Identification of a Novel Mechanism for the Removal of Glucose Residues from High Mannose-type Oligosaccharides*

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The role of glucosylated oligosaccharides in the biogenesis of the glycoprotein (G protein) of vesicular stomatitis virus was studied in PhaR2.7, a mouse lymphoma cell line deficient in glucosidase II activity. As expected, the great majority of cell-associated G protein remained glucosylated in PhaR2.7, and the G protein was rapidly deglucosylated in BW5147, the parental cell line. Despite these differences in glucosylation, the rates of G protein trimerization and transport to the cell surface were as rapid and efficient in the PhaR2.7 mutant as in BW5147. Surprisingly, greater than 73% of the oligosaccharides on G proteins recovered from released virions were complex-type units. The efficient processing of the G protein oligosaccharides coincided with the efficient removal of glucose residues from its oligosaccharides. After treatment with deoxynojirimycin, an inhibitor of endoplasmic reticulum (ER) glucosidases I and II, the total percentage of G protein-associated high mannose-type oligosaccharides increased more in the parental cells than in the mutant cells. Furthermore, when the G protein was retained in the ER of PhaR2.7 cells by depletion of the cellular ATP pools with m-chlorophenylhydrazine, its oligosaccharides remained glucosylated. Under identical conditions, BW5147 cells removed the glucose residues from >90% of the retained G protein’s oligosaccharides. Thus, PhaR2.7 cells efficiently remove glucose residues from high mannose-type oligosaccharides of selected proteins using a deoxynojirimycin-insensitive enzyme located in a post-ER compartment. The existence of a second mechanism for the deglucosylation of N-linked oligosaccharides provides evidence for the important role of glucose removal in glycoprotein maturation.

N-linked glycosylation occurs cotranslationally by the en bloc transfer of a preformed oligosaccharide from a dolichol-linked donor to nascent polypeptides within the rough endo-

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The abbreviations used are: ER, endoplasmic reticulum; VSV, vesicular stomatitis virus; MEM, minimal essential medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; ConA, concavalin A; HPLC, high performance liquid chromatography; Mes, 4-morpholineethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)tetraacetic acid; dNM, deoxynojirimycin; CCCP, carbonyl cyanide m-chlorophenylhydrazone; endo H, endoglucosidase H; TBS, Tris-buffered saline.

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N-linked glycosylation occurs cotranslationally by the en bloc transfer of a preformed oligosaccharide from a dolichol-linked donor to nascent polypeptides within the rough endo-
MATERIALS AND METHODS

Cell Culture—PhaR77 cells and BW5147 cells were grown in α-MEM containing 0.5% fetal bovine serum, 20 mg/ml Heps, 100 units/ml penicillin, and 100 μg/ml streptomycin and maintained in an atmosphere of 5% CO2 at 37°C. Viral Infection—Cells (1–2 × 10^7 cells/ml) were mixed with 20 plaque-forming units/cell of wild-type VSV strain WT-2B in growth medium and incubated for 30 min at 32°C in an atmosphere containing 5% CO2. Touniform viral adsorption, the cell suspension was shaken every 10 min. The cells were harvested by centrifugation at 1,000 rpm for 5 min, and nonsorbed virus was discarded. The pellet was resuspended in growth medium at 1-5 × 10^7 cells/ml and incubated at 37°C in an atmosphere containing 5% CO2 for 4 h. Pronase E digestion of the G protein-expressing cells was harvested by centrifugation, and the resulting pellet was washed twice with glucose-free α-MEM. The cell pellet (1-5 × 10^7 cells) was resuspended at 10^5 cells/ml in 5 ml of the labeling medium (glucose-free α-MEM, 10% dialyzed fetal bovine serum, and 0.5–1 mM) of [2-14C]mannoto (American Radiolabeled Chemicals Inc., St. Louis, MO; 14 Ci/mmol) and incubated for 30 min at 37°C in 5% CO2. At the end of the labeling period, the cell suspension was diluted with α-MEM containing 10% fetal bovine serum, 1 mM sodium pyruvate, 10 mM glucose, 10 mM mannose, and 150 μM cycloheximide. When G protein was to be recovered from the chase medium, the final concentration of serum was reduced to 1% to minimize its interference with the immunoprecipitation. At each time point, the labeled cells were harvested by centrifugation, and the resulting pellet was washed twice with 5 ml of PBS at 4°C. The pelleted cells then were solubilized with 1 ml of lysis buffer containing 1% Triton X-100, 0.1% sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride in PBS. Chase also were centrifuged at 30,000 rpm for 60 min in a Beckman Ti-70.1 rotor to isolate virus-associated G protein. The resulting pellet was resuspended in Laemmli sample buffer (21).

Immunoprecipitation of G Protein—G protein was recovered from cell lysates as described previously (9). To immunoprecipitate extracellular G protein, media samples were adjusted to 0.1% Triton X-100, 0.04% deoxycholate, and 0.01% SDS and incubated on ice with 10 μg/ml rabbit anti-G protein. Antigen-antibody complexes were recovered by the addition of 100 μl of fixed Staphylococcus aureus cells/ml of the culture medium (a 10% suspension from Calbiochem). After a 30-min incubation on ice, the bacteria were collected by centrifugation at 12,000 × g for 20 min, washed five times with 10 mM Tris, pH 8, 10 mM EDTA, 1% Triton X-100, 0.4% deoxycholate, and 0.1% SDS, and washed once with 10 mM Tris, pH 8, 1 mM EDTA.

To immunoprecipitate G protein that had reached the plasma membrane, the cells were harvested by centrifugation, washed twice with 0.1% Triton X-100, 0.1% SDS, and resuspended from the disrupted cells with 20 μg of affinity-purified rabbit antibody to the VSV G protein. After incubation on ice for 50 min, the cells were harvested by centrifugation, washed twice in PBS containing 2 mg/ml bovine serum albumin, and solubilized in 200 μl of lysis buffer. Nuclei were removed by centrifugation in an Eppendorf microcentrifuge for 1 min. The antigen-antibody complexes were recovered as described above.

To quantify the amount of extracellular, cell surface, and total cell-associated G protein simultaneously, cells were separated from the chase medium by centrifugation. The G protein was recovered from the medium fraction as described above, and the cell fraction was washed twice with ice-cold PBS and split. Half of the cells were suspended in 1 ml of lysis buffer, and total cell-associated G protein was immunoprecipitated. The other half was used for the determination of cell surface G protein as detailed above. All immunoprecipitations were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography as described previously (8).

Isolation and Analysis of G Protein Oligosaccharides—Regions of the dried gels that contained radiolabeled G protein were excised, and radioactive glycopeptides were prepared by Pronase digestion. The glycopeptides were fractionated on concanavalin A (ConA)-Sepharose (Pharmacia LKB Biotechnology, Inc.) as described previously (22).

To quantitate the percentage of each fraction recovered from ConA-Sepharose, the average number of mannose residues present on the high mannose-type oligosaccharides was estimated using data from HPLC analysis of the α-mannosidase digestion products of high mannose-type oligosaccharides (see below). This analysis yielded essentially the same results for high mannose-type oligosaccharides isolated from affinity-purified or immunoprecipitated G protein, demonstrating that the normal glycosylated forms of the N-linked oligosaccharides are transferred to nascent G proteins in PhaR77 cells (not shown). The data on high mannose-type oligosaccharides isolated from extracellular G protein have therefore been presented (see Fig. 5). Assuming that the glycosylated and nonglycosylated high mannose-type structures contained the same number of mannose residues, the formula for the average number of mannose residues would be

\[
\text{Mann} = 1 + 3/4 \times \text{ManA} + 3/4 \times \text{ManB}
\]

where ManA are the cpm in the manninitol peak (fractions 20–25), ManG are the cpm in the mannose-N-acetylgalactosaminol peak (fractions 30–35), and ManB are the cpm in the Glc2Man7N-acetylgalactosaminol peak (fractions 70–90). Based on this formula, the high mannose-type oligosaccharides in BW5147 contained on average 7.3 mannose residues, and those in PhaR77 contained 6.4 mannose residues. Further, assuming that complex-type oligosaccharides (peaks I and II) contained 3 labeled mannose and 1 labeled fucose residue (the H is retained when mannose is converted to fucose), one must divide the amount of radioactivity in peak III by 1.8 (for BW5147) or 1.6 (for PhaR77) before estimating the percentage of the oligosaccharides that were processed to complex-type units.

High mannose-type glycopeptides (peak III from ConA-Sepharose) were dissolved in 0.250 ml of citrate-phosphate buffer, pH 5.6, and endoglycosidase H (endo H) was added to a final concentration of 50 milliunits/ml. Digestion of the high mannose-type oligosaccharides with jack bean α-mannosidase was performed as described previously (22). Exoglycosidase digests were analyzed by descending paper chromatography in ethyl acetate/pyridine/acetic acid/H2O (5/5/1/3). The dried chromatograms were cut into 1-cm strips, and the radioactivity associated with each strip was determined by liquid scintillation counting. HPLC analysis was performed as described previously (9).

Trimization Assay—Virus-infected cells (5 × 10^5 in 0.5 ml of α-MEM) were labeled with [35S]methionine for 3 min and incubated for 6 min in chase medium (α-MEM containing 10% fetal bovine serum, 10 mM glucose, 10 mM mannose, 1 mM sodium pyruvate, 100 units/ml penicillin, and 100 μg/ml streptomycin) at 37°C. At the end of the chase, the labeled cell suspension was diluted with an equal volume of lysis buffer (40 mM Mes, 60 mM Tris, 2.5 mM EDTA, 2 mM EGTA, pH 5.5) as described by Doms et al. (23). Nuclei were pelleted by centrifugation in an Eppendorf microcentrifuge for 1 min. 350-μl fractions were collected. The VSV proteins were immunoprecipitated from each fraction and analyzed by polyacrylamide gel electrophoresis as described above. The locations of the monomer and trimers were determined by autoradiography of N/N acetylated N-acetylgalactosaminoglycans gradients. Radioactivity in the G protein bands was quantified by Pronase digestion followed by scintillation counting.

RESULTS

Kinetics of Trimerization and Secretion of G Protein by PhaR77 and BW5147 Cells—To determine whether glucosidase II deficiency inhibited the transport of wild-type G protein, VSV infected PhaR77 and BW5147 cells were pulse labeled with [35S]methionine and chased in the absence of the isotope. The distribution of the [35S]-labeled G protein was determined with respect to the cell surface and the total cell-associated fraction by selective immunoprecipitation (see “Materials and Methods”). At the end of the 10-min pulse, no radiolabeled G proteins were recovered from either the medium or the cell surface (Fig. 1, A and B). However, [35S]-labeled G proteins were detected at the cell surface and in the medium of both the wild-type (Fig. 1A) and the PhaR77 mutant (Fig. 1B) cells after 30 min of chase. The amounts of radiolabeled G protein recovered in each of the three fractions was quantified after excision of the radioactive regions of the gel (Fig. 2). The amount of radioactive G protein produced was higher in BW5147 cells than in PhaR77 cells (compare the zero time points in Figs. 2, A and B). This difference was seen consistently and may be caused by different rates of methionine uptake by the mutant and wild-type cells or differences in their infectibility. The kinetics of G protein transport to the
cell surface were very similar in the two cell types. Maximal levels of G protein surface expression were detected after 60 min of chase, when 17% of the total G protein synthesized by BW5147 cells and 19% of that synthesized by PhaR2.7 cells was surface-associated (Fig. 2 A and C). Much of the surface G protein was recruited into virus particles and released into the medium (Fig. 2 A and C).

Interestingly, G proteins appeared to assemble into budding virions more efficiently in PhaR2.7 than in BW5147 cells. G protein produced by the mutant cells entered virus particles immediately after it reached the cell surface (Fig. 2 C). In contrast, there was a lag of approximately 60 min between the time G protein reached the surface of BW5147 cells and the time that it entered virus particles (Fig. 2 A). The cause of this difference in budding efficiency is unknown.

Under normal conditions the newly synthesized VSV G proteins oligomerize into trimers before they reach the Golgi apparatus (23, 24). To determine whether G proteins produced by PhaR2.7 cells oligomerized normally, oligomerization was compared in the wild-type and mutant cell lines. VSV-infected cells that were labeled with [35S]methionine for 3 min and chased for 6 min were harvested and lysed, and the VSV proteins were fractionated on a sucrose density gradient (23). G proteins produced by the wild-type (Fig. 3 A and the mutant (Fig. 3 B) cell lines were distributed in a biperic pattern corresponding to trimers (fractions 7–9) and monomers (fractions 4–6). As seen previously by Doms et al. (23), the N/NS and M proteins sedimented as monomeric species (Fig. 3). In summary, G proteins produced by PhaR2.7 cells were assembled into trimers and transported to the cell surface as efficiently as those produced by BW5147 cells. In addition, surface G protein was assembled into virus particles more efficiently in PhaR2.7 cells than in their wild-type counterparts.

Characterization of the Oligosaccharides on G Proteins Released from PhaR2.7 Cells—We next investigated whether oligosaccharides associated with the rapidly transported G proteins were processed to complex-type units in PhaR2.7 and BW5147 cells. VSV-infected cells were labeled with [2-3H]mannose for 30 min and chased for 4 h at 37 °C after which G protein was isolated from the chase media. Glycopeptides were prepared by Pronase digestion and characterized by ConA-Sepharose chromatography. G proteins produced by the wild-type cells yielded primarily complex-type units that eluted in peaks I and II from ConA-Sepharose (Fig. 4 A). Surprisingly, G protein produced by PhaR2.7 cells also yielded glycopeptides that eluted as complex-type units (Fig. 4 B); 63% of the total radioactivity was recovered as complex-type units and 37% as high mannose-type units. Based on the number of mannose residues associated with each of these structural classes accounted for (see "Materials and Methods"), 73% of the oligosaccharides were complex-type units. By the same analysis, 98% of the G protein oligosaccharides produced by the wild-type cells were complex-type structures. Since glucose residues must be removed for an N-linked oligosaccharide to mature to a complex-type unit, the PhaR2.7 cells deglucosylated the majority of the G protein oligosaccha-
products yielded two peaks that corresponded to mannitol and protein oligosaccharides produced by the wild-type cells. Exoglycosidase releases only terminal α-linked mannose residues from these fractions were analyzed by gel electrophoresis and fluorography.

The high mannose-type glycopeptides (ConA-Sepharose peak III) were digested with endo H, and the released oligosaccharides were treated with jack bean α-mannosidase. The products of the α-mannosidase digestion were reduced with NaBH₄ and analyzed by HPLC. The HPLC profile of BW5147 cell products yielded two peaks that corresponded to mannitol, and the disaccharide mannose-N-acetylglucosaminitol (Fig. 5A; fractions 28–30); the β-linked mannose residue of the core oligosaccharide is resistant to α-mannosidase and, as a result, yields the disaccharide. All of the G protein oligosaccharides produced by the wild-type cells, therefore, were deglucosylated. In contrast, when the high mannose-type oligosaccharides derived from G protein released by PhaR².7 cells were subjected to α-mannosidase digestion, three products were produced that eluted in the positions expected for mannitol, were resistant to n-mannosidase digestion. Based on the assumption that the resistant species contained 4 mannose residues, 74% of the extracellular G protein produced by PhaR².7 cells was glucosylated. This represents less than 20% of all the G protein associated oligosaccharides in PhaR².7 cells.

Deoxynojiririmycin Does Not Prevent Deglucosylation of G Protein’s Oligosaccharides by PhaR².7 Cells—When assayed for glucosidase II using p-nitrophenyl α-D-glucopyranoside as a substrate, extracts of our PhaR².7 cell line contained less than 1% of the specific activity of BW5147 cells (not shown); this is consistent with previous estimates of residual glucosidase II activity within these cells (20). To ascertain whether this residual glucosidase II activity was responsible for deglucosylation of the G protein, the sensitivity of the viral glycoprotein’s maturation to deoxynojirimycin (dNM) was determined. dNM is a glucose analog that inhibits glucosidase II activity both in vitro and in vivo (25, 26). Two h after VSV infection, PhaR².7 or BW5147 cells were treated with dNM for 2 h and then labeled with [2-¹⁴C]mannose for 30 min. The cells subsequently were chased for 80 min in the presence of dNM.

G protein produced by dNM-treated BW5147 cells contained a greater fraction of high mannose-type oligosaccharides (Fig. 6B) than did G protein isolated from nontreated BW5147 cells (Fig. 6A and Table I). In the absence of dNM, only 43% of the radioactivity associated with ¹⁴C-labeled glycopeptides eluted from ConA-Sepharose as high mannose-type structures (Fig. 6A and Table I). Treatment of the cells with dNM increased the radioactivity associated with high mannose-type oligosaccharides recovered from cell-associated G proteins to 80% of the total (Fig. 6B and Table I). In contrast, dNM did not inhibit significantly the processing of the G protein-associated oligosaccharides in PhaR².7 cells. In the absence and presence of dNM, the radioactivity associated with high mannose-type units comprised 61 and 75%, respectively, of the total G protein-linked radioactive oligosaccharides (Fig. 6, C and D). Thus, dNM inhibited the maturation of cell-associated G protein more effectively in wild-type than in PhaR².7 cells.

To examine the glucosylation state of the high mannose-type oligosaccharides, these units were subjected to α-mannosidase digestion; the percentage of glucosylated high man-
The resulting glycopeptides were applied to ConA-Sepharose, and the columns were washed with TBS and 56 mM a-methyl glucoside in TBS. The oligosaccharides in turn were digested with a-mannosidase, reduced with NaBH₄, and analyzed by HPLC. The peaks of radioactivity eluted in the positions expected for mannitol (fractions 20-25, 2,330 cpm in panel A and 1,198 cpm in panel B), mannose-N-acetylglucosaminol (fractions 30-35, 358 cpm, panel A and 98 cpm, panel B), and the oligosaccharide Glc₃Man₉N-acetylglucosaminol (fractions 70-90, 98 cpm, panel A and 1,097 cpm, panel B).

FIG. 5. Virus-associated VSV G protein produced by PhaR²⁻⁷ cells contains glucosylated high mannose-type oligosaccharides. High mannose-type glycopeptides derived from G proteins released by BW5147 (A) or PhaR²⁻⁷ (B) cells were digested with endo H. The oligosaccharides in turn were digested with a-mannosidase, reduced with NaBH₄, and analyzed by HPLC. The peaks of radioactivity eluted in the positions expected for mannitol (fractions 20-25, 2,330 cpm in panel A and 1,198 cpm in panel B), mannose-N-acetylglucosaminol (fractions 30-35, 358 cpm, panel A and 98 cpm, panel B), and the oligosaccharide Glc₃Man₉N-acetylglucosaminol (fractions 70-90, 98 cpm, panel A and 1,097 cpm, panel B).

In summary, dNM treatment greatly inhibited the deglucosylation of the high mannose-type oligosaccharides attached to the G protein produced by BW5147 cells. However, dNM was less effective in reducing the processing of oligosaccharides in PhaR²⁻⁷ cells despite the fact that the specific activity of glucosidase II was less than 1% that present in the parental cells. The PhaR²⁻⁷ cells, therefore, must possess elevated levels of a compensatory dNM-insensitive deglucosylating activity.

PhaR²⁻⁷ Cells Remove the Glucose Residues from the G Protein in a Post-ER Compartment—To determine the intracellular location of this compensatory deglucosylating activity, the extent of deglucosylation of G proteins that were retained in the ER was assessed. PhaR²⁻⁷ and BW5147 cells were infected with VSV and labeled with [2-³H]mannose for 15 min. At the end of the labeling period, the cells either were harvested and lysed immediately or were chased for 90 min in the presence or absence of CCCP. CCCP is an uncoupler of oxidative phosphorylation that lowers cellular ATP levels sufficiently to prevent export of proteins from the ER (27, 28). Cycloheximide also was added to the chase medium to block subsequent protein synthesis and, thus, improve the effectiveness of the chase. G protein recovered from both cell types immediately after the labeling period contained only high mannose-type oligosaccharides (Table II). When BW5147 cells were chased for 90 min in the absence of CCCP, the total G protein-associated radioactivity declined; this loss reflects processing of the N-linked oligosaccharides, budding of G protein containing virions into the medium, and degradation. As expected, many of the oligosaccharides were processed to complex-type units during the chase; only 52% of the radioactivity associated with the G protein was recovered as high mannose-type oligosaccharides (Table II). When BW5147 cells were chased in the presence of CCCP, the G protein oligosaccharides remained primarily as high mannose-type structures (Table II). Again, the total radioactivity decreased, suggesting that G protein was degraded within the ER of the transport-impaired cells (29). The total amount of radioiodinated G protein isolated from PhaR²⁻⁷ cells also declined during the chase in the presence and absence of CCCP (Table II). In the absence of the ionophore the fraction of G protein oligosaccharides that were high mannose-type structures declined, but CCCP prevented the processing of the high mannose-type oligosaccharides to complex-type units.

FIG. 6. Deglucosylation of the G protein's high mannose-type oligosaccharides in PhaR²⁻⁷ cells is insensitive to dNM. VSV-infected BW5147 (A and B) and PhaR²⁻⁷ (C and D) cells were treated with (B and D) or without (A and C) dNM for 2 h (at 2 h postinfection), labeled with [³H]mannose for 30 min, and chased in the presence or absence of dNM for 80 min. G proteins were immunoprecipitated from extracts of the radioiodinated cells and analyzed by polyacrylamide gel electrophoresis. The regions of the dried gels containing G protein were excised, and the radioactivity was solubilized by Pronase digestion. The resulting glycopeptides were applied to ConA-Sepharose, and the columns were eluted with TBS (peak I), 10 mM a-methyl glucoside in TBS (peak II), and 100 mM a-methyl mannoside in TBS at 56 °C (peak III).

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The quantity of the various glycopeptide species recovered after ConA-Sepharose chromatography (Fig. 6) is indicated as the percentage of total cpm present in the oligosaccharides eluting in ConA-Sepharose peak III. High mannose-type glycopeptides (ConA-Sepharose peak III of Fig. 6) were digested with endo H and α-mannosidase. The digestion products were analyzed by descending paper chromatography, and the percentage of glucosylated oligosaccharides was calculated based on the amount of radioactivity recovered in the α-mannosidase-resistant fraction (R) and with the disaccharide mannose-N-acetylglucosamine peak (S) using the formula:

$$\% \text{ glucosylated} = \frac{100^* (R/4)}{(R+S)}$$

### Table I

**Differential effect of dNM on G protein oligosaccharide maturation**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Total cpm</th>
<th>% cpm in peak I</th>
<th>% cpm in peak II</th>
<th>% cpm in peak III</th>
<th>% cpm in peak IV</th>
<th>% cpm in peak V</th>
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<tr>
<td>BW5147</td>
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<tr>
<td>PhaR²⁻²¹</td>
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<td>98</td>
<td>79</td>
<td>12,542</td>
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### Table II

**Deglucosylation of G protein oligosaccharides by PhaR²⁻²¹ cells in a post-ER compartment**

VSV-infected cells were pulse labeled with [³H]mannose for 15 min and chased for 90 min in the presence (+) or absence (-) of CCCP. Glycopeptides were prepared from the immunoprecipitated G protein, and these were treated with endo H and α-mannosidase; the digestion products were analyzed by descending paper chromatography. The percentage of the high mannose-type units that were glucosylated was determined as detailed in Table I.

### Discussion

High mannose-type oligosaccharides attached to several mutant forms of the VSV G protein remain glucosylated within the ER. In part, these glucosylated structures are formed by the post-translational deglucosylation of the glucosylated precursor oligosaccharide (9). The function of this post-translational modification is unknown. However, the persistence of the glucosylated high mannose-type oligosaccharides on the aberrant G proteins in vivo coupled with the recent demonstration that denatured thyroglobulin is a better substrate than the native protein for deglucosylation in vitro (30) suggested that glucosylation serves as a component of a cellular proofreading machinery.

In this study, we found the wild-type G protein reaches the plasma membrane with normal kinetics in PhaR²⁻²¹ cells (Fig. 2). Moreover, the majority of the oligosaccharides attached to the extracellular G protein were complex-type units (Fig. 4). Since removal of glucose residues from the precursor high mannose-type oligosaccharide is required for the formation of complex-type structures, G proteins produced by the mutant cells must be deglucosylated efficiently. This was unexpected since our PhaR²⁻²¹ cells possess less than 1% of the glucosidase II activity of BW5147 cells (not shown), the wild-type cell line from which PhaR²⁻²¹ was derived. Previous studies indicated that PhaR²⁻²¹ cells contain a much higher level of glucosylated high mannose-type oligosaccharides on endogenous proteins than do BW5147 cells (20). The VSV G protein, therefore, must be an especially good substrate for the residual glucosidase II activity or for a novel enzyme that is present within these mutant cells.

We have presented two lines of evidence which indicate that the mutant cells contain a novel deglucosylating activity. First, removal of the glucose residues from the G protein required normal cellular ATP levels. When ATP synthesis was inhibited with CCCP, an uncoupler of oxidative phosphorylation, glucose residues were not removed from the high mannose-type oligosaccharides of G protein produced by PhaR²⁻²¹ cells. Glucosidase II does not require ATP in vitro (32), and CCCP-treated BW5147 cells continued to remove glucose residues efficiently from the G protein in vivo. Although the enzyme responsible for the removal of the glucose residues from the G protein in PhaR²⁻²¹ cells may require ATP for its activity, we believe that it is more likely that the deglucosylating enzyme resides within a post-ER compartment and that ATP is required for the G protein to reach this compartment. Second, glucosidase II is inhibited by the glucose analog dNM, and treatment of infected BW5147 cells with dNM increased the number of glucosylated high mannose-type oligosaccharides on G protein 2.8-fold. Similarly, Schlessinger et al. (31) showed that dNM prevents the deglucosylation of G protein in normal chick embryo fibroblasts. If residual glucosidase II were responsible for deglucosylation of with glucosylated oligosaccharides.
the G protein, then these cells would be expected to be more sensitive to dNM treatment than BW5147 cells. However, treatment with dNM only slightly impaired the removal of glucose residues from the G protein oligosaccharides produced by Pha<sup>R2.7</sup> cells.

Thus, our data suggest that Pha<sup>R2.7</sup> cells contain a dNM-insensitive enzyme that deglucosylates the G protein's oligosaccharides in a post-ER compartment. Since the dNM-treated Pha<sup>R2.7</sup> cells processed G proteins more efficiently than dNM-treated BW5147 cells, the mutant cells may have up-regulated this alternative pathway to compensate for their deficiency in glucosidase II activity. A possible candidate for this alternate activity is the endomannosidase described by Lubas and Spiro (33, 34). Using the in vitro processing activity by PhaR2.7 cells, we conclude that the G proteins released from PhaR2.7 cells contained primarily complex-type N-linked oligosaccharides that were deglucosylated in a post-ER compartment. Since the G protein was transported to the cell surface with normal kinetics in Pha<sup>R2.7</sup> cells, we conclude that glucose residues do not hinder the transport of the G protein to this post-ER compartment. An intriguing possibility suggested by recent work with GRP78/Bip (35) is that glucosylated proteins may migrate to an intermediate compartment between the ER and the Golgi apparatus but recycle to the ER unless the glucose residues are removed.

In conclusion, our data indicate that Pha<sup>R2.7</sup> cells have compensated for a deficiency in glucosidase II activity by upregulating a second system capable of efficiently deglucosylating glycoproteins in a post-ER compartment. These observations suggest that the removal of the glucose residues is of great importance to proper cell function.

**Acknowledgments—** We thank Linda Friedman and Edith Abreu for the photographic and graphic artwork.

**REFERENCES**