 8, 12, 29), giving rise to viruses with dual host cell tropisms.

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