The Synthesis of Complex-type Oligosaccharides

II. CHARACTERIZATION OF THE PROCESSING INTERMEDIATES IN THE SYNTHESIS OF THE COMPLEX OLIGOSACCHARIDE UNITS OF THE VESICULAR STOMATITIS VIRUS G PROTEIN

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The synthesis of the complex-type oligosaccharide units of the vesicular stomatitis virus G protein is initiated by the en bloc transfer of a high molecular weight oligosaccharide from a lipid carrier to the nascent polypeptide. Following transfer, the oligosaccharide precursor, a branched molecule containing 3 glucose, 9 mannose, and 2 N-acetylglucosamine residues (Li, E., Tabas, I., and Kornfeld, S. (1978) J. Biol. Chem. 253, 7762-7770) is processed to give rise to the mature complex-type oligosaccharide. To elucidate the sequence of processing, pulse-chase experiments were performed with virus-infected Chinese hamster ovary cells using labeled mannose, glucosamine, and galactose. The processing intermediates were isolated and characterized. At 20 to 30 min after labeling, the major processing intermediates are (Glc)(Man)(GlcNAc)2 and (Man)(GlcNAc)2. Small amounts of (Man)(GlcNAc)2 and (Man)(GlcNAc)2 were detected along with trace amounts of (Glc)(Man)(GlcNAc)2 and (GlcNAc)2. In addition, by 20 to 30 min completely processed oligosaccharides containing only 3 mannose residues were also present. These data demonstrate that processing is initiated by the rapid removal of 2 of the 3 glucose residues of the precursor oligosaccharide. At 20 to 30 min the last glucose residue is removed and processing proceeds rapidly with the ultimate removal of 6 of the 9 mannose residues and the addition of the outer branch sugars. Clone 15B cells, a line deficient in UDP-GlcNAc: glycoprotein N-acetylglucosaminyltransferase I, accumulate an intermediate with the structure Manα1→3 (Manα1→6) Manα1→6 (Manα1→3) Manβ1→4 GlcNAc β1→4 GlcNAc. This indicates that normal processing requires the transfer of a N-acetylglucosamine residue to the mannose residue linked α1→3 to the β-linked core mannose. Following transfer of the N-acetylglucosamine residue, an α-mannosidase removes the 2 mannose residues linked to the mannose which is linked α1→6 to the core mannose. Direct evidence for this reaction is presented in the following paper (Tabas, I., and Kornfeld, S. (1978) J. Biol. Chem. 253, 7779-7786). Complex oligosaccharide biosynthesis is then completed by the addition of the final outer N-acetylglucosamine residues and the galactosialic acid and fucose residues.

Several laboratories, including ours, have recently demonstrated that the biosynthesis of the two complex oligosaccharide units of the vesicular stomatitis virus G glycoprotein is initiated by the en bloc transfer of a high molecular weight oligosaccharide from a lipid carrier to the nascent polypeptide (1-3). In the previous paper we presented data which established that the structure of the lipid-linked oligosaccharide is as shown in Scheme 1 (4) where M = mannose and G = glucose. After transfer to the G protein, this oligosaccharide is processed to give rise to the completed complex oligosaccharide units which have the structure shown in Scheme 2 (5). We now report the characterization of the major processing intermediates and propose a scheme for the pathway of complex oligosaccharide biosynthesis.

EXPERIMENTAL PROCEDURES

Materials—D-[1-14C]Glucosamine (45 mCi/mmol) and D-[1-14C]Galactose (30 mCi/mmol) were purchased from New England Nuclear Corp. α-[2,3-3H]Mannose (2 Ci/mmol) was from Amersham. α-Mannosidase, hen oviduct phosphomannose isomerase, α-mannosidase, and α-mannosidase were from Grand Island Biological Co. The 3α70 scintillation mixture was from Research Products International Corp., Elk Grove Village, Ill. (Man,GlcNAc, (Man,GlcNAc, (Man,GlcNAc, and (Man,GlcNAc were prepared from glycopeptides isolated from clone 15B cells, a ricin-resistant Chinese hamster ovary cell line. The proposed structures of these compounds are given in the preceding paper (4). Structural analysis of these oligosaccharide alcohols will be described elsewhere. α-Mannosidase and α-mannosidase were prepared from glycopeptides isolated from the lipid-linked oligosaccharide (4). Bio-Gel P-2 (200 to 400 mesh) was from Bio-Rad. All other chemical reagents were of reagent grade and obtained from commercial sources.

Enzymes—Pronase was obtained from Calbiochem. Jack bean α-mannosidase was prepared by the method of Li and Li (6). Clostridium perfringens endo-β-N-acetylglucosaminidase C1 and C4 were purified as described by Ito et al. (6). Hen ovviduct β-mannosidase was prepared by the method of Saito et al. (6).

Cells—Monolayer cultures of Chinese hamster ovary cells were grown in a minimal essential medium supplemented with 10% fetal calf serum, 50 units of penicillin/ml, and 50 μg of streptomycin/ml. The lectin-resistant cells were isolated and characterized as previously described (9, 10). Clone 13, a wheat germ agglutinin-resistant Chinese hamster ovary cell line, is deficient in membrane sialic acid and galactose. Its complex-type oligosaccharides contain N-acetylglucosamine residues at their nonreducing ends. Clone 15B, a ricin-resistant line, lacks UDP-GlcNAc:glycoprotein N-acetylglucosaminyltransferase activity which results in the synthesis of membrane oligosaccharides which are deficient in sialic acid, galactose, and N-acetylglucosamine (10, 11).

The abbreviations used are: GlcNAc, N-acetylglucosaminioyl; VSV, vesicular stomatitis virus; SA, sialic acid.

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Radioactive Labeling of the G Protein and Extraction of VSV-infected Cells—The preparation of the VSV, Indiana strain, the infection of the cells, the radioactive labeling of the infected cells, and the extraction of the labeled cells have been previously described (2). The details of the individual experiments are given in the legends to the figures.

Preparation of Glycopeptides—The extracted cell pellets were brought to 1.0 ml with 0.1 M Tris buffer, pH 8.0, containing 2 mM CaCl₂. Pronase (2 mg) was added daily for 3 days and the incubation was carried out at 37°C under a toluene atmosphere. The digestion was terminated by boiling for 5 min and the soluble glycopeptide material was applied to a Bio-Gel P-6 (200 to 400 mesh) column (1.5 x 91 cm) using the following solvent systems: Solvent A, n-butyl alcohol/pyridine/H₂O (4:3:4) for 6 to 21 days; Solvent B, ethyl acetate/pyridine/acetic acid/H₂O (65:5:1:3) for 20 h. Radioactivity was monitored for radioactivity.

Reduction of Oligosaccharides—The oligosaccharides released by the endoglycosidase were reduced with 0.2 M NaBH₄ in 0.1 M NH₄HCO₃, pH 8.0, for 12 h. The reaction was stopped by the addition of 0.02 ml of glacial acetic acid. Borate was removed by repeated evaporation with methanol.

Exoglycosidase Digestion of Oligosaccharide Alcohols—The oligosaccharide alcohols were incubated with 1 unit of jack bean a-mannosidase in 0.02 ml of 0.05 M sodium citrate, pH 4.5, for 16 h at 37°C under a toluene atmosphere. Samples were incubated with 7 milliunits of hen ovoviduct a-mannosidase in 0.04 ml of 0.05 M citrate-phosphate, pH 6.5, for 20 h at 37°C under a toluene atmosphere.

Acetylation and Methylation—Acetylation and methylation analysis of the oligosaccharide alcohols were performed as described in the preceding paper (4).

Paper Chromatography—Labeled oligosaccharide alcohols were separated by descending paper chromatography on Whatman No. 1 using the following solvent systems: Solvent A, n-butyl alcohol/pyridine/H₂O (4:3:4) for 6 to 21 days; Solvent B, ethyl acetate/pyridine/acetic acid/H₂O (65:5:1:3) for 20 h. Radioactivity was monitored as described previously (2).

RESULTS

Characterization of Processing Intermediates Labeled with [³H]Mannose and [¹⁴C]Glucosamine—In our previous experiments, Chinese hamster ovary cells infected with VSV were pulsed with [³H]mannose for 3 to 5 min and then chased for up to 2 h. Glycopeptides were prepared from the cells following various chase intervals and treated with C. perfringens endo-β-N-acetylglucosaminidase Cn. These enzyme cleaves the di-N-acetylchitobiose unit of glycopeptides with the structure (Man)₃(GlcNAc)₂-Asn but will not act on (Man)₃(GlcNAc)₂-Asn or glycopeptides with complex-type oligosaccharides (7). Consequently it can be used to distinguish these two classes of glycopeptides and to follow the course of processing. These experiments demonstrated that the processing of the initial high mannose oligosaccharide to a complex-type oligosaccharide required about 30 min.

Identical results were obtained with clone 13 cells, a variant line lacking sialic acid and galactose. These cells were used in subsequent experiments to facilitate differential incorporation of radioactivity into glucose. In order to further characterize the processing intermediates, VSV-infected clone 13 cells were labeled for 5 min with [³H]mannose and [¹⁴C]glucosamine and chased for 25 min with unlabeled medium. The cells were harvested and glycopeptides prepared using pronase to digest the VSV G glycoprotein. As shown in Fig. 1A, one major glycopeptide fraction was obtained when the material was subjected to gel filtration on Bio-Gel P-6, although the failure of the [³H]mannose and [¹⁴C]glucosamine peaks to coincide indicated that there was, in fact, a mixture of glycopeptides. Treatment of the pooled glycopeptides with endo-β-N-acetylglucosaminidase Cn released a large amount of mannose-rich oligosaccharide material leaving behind the processed complex-type oligosaccharides which are enriched with [¹⁴C]glucosamine (Fig. 1B). Since endo-β-N-acetylglucosaminidase Cn would not cleave molecules with the composition (Man)₃(GlcNAc)₂-Asn the residual glycopeptide material in Fractions 56 to 66 was incubated with endo-β-N-acetylglucosaminidase Cn which acts on this glycopeptide. This treatment failed to release any oligosaccharide with the composition (Man)₃(GlcNAc)₂-Asn, confirming our previous finding that the mannose-rich oligosaccharide processing intermediates were predominantly of high molecular weight. The fully processed glycopeptide from VSV-infected clone 13 cells has been shown to have the composition (GlcNAc)₂-[³H]mannose-[¹⁴C]glucosamine-Asn.

The oligosaccharide material that was released by endo-β-N-acetylglucosaminidase Cn was pooled, reduced with NaBH₄, and subjected to paper chromatography in Solvent A for 6 days (Fig. 2A). The major oligosaccharide fraction (Ib and c) migrated with authentic (Man)₃GlcitolNAc. In addition, another oligosaccharide (II) migrated slightly faster than (Man)₃GlcitolNAc, a small amount of oligosaccharide migrated in the expected position of (Man)₃GlcitolNAc (III) and some oligosaccharide material (Ia) appeared to migrate slightly slower than the (Man)₃GlcitolNAc. A trace amount of oligosaccharide migrated with authentic (Man)₃GlcitolNAc.
Oligosaccharide Processing

The proposed structures of the oligosaccharides Ia, Ib, and_prepare the Bio-Gel P-6 column (B). VO was determined with

Treatment of oligosaccharides II and III with jack bean α-mannosidase produced free mannose and a disaccharide which co-chromatographed with Manβ1 → 4 GlcitolNAc in Solvent B (Fig. 4). The acetylated of oligosaccharide II differed from that obtained with oligosaccharide Ic in that the mannose residue from oligosaccharide Ib to form oligosaccharide Ic and indicated that the glucose to mannose linkage was 1 → 3.

The oligosaccharide alcohols were next subjected to acetolysis which specifically cleaves Manα1 → 6 Man linkages. The acetylated was analyzed by descending paper chromatography in Solvent B (see Fig. 6). The acetolysate of oligosaccharide Ic differs from that of oligosaccharide Ib only in the increased mobility of the largest fragment (designated d) which chromatographs in the position of (Man)4GlcitolNAc. These data taken together with the fact that the oligosaccharides are derived from the lipid-linked oligosaccharide support the proposed structures of the processing intermediates Ia and Ic shown in Fig. 3.

Methylation analysis of oligosaccharide Ib revealed the presence of 2,4-di-, 3,4,6-tri-, 2,4,6-tri-, and 2,3,4,6-tetra-O-methylmannose (see Fig. 5A). Since the oligosaccharides were obtained from a pulse-chase labeling experiment, the mannoses were probably not labeled equivalently and the relative ratios of the partially methylated mannoses could not be determined. Methylation analysis of oligosaccharide Ic revealed the presence of 2,4,6-di-, 3,4,6-tri-, and 2,3,4,6-tetra-O-methylmannose (see Fig. 5B). The disappearance of 2,4,6-tri-O-methylmannose and concomitant increase in 2,3,4,6-tetra-O-methylmannose was consistent with removal of a glucose residue from oligosaccharide Ib to form oligosaccharide Ic and indicated that the glucose to mannose linkage was 1 → 3.

The various oligosaccharides were then subjected to α-mannosidase treatment, methylation, and acetolysis in order to obtain more information about their structures. To aid the reader, the proposed structures of the oligosaccharides Ia, Ib, and Ic, are shown in Fig. 3 along with the expected products of methylation and acetolysis fragmentation. Treatment of oligosaccharide Ib with jack bean α-mannosidase resulted in the formation of free mannose and an oligosaccharide which migrated slightly slower than (Man)4GlcitolNAc in Solvent B (Fig. 4). In a separate experiment (data not shown), the oligosaccharide was shown to co-chromatograph with glucitolNAc prepared from the lipid-linked oligosaccharide (4). When oligosaccharide Ic was treated with α-mannosidase, free mannose, and a disaccharide which co-chromatographed with authentic Manβ1 → 4 GlcitolNAc in Solvent B were obtained (Fig. 4). On treating the disaccharide with hen oviduct β-mannosidase, 25% of the radioactivity was released as free mannose.

Methylation analysis of oligosaccharide Ib revealed the presence of 2,4-di-, 3,4,6-tri-, 2,4,6-tri-, and 2,3,4,6-tetra-O-methylmannose (see Fig. 5A). Since the oligosaccharides were obtained from a pulse-chase labeling experiment, the mannoses were probably not labeled equivalently and the relative ratios of the partially methylated mannoses could not be determined. Methylation analysis of oligosaccharide Ic revealed the presence of 2,4,6-di-, 3,4,6-tri-, and 2,3,4,6-tetra-O-methylmannose (see Fig. 5B). The disappearance of 2,4,6-tri-O-methylmannose and concomitant increase in 2,3,4,6-tetra-O-methylmannose was consistent with removal of a glucose residue from oligosaccharide Ib to form oligosaccharide Ic and indicated that the glucose to mannose linkage was 1 → 3.

The oligosaccharide alcohols were next subjected to acetolysis which specifically cleaves Manα1 → 6 Man linkages. The acetylated was analyzed by descending paper chromatography in Solvent B (see Fig. 6). The acetolysate of oligosaccharide II differed from that obtained with oligosaccharide Ic in that the mannose residue from oligosaccharide Ib to form oligosaccharide Ic and indicated that the glucose to mannose linkage was 1 → 3.

The various oligosaccharides were then subjected to α-mannosidase treatment, methylation, and acetolysis in order to obtain more information about their structures. To aid the reader, the proposed structures of the oligosaccharides Ia, Ib, and Ic, are shown in Fig. 3 along with the expected products of methylation and acetolysis fragmentation. Treatment of oligosaccharide Ib with jack bean α-mannosidase resulted in the formation of free mannose and an oligosaccharide which migrated slightly slower than (Man)4GlcitolNAc in Solvent B (Fig. 4). In a separate experiment (data not shown), the oligosaccharide was shown to co-chromatograph with glucitolNAc prepared from the lipid-linked oligosaccharide (4). When oligosaccharide Ic was treated with α-mannosidase, free mannose, and a disaccharide which co-chromatographed with authentic Manβ1 → 4 GlcitolNAc in Solvent B were obtained (Fig. 4). On treating the disaccharide with hen oviduct β-mannosidase, 25% of the radioactivity was released as free mannose.

Methylation analysis of oligosaccharide Ib revealed the presence of 2,4-di-, 3,4,6-tri-, 2,4,6-tri-, and 2,3,4,6-tetra-O-methylmannose (see Fig. 5A). Since the oligosaccharides were obtained from a pulse-chase labeling experiment, the mannoses were probably not labeled equivalently and the relative ratios of the partially methylated mannoses could not be determined. Methylation analysis of oligosaccharide Ic revealed the presence of 2,4,6-di-, 3,4,6-tri-, and 2,3,4,6-tetra-O-methylmannose (see Fig. 5B). The disappearance of 2,4,6-tri-O-methylmannose and concomitant increase in 2,3,4,6-tetra-O-methylmannose was consistent with removal of a glucose residue from oligosaccharide Ib to form oligosaccharide Ic and indicated that the glucose to mannose linkage was 1 → 3.
Oligosaccharide Processing

Fig. 3. Scheme for characterization of the endo-β-N-acetylglucosaminidase Cb-released oligosaccharides.

Fig. 4. Paper chromatography of α-mannosidase-treated oligosaccharides Ib, Ic, II, and III. The enzyme digests were applied directly to Whatman No. 1 paper and the chromatogram was developed for 20 h in Solvent B. The markers are: I, (Man)6GlcitolNAc; 2, mannose.

Characterization of Processing Intermediates Labeled with [3H]Mannose and [14C]Glucose—To provide more information about the structure of the processing intermediates, VSV-infected clone 13 cells were labeled with [3H]mannose and [14C]galactose for 3 min followed by a 20-min chase. The [14C]galactose was used to label the glucose residues of the oligosaccharides (4). Glycopeptides were then prepared, treated with endo-β-N-acetylglucosaminidase Cb, and the released oligosaccharides were reduced with NaBH₄ and chromatographed on paper in Solvent A for 6 days. As shown in Fig. 8A, while the pattern of the [3H]mannose-labeled oligosaccharides was very similar to that observed in Fig. 2, the major [14C]-labeled oligosaccharide migrated slightly slower than the major [3H]mannose-labeled oligosaccharide. In addition one larger and several smaller [14C]-labeled oligosaccharides were present. The major oligosaccharides (Ia, Ib, Ic) were eluted and rechromatographed in Solvent A for 16 days. This resulted in the separation of three oligosaccharides (Fig. 8B). The smallest oligosaccharide (Ic), which contained only (Man)₆GlcitolNAc isomers, as well as some degree of under- and overdegradation during the acetolysis. These findings suggest that processing of the oligosaccharide proceeds in a relatively specific sequence. The reason why oligosaccharide II migrates faster than the (Man)₆GlcitolNAc standard in Solvent A is not known.
Oligosaccharide Processing

Fig. 5. Thin layer chromatogram of methylated sugars. Oligosaccharides Ib and Ic were methylated as described under "Experimental Procedures." The methylated sugars were separated on Silica Gel G plates in benzene/acetone/water/ammonium hydroxide (50:200:3:1.5). Segments (0.5 cm) of the silica gel were counted. A, oligosaccharide Ib; B, oligosaccharide Ic. The standards are (1), 2,3,4,6-tetra-Me-Man; (2), 2,4,6-tri-Me-Man; (3), 3,4,6-tri-Me-Man; (4), 2,4-di-Me-Man.

[$^3$H]mannose, co-migrated with authentic (Man)$_n$GlcitolNAc while the larger oligosaccharides (Ia and Ib) contained both [$^3$H]mannose and [$^{14}$C]glucose and migrated between the (Glc)$_3$(Man)$_n$GlcitolNAc and (Man)$_n$GlcitolNAc standards. The amount of Ia was quite small relative to Ib and Ic. When compared to the lipid-linked oligosaccharides, oligosaccharide Ib migrated with the (Glc)$_1$(Man)$_n$GlcitolNAc species while oligosaccharide Ic migrated faster (Fig. 9).

Oligosaccharides Ia, Ib, and Ic were each incubated with $\alpha$-mannosidase and Ib was subjected to acetolysis. Ic behaved as expected for a (Man)$_n$GlcitolNAc oligosaccharide, giving rise to free mannose and a disaccharide (Man $\rightarrow$ GlcitolNAc) following $\alpha$-mannosidase digestion (Fig. 10C). Ib was converted to a glucose- and mannose-containing hexasaccharide by $\alpha$-mannosidase with the release of free mannose (Fig. 10B). Acetolysis produced a similar hexasaccharide as well as a mannose-containing triasaccharide and disaccharide (Fig. 10D). Ia was converted to a glucose- and mannose-containing heptasaccharide by $\alpha$-mannosidase with free mannose being released (Fig. 10A). These data are consistent with the structures shown in Fig. 3 for Ia, Ib, and Ic.

[$^3$H]Glucose-containing Oligosaccharides after a 2-h Continuous Label—Glycopeptides were next prepared from VSV-infected clone 13 cells that had been incubated with [$^3$H]-galactose and [$^{14}$C]mannose for 2 h in order to incorporate more label into the glucose residues. When these glycopeptides were treated with endo-$\beta$-N-acetylglucosaminidase C$_1$ and the released oligosaccharides were reduced with NaBH$_4$ and chromatographed on paper in Solvent A for 3 weeks to ensure better resolution of the oligosaccharides, the pattern shown in Fig. 11A was obtained. Once again the major glucose-containing oligosaccharide (Ib) migrated slightly faster than authentic (Man)$_n$GlcitolNAc but in addition the two faster migrating glucose-containing oligosaccharides (Id and IIb) were well separated from the oligosaccharides which contained only labeled mannose (Ic and IIa). When oligosaccharides Ic and IIa were treated with $\alpha$-mannosidase, the resultant products were free mannose and a disaccharide which co-migrated in Solvent B with Man $\rightarrow$ GlcitolNAc, suggesting that these compounds were (Man)$_n$GlcitolNAc and (Man)$_n$GlcitolNAc. Following treatment of Ib, Id, and IIb with $\alpha$-mannosidase, a single oligosaccharide was obtained which migrated slightly faster than (Glc)$_2$(Man)$_n$GlcitolNAc (Fig.
Fig. 8. Paper chromatography of $[^{3}H]$mannose- and $[^{14}C]$glucose-labeled oligosaccharides. VSV-infected clone 13 cells were incubated with 300 $\mu$Ci of $[^{3}H]$mannose and 100 $\mu$Ci of $[^{14}C]$galactose in phosphate-buffered saline for 3 min followed by a 20-min chase in unlabeled complete medium. Glycopeptides were prepared from the cells by pronase digestion and purified by gel filtration on Bio-Gel P-6. The glycopeptide peak was incubated with endo-$\beta$-$N$-acetylglucosaminidase $C_{r}$ and then released oligosaccharides were reduced with NaBH$_4$, deionized, and subjected to descending paper chromatography in Solvent A for 6 days (A). The major oligosaccharide peak was eluted as noted and rechromatographed in Solvent A for 16 days (B). The markers are: 1, (Man)$_n$GlcitolNAc; 2, (Man)$_n$GlcitolNAc; 3, (Man)$_n$GlcitolNAc.

Fig. 9. Paper chromatography of oligosaccharides Ib and Ic and the lipid-linked oligosaccharides. The lipid-linked oligosaccharides were labeled with $[^{3}H]$mannose, isolated, subjected to mild acid hydrolysis endo-$\beta$-$N$-acetylglucosaminidase $C_{r}$ digestion, and reduced as described in Ref. 4. Three species were obtained corresponding to (Glc)$_n$(Man)$_n$GlcitolNAc, (Glc)$_n$(Man)$_n$GlcitolNAc, and (Glc)$_n$(Man)$_n$GlcitolNAc (A). The chromatogram was developed in Solvent A for 16 days.

11B). When these hexasaccharides were subjected to methylation analysis, the only methylated species detected were tetramethylglucose and trimethylmannose, indicating that the hexasaccharides had the sequence Glc $\rightarrow$ Man $\rightarrow$ Man $\rightarrow$ Glc.

Fig. 10. Paper chromatography of the products of $\alpha$-mannosidase treatment and acetylation of $[^{3}H]$mannose- and $[^{14}C]$glucose-labeled oligosaccharides. The $\alpha$-mannosidase digests of oligosaccharides Ia, Ib, and Ic were applied directly to Whatman No. 1 paper and the chromatogram was developed for 20 h in Solvent B (Panels A, B, and C). The acetylation products of Ib were chromatographed in Solvent B for 20 h (D). The markers are: 1, (Man)$_n$GlcitolNAc; 2, (Man)$_n$GlcitolNAc; 3, (Man)$_n$GlcitolNAc; 4, mannose.

Fig. 11. Products of VSV-infected clone 13 cells labeled for 2 h with $[^{3}H]$galactose and $[^{14}C]$mannose. VSV-infected clone 13 were incubated with 250 $\mu$Ci of $[^{3}H]$galactose and 100 $\mu$Ci of $[^{14}C]$mannose in complete medium for 2 h. Glycopeptides were prepared, isolated on Bio-Gel P-6, and treated with endo-$\beta$-$N$-acetylglucosaminidase $C_{r}$. The released oligosaccharides were reduced with NaBH$_4$, and subjected to descending paper chromatography in Solvent A for 21 days (A). The separated oligosaccharides were eluted as noted. Oligosaccharides Ib, Id, and Ib were digested with $\alpha$-mannosidase and the products chromatographed for 20 h in Solvent B (B). The markers are: 1, (Man)$_n$GlcitolNAc; 2, (Man)$_n$GlcitolNAc; 3, (Glu)$_n$(Man)$_n$GlcitolNAc; 4, (Glu)$_n$(Man)$_n$GlcitolNAc; 5, (Man)$_n$GlcitolNAc; 6, mannose.
Man → Man → GlcitolNAc (data not shown). This indicates that the three original oligosaccharides differ in mannose content and most likely have the structures (Glc)7-Man9-GlcitolNAc (Id), (Glc)7-Man9-GlcitolNAc (Ie), and (Glc)7-Man9-GlcitolNAc (Ih).

(Man)9(GlcNAc)2Asn Is a Processing Intermediate in 15B Cells—We have previously shown that the oligosaccharide units of the G protein of purified VSV grown in 10B cells have the composition (Man)9(GlcNAc)2-Asn, indicating that there is a block in processing in this cell line (9). To determine whether other processing intermediates accumulate in VSV-infected 15B cells, the cells were labeled for 10 min with [3H]mannose and [14C]glucosamine and then chased for 2 h. Glycopeptides prepared from such cells migrated primarily as a single peak on Bio-Gel P-6 (Fig. 12A). When this material was treated with endo-β-N-acetylgalactosaminidase C9, the major oligosaccharide that was released migrated on paper chromatography in Solvent A with authentic (Man)9(GlcNAc)2-Asn (Fig. 12B). No significant amount of (Man)9GlcitolNAc could be detected. NaBH4 reduction of the released oligosaccharide and acetylation of the resulting oligosaccharide alcohol gave rise to free mannose, a mannose-containing disaccharide, and a mannose- and glucosamine-containing trisaccharide (Fig. 12C). Digestion of the oligosaccharide with α-mannosidase produced free mannose and Man → GlcitolNAc (data not shown).

Evidence obtained in this laboratory and in others has demonstrated that the glycosylation of the VSV G protein is initiated by the en bloc transfer of a high molecular weight oligosaccharide from a lipid carrier to the newly synthesized protein (1-3). This oligosaccharide is then processed to give rise to the final complex-type oligosaccharide. In the preceding paper, the composition of the precursor oligosaccharide was shown to be (Glc)3(Man)9(GlcNAc)2 and its complete structure was elucidated (4). The present experiments provide data about the structure of the major processing intermediates and from this information a scheme for the sequence of oligosaccharide processing can be proposed. As shown in Fig. 13, the initial glycosylation involves the transfer of the glucose-containing oligosaccharide from the lipid carrier to the nascent G protein. Using an in vitro system it has been shown that glycosylation of G occurs prior to completion of the poly-peptide chain (12). Within a few minutes of transfer, processing of the oligosaccharide begins with the removal of the glucose residues (2). By 20 to 25 min the major species are (Glc)3(Man)9(GlcNAc)2 and (Man)9(GlcNAc)2. In parent cells, the (Man)9(GlcNAc)2 intermediate is then rapidly processed to give rise to the complex-type oligosaccharides. However, small amounts of (Man)9(GlcNAc)2 and (Man)9(GlcNAc)2 and a trace of (Man)9(GlcNAc)2 were detected in the cells at 20 to 30 min. The acetolysis fragmentation pattern of the intermediates suggest that the sequence of events in processing is relatively specific. A minor processing pathway may involve the conversion of (Glc)3(Man)9(GlcNAc)2 to (Glc)3(Man)9(GlcNAc)2 and (Glc)3(Man)9(GlcNAc)2. The studies with 15B cells indicate that Man1 → 3 (Man1 → 6) Man1 → 6 (Man1 → 3) Manβ1 → 4 GlcNAcβ1 → 4 GlcNAc → Asn.

**Discussion**

Evidence obtained in this laboratory and in others has demonstrated that the glycosylation of the VSV G protein is initiated by the en bloc transfer of a high molecular weight oligosaccharide from a lipid carrier to the newly synthesized protein (1-3). This oligosaccharide is then processed to give rise to the final complex-type oligosaccharide. In the preceding paper, the composition of the precursor oligosaccharide was shown to be (Glc)3(Man)9(GlcNAc)2 and its complete structure was elucidated (4). The present experiments provide data about the structure of the major processing intermediates and from this information a scheme for the sequence of oligosaccharide processing can be proposed. As shown in Fig. 13, the initial glycosylation involves the transfer of the glucose-containing oligosaccharide from the lipid carrier to the nascent G protein. Using an in vitro system it has been shown that glycosylation of G occurs prior to completion of the poly-peptide chain (12). Within a few minutes of transfer, processing of the oligosaccharide begins with the removal of the glucose residues (2). By 20 to 25 min the major species are (Glc)3(Man)9(GlcNAc)2 and (Man)9(GlcNAc)2. In parent cells, the (Man)9(GlcNAc)2 intermediate is then rapidly processed to give rise to the complex-type oligosaccharides. However, small amounts of (Man)9(GlcNAc)2 and (Man)9(GlcNAc)2 and a trace of (Man)9(GlcNAc)2 were detected in the cells at 20 to 30 min. The acetolysis fragmentation pattern of the intermediates suggest that the sequence of events in processing is relatively specific. A minor processing pathway may involve the conversion of (Glc)3(Man)9(GlcNAc)2 to (Glc)3(Man)9(GlcNAc)2 and (Glc)3(Man)9(GlcNAc)2. The studies with 15B cells indicate that Man1 → 3 (Man1 → 6) Man1 → 6 (Man1 → 3) Manβ1 → 4 GlcNAcβ1 → 4 GlcNAc → Asn.
Oligosaccharide Processing

intermediate in the formation of complex oligosaccharide units. We have proposed that this molecule is the physiological substrate for the UDP-GlcNAc:glycoprotein N-acetylgalactosaminyltransferase which is deficient in 15B cells (13). We suggest that the transfer of a N-acetylglucosamine residue to the mannose residue linked α1 → 3 to the β-linked core mannose serves as the signal for an α-mannosidase to remove the 2 mannose residues linked to the mannose which is linked α1 → 6 to the core mannose. In the following paper we present evidence from in vitro experiments which provides direct support for this pathway (14). Following removal of the mannose residues, the final 2 outer N-acetylglucosamine residues are transferred to the oligosaccharide and then glycosylation is completed by the addition of galactose, sialic acid, and fucose residues. This scheme indicates that two α-mannosidases may be involved in processing. The first enzyme would cleave mannoses linked α1 → 2 and therefore convert the (Man)₉(GlcNAc)₂ to (Man)₉(GlcNAc)₂. The second enzyme would cleave mannose residues linked α1 → 3 and α1 → 6. The finding of Opheim and Touster (15) that a purified rat liver Golgi α-mannosidase hydrolyzes 1 → 2 but not 1 → 3 linkages is consistent with this postulation. It is also possible that a single α-mannosidase could carry out the processing if its specificity for α1 → 6 and α1 → 3 linkages is altered by the addition of a N-acetylglucosamine residue to the oligosaccharide.

It should be emphasized that these studies have only dealt with the synthesis of complex-type oligosaccharides. It has yet to be determined whether high mannose oligosaccharides are formed by incomplete action of the processing enzymes involved in complex-type oligosaccharide synthesis or if a different series of processing enzymes are responsible for high mannose oligosaccharide synthesis. Our current experiments are designed to resolve this point.

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