Selective Retention of Monoglucosylated High Mannose Oligosaccharides by a Class of Mutant Vesicular Stomatitis Virus G Proteins

Kyungsun Suh, John E. Bergmann, and Christopher A. Gabel

Department of Anatomy and Cell Biology, College of Physicians and Surgeons, Columbia University, New York 10032

Abstract. Cells infected with a temperature-sensitive mutant of vesicular stomatitis virus, ts045, or transfected with the plasmid vector pdTM12 produce mutant forms of the G protein that remain within the ER. The mutant G proteins were isolated by immunoprecipitation from cells metabolically labeled with [2-^{3}H]-mannose to facilitate analysis of the protein-linked oligosaccharides. The ^{3}H-labeled glycopeptides recovered from the immunoprecipitated G proteins contained high mannose-type oligosaccharides. Structural analysis, however, indicated that 60–78% of the ^{3}H-mannose-labeled oligosaccharides contained a single glucose residue and no fewer than eight mannose residues. The ^{3}H-labeled ts045 oligosaccharides were deglucosylated and processed to complex-type units after the infected cells were returned to the permissive temperature. When shifted to the permissive temperature in the presence of a proton ionophore, the G protein oligosaccharides were deglucosylated but remained as high mannose-type units. The glucosylated state was observed, therefore, when the G protein existed in an altered conformation. The ts045 G protein oligosaccharides were deglucosylated in vitro by glucosidase II at both the permissive and nonpermissive temperatures. G protein isolated from ts045-infected cells labeled with [6-^{3}H]galactose in the presence of cycloheximide contained ^{3}H-glucose-labeled monoglucosylated oligosaccharides, indicating that the high mannose oligosaccharides were glucosylated in a posttranslational process. These results suggest that aberrant G proteins are selectively modified by resident ER enzymes to retain monoglucosylated oligosaccharides.

THE processing of asparagine-linked oligosaccharides from high mannose-type units to mature complex-type structures occurs in a highly ordered series of reactions (21, 27). Enzymes involved in the processing reactions reside within the ER and Golgi apparatus, and biochemical as well as morphological studies indicate that they are restricted to defined compartments along the secretory pathway (13, 14, 16, 18, 32, 46, 47). The physical location of an enzyme within the pathway generally corresponds with the temporal order in which it functions (21, 27). In a previous study (16) we took advantage of the ordered processing reactions to define the intracellular compartments that contained mutant forms of the G protein of vesicular stomatitis virus (VSV). The mutant G proteins were synthesized in COS-1 cells after transfection with plasmids that coded for proteins whose carboxy termini possessed deletions or alterations. As a result of the alterations, the proteins accumulated at intracellular sites rather than at the cell surface (43, 44). The structure of the oligosaccharides attached to the mutant G proteins reflected the site of accumulation within the cells. For example, the G protein encoded by Δ1473 was shown to accumulate in the ER by immunofluorescence (44), and its asparagine-linked oligosaccharides were processed to high mannose-type units containing eight mannose residues (16). In contrast, many of the G proteins encoded by Δ1554 were transported to the medial compartment of the Golgi apparatus where the oligosaccharides were processed to complex-type units that terminated in *N*-acetylglucosamine (16). As a result of their abnormal accumulation at distinct intracellular locations, the altered G proteins served as useful reporter molecules with which to identify the biosynthetic capabilities of the various intracellular compartments.

In this report two additional mutant G proteins are analyzed. ts045 is a temperature-sensitive mutant of VSV; the temperature sensitive phenotype is due to a single point mutation in the G protein (17). At the nonpermissive temperature the newly synthesized mutant G protein remains in the ER; reducing the temperature to 32°C allows the G protein to move out of the ER and to the cell surface (6, 25). The form of the G protein encoded by the plasmid vector pdTM12 lacks a portion of the normal transmembrane domain and also accumulates within the ER (1). As expected, oligosaccharides isolated from the two proteins are high mannose-type units. However, unlike the oligosaccharides attached to the G protein encoded by Δ1473 (16), the ts045 and pdTM12 G protein oligosaccharides possess a single glucose residue at a nonreducing terminus. The ability of different

forms of the VSV G protein to exist within the ER in either a glucosylated or nonglucosylated state suggests that the proteins are differentially processed by resident ER enzymes. The significance of this unequal processing and the mechanism by which the glucosylated species persist are addressed.

Materials and Methods

Cell Culture

COS-1 cells were grown in a 1:1 mixture of DME and Ham's F12 containing 5% FBS, 20 mM Hepes, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cultures were maintained in an atmosphere of 5% CO_2 at 37°C. The wheat germ agglutinin resistant Chinese hamster ovary (CHO) cell line Lec 8 was obtained from the American Type Culture Collection (Rockville, MD) and was maintained in α MEM containing 10% FBS, 20 mM Hepes, 100 U/ml penicillin, 100 μg/ml streptomycin, and 40 mg/liter proline.

Expression of the G Proteins

The procedure for the transfection of the COS-1 cells was as described (16, 43), except that the cDNA in the plasmid was pdTM12 (a generous gift of Dr. J. K. Rose); this cDNA has been shown to code for a G protein that contains an altered transmembrane domain (1).

The COS-1 and Lec 8 cells were infected with the temperature-sensitive mutant of VSV (ts045) as follows. Cells were plated on 10-cm dishes 1 d before the infection. 4×10^8 pfu of ts045 suspended in 1 ml of DME containing 10% FBS and 20 mM Hepes were added to each dish and the virus was allowed to adsorb to the cells for 30 min at 32°C; the plates were rocked occasionally during this period. The medium containing nonadsorbed virus was removed, 5 ml of fresh medium was added, and the cells were shifted to 37°C and incubated for 4 h.

Metabolic Labeling of the G Protein

The medium from the ts045-infected cells was discarded and the cells were washed with 5 ml of 10 mM sodium phosphate, 150 mM NaCl, pH 7.4 (PBS), prewarmed to 41°C. 5 ml of glucose-free α MEM containing 0.4 mCi of [2-^{3}H]mannose (American Radiolabeled Chemicals Inc., St. Louis, MO; 14 Ci/mmol), 20 mM Hepes, pH 7, and 10% dialyzed FBS was added to each dish, and the cells were incubated at 41°C for 30 min. At the end of the pulse the medium was replaced with 5 ml of isotope-free MEM containing 25 mM mannose, 25 mM glucose, and 10% FBS; the chase medium was prewarmed to either 32°C (permissive) or 41°C (nonpermissive). The medium was discarded after the chase and the cells were rinsed twice with 5 ml of cold PBS. The labeled cells were solubilized with 2 ml of extraction buffer (PBS containing 1% Triton X-100, 0.4% deoxycholate, and 1 mM PMSF) and the extracts were clarified in an Eppendorf centrifuge for 10 min.

The resulting supernatants were incubated with 0.05 ml of a 10% suspension of fixed *Staphylococcus aureus* cells (Calbiochem-Behring Corp., La Jolla, CA) for 15 min on ice. The bacteria were removed by centrifugation and 10 μg of affinity-purified rabbit anti-G protein antibody was added. The extracts were incubated on ice for 30 min and the antigen-antibody complexes were recovered by the addition of 0.05 ml of *S. aureus*; the time of incubation with the antibody was minimized to prevent potential alterations of the G protein oligosaccharides by glycosidases within the extracts. After 30 min on ice, the bacteria were collected by centrifugation and washed five times with 10 mM Tris, pH 8, 10 mM EDTA, 1% Triton X-100, 0.4% deoxycholate, 0.1% SDS, and once with 10 mM Tris, pH 8, 1 mM EDTA. The final pellet was suspended in sample buffer (29) and boiled for 3 min; the suspensions were clarified by centrifugation and the disaggregated supernatants were stored at −70°C until analyzed by SDS gel electrophoresis (29) and fluorography. The gels were soaked in Amplify (Amersham Corp., Arlington Heights, IL) before drying.

COS-1 cells (confluent monolayers on 6-cm dishes) transfected with pdTM12 were labeled (48 h after transfection) for 6 h with 1 mCi of [2-^{3}H]mannose in 5 ml of glucose-free MEM containing 10% FBS and 20 mM Hepes, pH 7. The cells were solubilized and the G protein was isolated as described above.

Isolation and Analysis of [2-^{3}H]Mannose-labeled Glycopeptides

Regions of the dried gel containing the radiolabeled G proteins were excised and the radioactivity was solubilized by digestion of the gel pieces with pronase (16). The resulting glycopeptides were fractionated by Con A-Sepharose chromatography as previously outlined (16). The high mannose-type glycopeptides (Peak III from the Con A-Sepharose column) were dissolved in 0.025 ml of citrate-phosphate buffer, pH 5.6, and 0.025 ml of endoglycosidase H (endo H; 50 mU/ml in citrate-phosphate buffer, pH 5.6) were added. The digests were incubated overnight at 37°C, after which they were diluted with 1 ml of H_2O and applied to 2-ml columns of Amberlite MB3 resin. The neutral endo H-released oligosaccharides were eluted with 10 ml of H_2O and dried by rotary evaporation.

Digestion of the high mannose oligosaccharides with jack bean α-mannosidase was performed as previously described (16). In some cases, the high mannose oligosaccharides were treated with 2 mU of glucosidase II (in 0.025 ml of 50 mM potassium phosphate, pH 7, 0.1% Triton X-100, 5 mM β-mercaptoethanol, 1 mM EDTA) before the mannosidase digestion. The glucosidase was purified from rat liver as described (20). The purified enzyme had a specific activity of 0.76 U/mg against *p*-NO_2-phenyl-α-glucoside, and contained two Coomassie staining bands at 103 and 83 kD when analyzed by SDS gel electrophoresis; these properties are comparable to those previously reported (8, 9, 20). The glucosidase digests were applied to Amberlite MB3 minicolumns and the oligosaccharides were eluted with 10 ml of H_2O, dried, and digested with α-mannosidase.

Endo H-released oligosaccharides were analyzed by HPLC using an AX-5 micropak column (Varian Associates, Inc., Palo Alto, CA) as previously described (34). When α-mannosidase digests were analyzed by HPLC, the reaction products were reduced with $NaBH_4$ before their injection onto the column, and the gradient was changed to 40–70% in H_2O. Exoglycosidase digests were also separated by descending paper chromatography in ethyl acetate/pyridine/acetic acid/H_2O (5:5:1:3); the dried chromatograms were cut into 1-cm strips, and the associated radioactivity was determined by liquid scintillation counting.

Results

Processing of the ts045 G Protein Oligosaccharides

COS-1 cells infected with ts045 VSV were labeled for 30 min at the nonpermissive temperature with [2-^{3}H]mannose after which they were shifted to 32°C (permissive temperature) and chased for various times. The G protein was immunoprecipitated from detergent extracts of the cells and was digested with pronase. The resulting ^{3}H-mannose-labeled glycopeptides fractionated into three peaks when chromatographed on Con A-Sepharose corresponding to tri- and/or tetraantennary complex-type (Peak I), biantennary complex-type (Peak II), and high mannose-type (Peak III) units. G protein derived from cells labeled at the nonpermissive temperature contained predominantly high mannose-type glycopeptides (Fig. 1 *A*), and these oligosaccharides remained as high mannose units when the cells were chased at 41°C. After the shift to 32°C, however, the amount of radioactivity recovered as complex-type units increased and a corresponding decrease occurred in the quantity of high mannose-type units (Fig. 1, *B* and *C*). To confirm that the radioactivity in Peaks I and II corresponded to complex type glycopeptides, the immunoprecipitated G proteins were treated with endo H before pronase digestion. Complex-type asparagine-linked oligosaccharides are resistant to endo H (26, 33). The endo H-treated samples lacked radiolabeled high mannose-type glycopeptides but contained the same quantities of radioactivity in Peaks I and II as their nontreated counterparts (compare Fig. 1, *B* and *C* with *E* and *F*). After 180 min of chase at the permissive temperature the Con A-Sepharose profiles were similar before and after endo H digestion (Fig. 1, *D* and

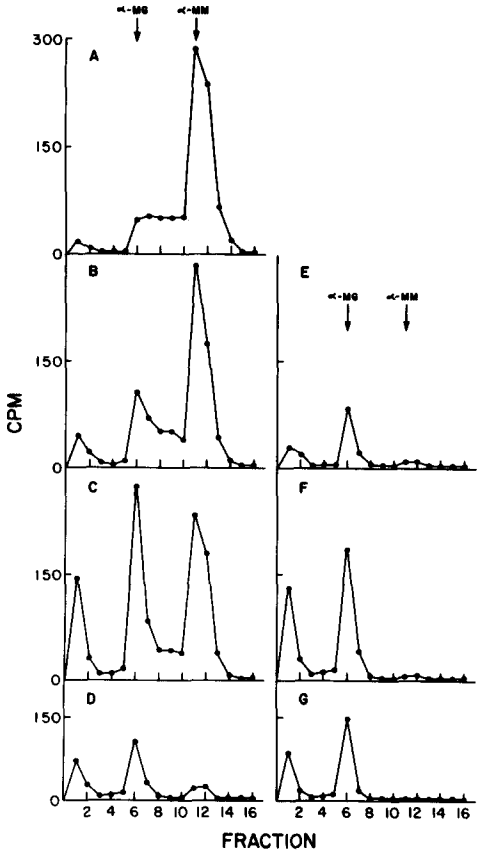

Figure 1. Con A–Sepharose chromatography of the G protein ^{3}H-mannose–labeled glycopeptides. G protein was immunoprecipitated from ^{3}H-mannose–labeled COS-1 cells and analyzed by SDS gel electrophoresis. The regions of the dried gel containing the radiolabeled G protein were excised and the radioactivity was solubilized by pronase digestion. The ^{3}H-labeled glycopeptides were fractionated by Con A–Sepharose chromatography; the columns were eluted sequentially with TBS (Peak I), 10 mM α-methylglucoside (*α-MG*) in TBS (Peak II), and 100 mM α-methylmannoside (*α-MM*) in TBS (Peak III). The chromatograms show the distribution of the ^{3}H-mannose–labeled glycopeptides recovered from the G protein after a 30-min pulse at 41°C and (*A*) no chase, (*B* and *E*) 20-min chase at 32°C, (*C* and *F*) 40-min chase at 32°C, and (*D* and *G*) 180 min chase at 32°C. The glycopeptides in *E*, *F*, and *G* were isolated from G proteins that were digested with endo H before SDS gel electrophoresis.

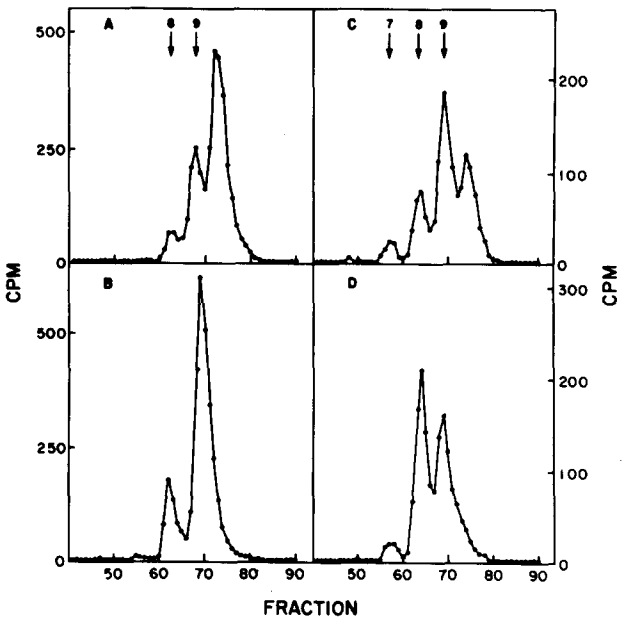

Figure 2. High performance liquid chromatography of ^{3}H-mannose–labeled oligosaccharides. The high mannose oligosaccharides recovered from the ts045 G protein (after a 30-min pulse labeling of the infected COS-1 cells at 41°C) and from the pdTM12 encoded G protein were analyzed by HPLC using an AX-5 micropak column. The profiles show the distribution of the ts045 G protein oligosaccharides before (*A*) and after (*B*) digestion with rat liver glucosidase II, and the pdTM12 G protein oligosaccharides before (*C*) and after (*D*) glucosidase II digestion. The arrows in *A* and *C* denote the elution position of high mannose oligosaccharides composed of $Man_7GlcNAc$ (*7*), $Man_8GlcNAc$ (*8*), and $Man_9GlcNAc$ (*9*).

G). The high mannose–type asparagine-linked oligosaccharides attached to the G protein at the nonpermissive temperature, therefore, matured to complex type units during the 32°C chase. These results are in complete agreement with previous studies showing that the ts045 mutation is reversible (3, 17, 25, 42), and indicate that the mutant G protein oligosaccharides are processed by the COS-1 cells as in other cell types (23, 38, 49, 52).

Characterization of the High Mannose–Type Oligosaccharides

High mannose–type glycopeptides recovered from the ts045 G protein were digested with endo H and the released oligosaccharides were analyzed by HPLC. The oligosaccharides eluted from the HPLC column in the positions expected for oligosaccharides containing 8 ($Man_8GlcNAc$; 6% of the radioactivity) and 9 mannose residues ($Man_9GlcNAc$; 28% of the radioactivity) (Fig. 2 *A*). The bulk of the radioactivity (66%), however, eluted in the position expected for glucosylated high mannose–type units. To confirm that the ts045 G proteins produced at the nonpermissive temperature contained glucosylated oligosaccharides, the endo H–released units were treated with glucosidase II; this enzyme removes the α1,3-linked glucose residues from the high mannose oligosaccharide precursor (9, 19, 35, 48). Glucosidase II simplified the HPLC profile (Fig. 2 *B*); after the digestion, the radioactivity eluted only as the $Man_8GlcNAc$ (18%) and $Man_9GlcNAc$ (79%) species. Since the glucosidase-treated fraction showed an increase in both the $Man_8GlcNAc$ and $Man_9GlcNAc$ species relative to the nondigested oligosaccharides (Fig. 2 *A*), some of the glucosylated oligosaccharides had lost one mannose residue from the initial precursor molecule.

The percentage of the oligosaccharides that remained glucosylated was confirmed by digestion of the endo H–released oligosaccharides with α-mannosidase. Oligosaccharides isolated from the ts045 G protein produced at 41°C yielded free mannose, the disaccharide mannose-*N*-acetylglucosamine, and an α-mannosidase–resistant species (Table I). Predigestion of the G protein oligosaccharides with glucosidase II led to the loss of the resistant species (Table I). Assuming that the radioactivity associated with the α-mannosidase–resis-

Table I. α-Mannosidase Digestion of High Mannose Oligosaccharides

Source of oligosaccharide	Pretreatment with glucosidase II	CPM recovered as			
		Resistant	Man-GlcNAc	Man	Glucosylated
					%
G protein	No	358	25	746	78
G protein	Yes	39	141	1,278	7
Non-G protein	No	227	226	1,763	20

ts045 VSV-infected COS-1 cells were labeled for 30 min with [2-^{3}H]mannose at 41°C. The cells were solubilized with Triton X-100 and the G protein was immunoprecipitated and analyzed by SDS gel electrophoresis; the non-G proteins remaining in the postimmunoprecipitate supernatant were precipitated with 90% acetone. High mannose-type glycopeptides were isolated by Con A-Sepharose chromatography after pronase digestion of the G protein and the acetone precipitate, and the oligosaccharides were released from their amino acid constituents by endo H. The released oligosaccharides were digested with α-mannosidase and the digestion products were analyzed by descending paper chromatography. Three peaks of radioactivity were detected in the chromatograms corresponding to α-mannosidase-resistant species (*Resistant*), the disaccharide mannose-*N*-acetylglucosamine (*Man-GlcNAc*), and free mannose (*Man*). The percentage of glucosylated high mannose oligosaccharides was calculated based on the amount of radioactivity recovered in the α-mannosidase-resistant species (R_{cpm}) and in the mannose-*N*-acetylglucosamine peak (S_{cpm}), using the following formula:

$$\text{percent glycosylated} = (R_{cpm}/4)/[(R_{cpm}/4) + S_{cpm}] \times 100.$$

tant species represented glucosylated oligosaccharides containing four mannose residues, we estimated that 78% of the G protein asparagine-linked high mannose oligosaccharides remained glucosylated at the nonpermissive temperature (see legend to Table I for calculation). The glucosylation state of the high mannose oligosaccharides recovered from the postimmunoprecipitate supernatant was also analyzed; this fraction contained all of the non-G proteins produced by the cells during the 30-min pulse-labeling. The nonviral glycoprotein oligosaccharides were not highly glucosylated as 80% of the oligosaccharides were degraded by α-mannosidase (Table I). The glucose residues, therefore, were enriched in the G protein oligosaccharides, and their presence was not a general result of labeling the cells at 41°C.

Since predigestion with glucosidase II allowed α-mannosidase to completely degrade the oligosaccharides, it was likely that the G protein structures contained one or two α1,3-linked glucose residues (9, 19, 35, 48). To determine the actual number, the α mannosidase digests were reduced with $NaBH_4$ and analyzed by HPLC, and the elution position of the resistant fragment was compared to the position of $Glc_2Man_4GlcNAc_{itol}$. The latter standard was prepared by α-mannosidase digestion of the high mannose oligosaccharides isolated from glucosidase II-deficient mouse lymphoma cells (PHA^{R}2.7; reference 39). Three peaks of radioactivity were detected after α-mannosidase digestion of the ts045 G protein oligosaccharides (Fig. 3 *A*); 63% of the radioactivity eluted with mannitol, 4% eluted with mannose-*N*-acetylglucosaminitol, and 32% eluted with a single high molecular weight species. The high molecular weight fragments isolated from the G protein oligosaccharides eluted earlier than $Glc_2Man_4GlcNAc_{itol}$ (Fig. 3 *B*). To confirm the difference in the elution positions, the digestion products of ts045 G protein and PHA^{R}2.7 cell oligosaccharides were combined and analyzed. The resistant fragments eluted as

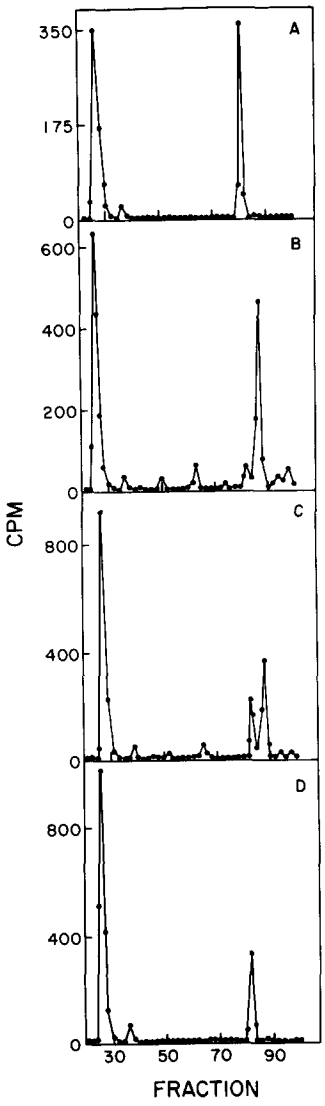

Figure 3. Characterization of the α-mannosidase resistant fragments. The high mannose oligosaccharides isolated from the ts045 G protein (*A*), from the pdTM12 encoded G protein (*D*), and from the PHA^R-2.7 mutant mouse lymphoma cells (*B*) were digested with α-mannosidase. The reaction products were reduced with $NaBH_4$ and analyzed by HPLC. The peaks of radioactivity eluted in the positions expected for mannitol (fractions 25–30), mannose-*N*-acetylglucosaminitol (fractions 35–40), and α-mannosidase-resistant fragments (fractions 79–86). *C* shows the chromatogram obtained after mixing the α-mannosidase digest of the ts045 G protein and the PHA^{R}2.7 oligosaccharides.

two well-resolved peaks (Fig. 3 *C*). The G protein α-mannosidase-resistant fragments yielded mannose and mannose-*N*-acetylglucosamine in a 3.2:1.0 ratio after sequential digestion with glucosidase II and α-mannosidase (data not shown). Thus, the difference in size of the resistant fragments is not attributable to mannose residues (each contains four) and it is concluded that the G protein oligosaccharides contain only one glucose residue.

G protein oligosaccharides produced at the nonpermissive temperature were converted to complex-type units when the infected COS-1 cells were subsequently chased at the permissive temperature (Fig. 1). After 40 min of chase at 32°C the total quantity of ^{3}H-mannose-labeled G protein recovered from the infected cells declined as a result of this oligosaccharide processing and the release of virions to the medium. The number of glucosylated oligosaccharides also declined, and only 35% of the original quantity of these oligosaccharides was recovered in the G protein immunoprecipitate after the 40-min chase (Table II). When the temperature shift was carried out in the presence of CCCP to block transport of the G protein to the Golgi apparatus (2), no complex-type oligosaccharides were formed (Table II). The

Table II. Effect of Chase on the Glucosylation of the ts045 G Protein Oligosaccharides

Chase	Temperature	CCCP	Total	High mannose	Glucosylated high mannose	
min	*°C*		*cpm*	*%*	*cpm*	*% of initial*
0	32	−	219,000	85	109,930	100
40	32	−	129,000	70	37,930	35
40	32	+	319,000	86	41,150	37
180	32	−	91,000	72	34,070	31
180	41	−	492,000	90	301,720	270

ts045-infected COS-1 cells were labeled for 30 min at 41°C with [2-^{3}H]mannose. At the end of the pulse, the cells received fresh medium and were chased at either 32 or 41°C. When present, CCCP was added to the chase medium at a final concentration of 20 μM. G protein was recovered from cell extracts by immunoprecipitation and analyzed by SDS gel electrophoresis and fluorography. The radioactive regions of the dried gel were excised and the radioactivity was solubilized by pronase digestion. The digests were fractionated by Con A–Sepharose chromatography and the high mannose–type glycopeptides were recovered and digested with endo H. The endo H–released oligosaccharides were subsequently digested with α-mannosidase, the products were separated by HPLC, and the percentage of glucosylated oligosaccharides was calculated as described in the legend to Table I.

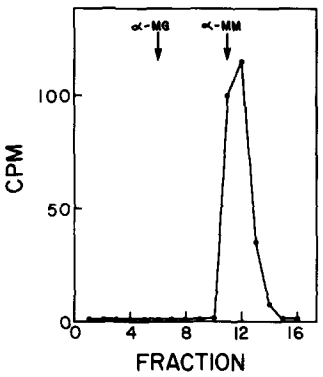

Figure 4. Con A–Sepharose chromatography of the ^{3}H-mannose–labeled glycopeptides isolated from the pdTM12 encoded G protein. COS-1 cells transfected with pdTM12 were labeled for 6 h with [2-^{3}H]mannose, after which the cells were solubilized and the G protein was isolated by immunoprecipitation. The immunoprecipitate was analyzed by SDS gel electrophoresis and autoradiography; the region of the dried gel containing the G protein was excised and the radioactivity was solubilized by pronase digestion. The glycopeptides were applied to Con A–Sepharose and the column was eluted stepwise with TBS, 10 mM α methylglucoside (*α-MG*) in TBS, and 100 mM α methylmannoside (*α-MM*) in TBS.

total quantity of high mannose oligosaccharides recovered from the CCCP-treated cells was greater than in the initial pulse, indicative of a continued incorporation of ^{3}H-mannose into the G protein from the lipid linked precursor pool during the chase. However, the number of glucosylated molecules was identical to that present after 40 min of chase in the absence of the inhibitor (Table II). The G proteins that remained intracellular after 180 min of chase at the permissive temperature still contained glucosylated high mannose-type oligosaccharides (Table II), suggesting that these molecules were not rescued by the temperature shift. When the infected cells were chased at the nonpermissive temperature the percentage of glucosylated high mannose oligosaccharides remained high after the 3-h chase (68%), and the overall quantity was again greater than was initially present after the pulse (Table II).

Analysis of the G Protein Produced by pdTM12-transfected Cells

COS-1 cells transfected with pdTM12 were labeled for 6 h with ^{3}H-mannose, after which the cells were harvested and the G protein was isolated by immunoprecipitation. Greater than 98% of the ^{3}H-labeled oligosaccharides associated with the G protein bound to Con A–Sepharose and eluted with 0.1 M α-methylmannoside (Fig. 4). The oligosaccharides ranged in size from species containing seven mannose residues to larger species that eluted in the region of glucosylated structures (Fig. 2 *C*). After digestion with glucosidase II, the majority of the larger forms disappeared with a concomitant increase in the $Man_8GlcNAc$ and $Man_9GlcNAc$ species (Fig. 2 *D*). Overall, the $Man_8GlcNAc$ species increased from 18% of the radioactivity associated with the nonglucosidase-treated sample to 47% of the glucosidase-treated profile, and the $Man_9GlcNAc$ species increased from 41 to 48%. In contrast, the percentage of the radioactivity that eluted with the $Man_7GlcNAc$ species did not change after glucosidase digestion. Thus, as observed for ts045 G protein oligosaccharides, glucose residues are associated only with the $Man_8GlcNAc$ and $Man_9GlcNAc$ species.

The percentage of the G protein oligosaccharides that were glucosylated was again determined by α-mannosidase digestion. The undigested oligosaccharides remained near the origin of a paper chromatogram (Fig. 5 *A*). After α-mannosidase treatment, 53% of the radioactivity migrated as free mannose, 7% as the disaccharide mannose-*N*-acetylglucosamine, and 40% remained near the origin (Fig. 5 *B*). Treatment of the intact oligosaccharides with glucosidase II did not alter their migration (Fig. 5 *C*). Treatment with both glucosidase II and α-mannosidase, however, reduced the amount of radioactivity that remained near the origin to 5% of the total, and the bulk of the radioactivity migrated with mannose-*N*-acetylglucosamine (11%) and mannose (84%) (Fig. 5 *D*). The α-mannosidase–resistant fragments eluted as expected for a $Glc_1Man_4GlcNAc_{itol}$ oligosaccharide from the HPLC column (Fig. 3 *D*). Thus, as with the G protein produced by ts045, 59% of the high mannose oligosaccharides associated with the pdTM12-encoded G proteins retained a single glucose residue.

In Vitro Deglucosylation of the ts045 G Protein

To determine the accessibility of the protein-bound oligosaccharides to exogenous glycosidases, ts045-infected COS-1 cells were pulse-labeled with ^{3}H-mannose for 30 min, the cells were extracted with Triton X-100, and the extract was divided into three equal fractions. One fraction (control) was placed on ice. The other two fractions were treated with purified glucosidase II; one tube was incubated at 32°C (glucosidase-treated/32°C) for 60 min and the other at 41°C (glucosidase-treated/41°C) for 60 min. At the end of the incubation, the G protein was recovered by immunoprecipitation and glycopeptides were prepared and fractionated on Con A–Sepharose. In each case, 85–88% of the radioactivity bound to Con A–Sepharose and eluted as high mannose-type glycopeptides. 68% of the high mannose oligosaccharides isolated from the control sample were glucosylated (Fig. 6 *A*). However, the majority of the G protein oligosaccharides recovered from the glucosidase-treated extracts were deglucosylated. The number of α-mannosidase–resistant oligosaccharides recovered from the glucosidase-treated/32°C

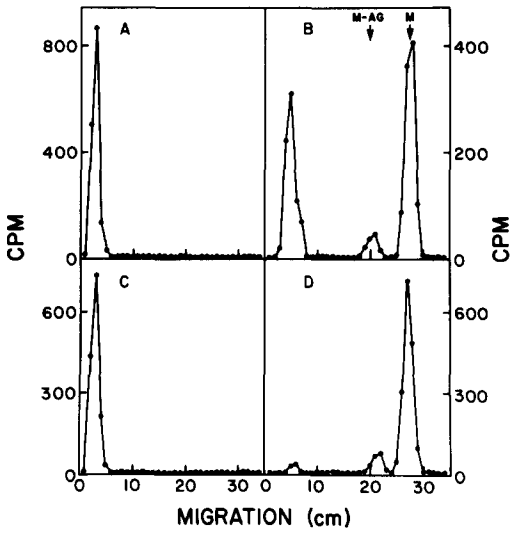

Figure 5. Sensitivity of the pdTM12 encoded G protein oligosaccharides to exoglycosidase digestion. The endo H–released high mannose oligosaccharides recovered from the pdTM12 encoded G protein were analyzed by descending paper chromatography after (*A*) no treatment, (*B*) digestion with jack bean α-mannosidase, (*C*) digestion with rat liver glucosidase II, and (*D*) digestion with a combination of glucosidase II and α-mannosidase. The mobility of the undigested oligosaccharide is shown in *A*. The arrows indicate the migration position of mannose (*M*) and mannose-*N*-acetylglucosamine (*M-AG*).

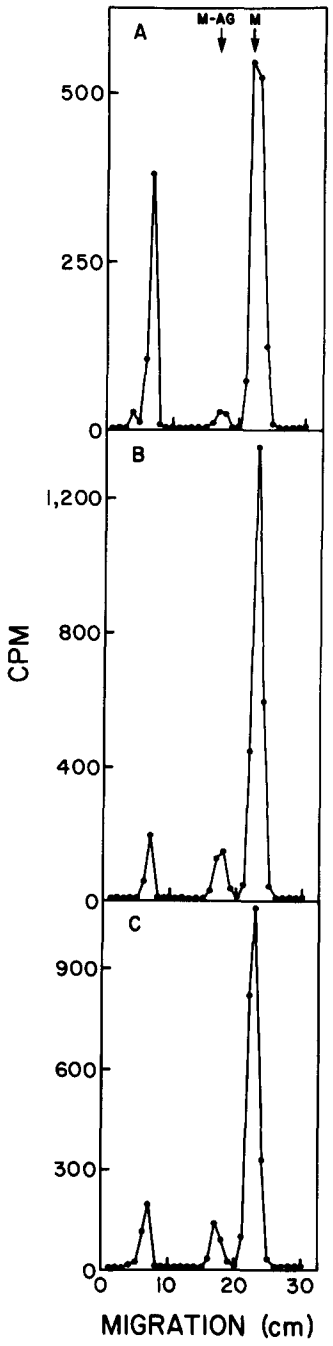

Figure 6. Accessibility of the ts045 G protein oligosaccharides to exogenous glucosidase II. COS-1 cells infected with ts045 VSV were labeled for 30 min at 41°C. The cells were harvested and solubilized with Triton X-100; the detergent extract was subsequently divided into three equal aliquots. The control fraction (*A*) was placed on ice. The other two fractions received 21 mU of rat liver glucosidase II (in a total volume of 0.35 ml of 50 mM potassium phosphate, pH 7, 0.4% deoxycholate, 5 mM β-mercaptoethanol, 1 mM EDTA, and 1% Triton X-100) and they were incubated at either 32 (*B*) or 41°C (*C*) for 60 min. At the end of the incubation, the G protein was immunoprecipitated from the three fractions; the high mannose oligosaccharides associated with the immunoprecipitates were recovered and were digested with α-mannosidase. The digestion products were subsequently analyzed by descending paper chromatography. The arrows in *A* indicate the migration position of mannose (*M*) and mannose-*N*-acetylglucosamine (*M-AG*).

and glucosidase-treated/41°C samples was reduced to 17 and 23%, respectively (Fig. 6, *B* and *C*). In all cases, when the isolated oligosaccharides were treated with glucosidase II and α-mannosidase the radioactivity recovered in the resistant peak was reduced to <3% of the total (not shown).

Posttranslational Glucosylation of the ts045 G Protein

Previous studies by Parodi et al. (36) described an unusual posttranslational attachment of glucose residues to protein-bound high mannose oligosaccharides. To determine whether this reaction occurred to the G proteins, ts045-infected cells were labeled with [6-^{3}H]galactose in the presence of cycloheximide to block protein synthesis. [6-^{3}H]galactose was used as the precursor since it is incorporated into high mannose-type oligosaccharides as glucose (22, 30). In addition, we used a mutant CHO cell line (Lec 8) deficient in the translocation of UDP-galactose across Golgi membranes (10, 50) to minimize the amount of label incorporated as a result of the posttranslational attachment of galactose residues to complex-type oligosaccharides (21, 27). Cycloheximide effectively halted protein synthesis in ts045-infected Lec 8 cells, as the amount of [^{35}S]methionine incorporated into the G protein was reduced >30-fold in the presence of the inhibitor (Table III). In contrast, the incorporation of ^{3}H-galactose into the G protein was reduced less than twofold by cycloheximide (Table III). G protein isolated from the ^{3}H-galactose–labeled cells contained radiolabeled high mannose–type oligosaccharides (Peak III in Table III). The absolute amount of radioactivity recovered in the high mannose fraction of the cycloheximide-treated cells was comparable to that isolated from the nontreated cells after the pulse (561 and 744 cpm, respectively). During a subsequent 60-min chase at the nonpermissive temperature the absolute amount of radioactivity associated with the G protein high mannose oligosaccharides declined both in the presence and absence of cycloheximide (Table III). Radioactivity was also recovered in Peak I from Con A–Sepharose (Table III); the nature of this material is unknown. The ^{3}H-labeled high mannose oligosaccharides isolated from the G protein were degraded by acid hydrolysis and the resulting monosaccharides were separated by paper chromatography; >90% of the radioactivity comigrated with glucose (not shown). Fragments generated from these oligosaccharides after α-mannosidase digestion yielded a single radioactive peak that eluted from the HPLC column in the position expected for GLc_1Man_4-

Table III. Posttranslational Glucosylation of ts045 G Protein

Label	Cyclo-heximide	Chase	Total cpm	cpm in Con A peaks I	II	III
		min				
^{35}S	−	0	22,227	−	−	−
	+	0	798	−	−	−
^{3}H	−	0	1,015	223	48	744
	+	0	671	95	15	561
	−	60	835	465	68	302
	+	60	545	223	30	292

ts045-infected Lec 8 cells were labeled with [6-^{3}H]galactose (0.05 mCi/ml) or [^{35}S]methionine (0.05 mCi/ml) in the presence or absence of 180 μM cycloheximide; before labeling, the cells were preincubated with the protein synthesis inhibitor for 10 min. After a 15-min pulse, the cells either were harvested or chased for 60 min at 41°C in the continued presence of cycloheximide but absence of radiolabeled monosaccharide. G protein was isolated from cell extracts by immunoprecipitation and analyzed by SDS gel electrophoresis and fluorography. The radioactive regions were excised from the dried gel, and the radioactivity was solubilized by pronase digestion; in the case of the ^{3}H-labeled samples, the digests were subsequently chromatographed on Con A–Sepharose. Radioactivity recovered in the runthrough of the Con A–Sepharose column (*I*), the biantennary fraction (*II*), and the high mannose fraction (*III*) is indicated.

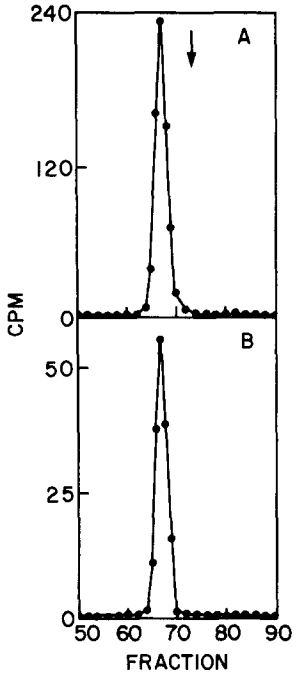

Figure 7. Characterization of the ^{3}H-glucose-labeled oligosaccharides generated in the presence of cycloheximide. High mannose oligosaccharides isolated from G protein immunoprecipitated from ^{3}H-galactose-labeled ts045-infected Lec 8 cells were digested with α-mannosidase and the reaction products were reduced with $NaBH_4$ and analyzed by HPLC. The chromatograms show the elution of the oligosaccharides recovered after the 15-min pulse (*A*) and 60-min chase (*B*). The elution position of $Glc_2Man_4GlcNAc_{itol}$ is indicated in *A* (*arrow*).

$GlcNAc_{itol}$ (Fig. 7). Thus, high mannose-type oligosaccharides attached to the mutant G protein were glucosylated in a posttranslational reaction, and the glucose residues were lost slowly during a subsequent chase.

Discussion

The wild-type G protein of VSV accumulates at the surface of virally infected cells where it is packaged into virions. During transport from its site of synthesis in the rough ER to the surface, the two asparagine-linked oligosaccharides attached to the G protein (at positions 178 and 335; reference 45) are processed to complex-type units (23, 38, 49, 52). In contrast to the wild-type protein, forms of the G protein encoded by the ts045 mutant of VSV (produced at the nonpermissive temperature) and the plasmid vector pdTM12 do not reach the plasma membrane. Both G proteins accumulate within the ER, and their asparagine-linked oligosaccharides remain in endo H–sensitive forms (1, 3). ^{3}H-Mannose–labeled oligosaccharides isolated from the two mutant forms of the G protein are high mannose–type units. However, unlike the oligosaccharides attached to other mutant forms of the G protein that accumulate in the ER (e.g., the proteins encoded by Δ1473 and Δ1554) most of the ts045 and pdTM12 G protein oligosaccharides possess a single glucose residue at a nonreducing terminus. Previous studies have also reported that the oligosaccharides associated with the ts045 G protein are larger than expected for deglucosylated high mannose structures (4, 54).

The initial glycosylation of an asparagine residue occurs by the en bloc transfer of a preformed oligosaccharide from dolichol pyrophosphate to the nascent protein chain within the rough ER; the oligosaccharide is invariant and contains 3 glucose, 9 mannose, and 2 *N*-acetylglucosamine residues (30, 51). The three glucose residues are located at a nonreducing terminus of the oligosaccharide and are linked Glcα1,2Gluα1,3Glcα1,3Man (51). Two separate glucosidase activities have been implicated in the removal of the glucose residues during the subsequent maturation of the glycoprotein (15, 19, 22, 35, 53). Glucosidase I is responsible for the removal of the terminal α1,2-linked residue, and glucosidase II is responsible for the removal of the inner two α1,3-linked residues. In view of the dual responsibility of glucosidase II and the previous observation that nascent polypeptide chains are substrates for glucosidase I and II (2), it was unexpected to find a single glucose residue associated with the G protein oligosaccharides.

The mechanism by which the mutant G proteins retain one glucose residue on their oligosaccharides while other ER proteins do not is unknown. The glucosylated oligosaccharides may arise (*a*) from an inability of the processing glucosidase to act due to the sequestration of the oligosaccharides within an altered conformation of the protein, (*b*) from the segregation of the proteins themselves to a region of the ER devoid of the processing glucosidase, or (*c*) from the selective posttranslational reglucosylation of the mutant G proteins within the lumen of the ER. Our results are most consistent with the latter two possibilities. It is difficult to envision how the dissimilar mutations encoded by pdTM12 and ts045 (pdTM12 has an eight-amino acid deletion within its transmembrane domain, and ts045 has a point mutation in the ectodomain of the G protein 268 amino acids from the pdTM12 deletion [1, 17]) could lead to conformations of the G protein that would allow for the removal of the outer two glucose residues yet sterically hinder the removal of the third residue. Moreover, since the ts045 oligosaccharides were deglucosylated with equal efficiency by rat liver glucosidase II in vitro at both the permissive and nonpermissive temperatures, the oligosaccharides are likely to be accessible to the glycosidase in vivo.

The possibility that the glucosylated G proteins reside within a region of the ER devoid of the glycosidase is more difficult to rule out. Several mutant membrane glycoproteins

that do not exit from the ER have been shown by immunocytochemical techniques to accumulate within subsets of ER cisternae (37, 40). In addition, evidence that the ER is compartmentalized has been provided previously from the posttranslational maturation of oligosaccharides attached to rotavirus glycoproteins. Kabcenell and Atkinson (24) reported that an early region of the ER contains an α-mannosidase activity that removed a single mannose residue from a $Man_9GlcNAc$ oligosaccharide attached to the rotavirus glycoprotein VP7, but further mannose trimming occurred only after movement of the glycoprotein to a separate region of the organelle. The glucosylated mutant G protein oligosaccharides were not processed by an ER mannosidase to units possessing fewer than eight mannose residues. It is likely, therefore, that the mutant G proteins produced by the COS-1 cells did not migrate beyond the compartment containing the initial processing α-mannosidase. While we can not exclude the possibility that the G proteins were segregated to a region of the ER devoid of glucosidase II, we believe this to be unlikely for the following reasons. First, ts045 G protein has been localized previously by immunoelectron microscopy, and the protein was found in all cisternae of the rough ER when the infected cells were maintained at the nonpermissive temperature (5, 6). Second, glucosidase II has been localized throughout the cisternae of the rough and smooth ER (32). Third, as discussed below, our data suggests that the glucose residues turn over.

The final mechanism to account for the glucose residue is that the G protein oligosaccharides are initially deglucosylated by glucosidases I and II, and then reglucosylated by the posttranslational addition of a single glucose residue. An enzyme capable of such a modification has been identified in rat liver slices by Parodi et al. (36). Since the ts045 G protein oligosaccharides were labeled with glucose under conditions where protein synthesis was arrested, the reglucosylation reaction must account for part, if not all, of the glucosylated oligosaccharides. When the galactose-labeled cells were chased at the nonpermissive temperature the specific radioactivity of the G protein oligosaccharides declined. Under identical conditions, however, the percentage of the G protein oligosaccharides that retained a glucose residue remained constant as assessed with the ^3H-mannose–labeled oligosaccharides. Thus, glucose residues associated with the mutant G protein high mannose oligosaccharides turn over more rapidly than do the glucosylated oligosaccharides, suggesting that the high mannose oligosaccharides cycle between glucosylated and nonglucosylated states.

Unlike the two G proteins analyzed in this study, other mutant forms of the viral glycoprotein do not contain the glucosylated oligosaccharides even though they reside within the ER (16). For example, the G protein encoded by plasmid Δ1473 contains an altered carboxy-terminal cytoplasmic domain and accumulates within the ER. The oligosaccharides associated with this G protein are nonglucosylated high mannose-type structures with eight mannose residues (16). Moreover, when cells infected with ts045 were labeled at the nonpermissive temperature and shifted to the permissive temperature in the presence of CCCP, the majority of the G protein oligosaccharides lost the glucose residues despite their persistence as high mannose–type structures. Residence of a G protein within the ER, therefore, is not sufficient for the oligosaccharides to be glucosylated. Rather, it appears that G proteins encoded by ts045 at the nonpermissive temperature and pdTM12 are selected to remain glucosylated. The reaction is not unique to mutant G proteins, however, as a recent report by Rizzolo and Kornfeld noted that a hybrid protein composed of rat growth hormone and the influenza hemagglutinin contained a high percentage of glucosylated high mannose oligosaccharides (41). Like the mutant G proteins, this hybrid molecule was restricted to the ER (40).

The selectivity of the modification suggests that G proteins which remain glucosylated within the ER are different from those forms that do not express the modification. The wild-type G protein trimerizes and this oligomerization precedes exit from the ER (11). The G protein encoded by Δ1473 trimerizes normally, but does not exit from the ER (12). In contrast, the G protein produced by ts045-infected cells at the nonpermissive temperature does not trimerize but, rather, assembles into higher molecular weight aggregates (11). At the permissive temperature the ts045 G protein disassembles from the aggregate and trimerizes normally (11, 28). Likewise, the G protein encoded by pdTM12 does not trimerize and assembles into aggregates (12). The two forms of the G protein that contain glucosylated oligosaccharides, therefore, possess the most abnormal structures as evidenced by their inability to trimerize. The glucosylated oligosaccharides may arise as a consequence of the accumulation of these aggregated G proteins within the ER. Alternatively, the glucose residue may reflect that these mutant G proteins were recognized by the ER-associated machinery as aberrant structures. In this regard, Lodish and Kong observed previously that inhibition of glucose removal from newly synthesized glycoproteins disrupts their transport from the ER (31). Thus, the selective glucosylation of aberrant proteins may provide a mechanism by which defective products are prevented from leaving the ER. A similar retention mechanism is provided by the binding protein BIP (7).

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