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Biochemistry

Oligosaccharide chains are trimmed during synthesis of the envelope glycoprotein of vesicular stomatitis virus

(glycoprotein biosynthesis/glycosylation/precursor glycopeptides/endoglycosidases)

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ABSTRACT **The biosynthesis and maturation of the oligosaccharide moieties of the envelope glycoprotein of vesicular stomatitis virus were investigated in virus-infected HeLa and BHK21 cells after pulse labeling with [2-^{3}H]mannose. Two major forms of the virus glycoprotein were detected by polyacrylamide gel electrophoresis, which appear to correspond to the viral glycoprotein with either "precursor" or "mature" oligosaccharide chains. The precursor chains in both HeLa and BHK21 cells infected with vesicular stomatitis virus obtained after a 30-min pulse were large oligomannose structures containing approximately 7–9 mannose residues as estimated by gel filtration analysis. The size of the oligomannose structures initially transferred to the protein may have been even larger. Mature, virus-size oligosaccharide chains, which could be detected after a 20- to 30-min delay, contained only three mannose residues and, in addition, contained branch structures terminating in sialic acid. A precursor–product relationship of these two forms of oligosaccharide chains was demonstrated by pulse–chase labeling of virus-infected HeLa cells. These studies indicated that the large oligomannosyl core structures initially added to the glycoprotein were being "trimmed" by the removal of mannose residues prior to (and/or during) the addition of the branch chains terminating in sialic acid.**

Vesicular stomatitis virus (VSV) is an enveloped, RNA-containing virus of the rhabdovirus group which matures by budding through host cell membranes that have been modified by replacement of host proteins with two virus-specified proteins, one of which is a glycoprotein (1). The VSV-infected animal cell is an excellent experimental system for elucidating the molecular and subcellular mechanisms involved in the biosynthesis and maturation of both cell membrane and virus envelope glycoproteins (2–8).

The VSV G polypeptide contains two major glycosylation sites with complex oligosaccharides that are linked *N*-glycosidically to asparagine (9–11). As expected for the biosynthesis of a membrane glycoprotein, mRNA coding for VSV G is localized on membrane-bound polyribosomes in infected cells (12, 13), and newly synthesized G is found predominantly in rough endoplasmic reticulum membranes (14). The sugar residues in the oligomannosyl core [$Man_n(GlcNAc)_2$] are apparently added to the G polypeptide in the rough endoplasmic reticulum, whereas the branch sugars (NeuNAc-Gal-GlcNAc) and fucose are apparently added in smooth internal membranes prior to the appearance of the mature glycoprotein at plasma membranes (15). The initial glycosylation event probably occurs by the *en bloc* transfer of a preformed oligomannosyl core structure from a lipid intermediate (16).

We have examined the product of the initial glycosylation of the VSV G glycoprotein and followed its conversion to mature, virus-size oligosaccharide chains in VSV-infected HeLa and BHK21 cells. Our results indicate that a large oligomannosyl core structure [$Man_{\geq 7}(GlcNAc)_2$] is initially added to the glycoprotein. Either during or shortly before the addition of the distal branch sugars at another intracellular site, these large oligomannosyl core structures are "trimmed" to virus-size oligomannosyl cores [$Man_3(GlcNAc)_2$] by the removal of mannose residues.

This finding demonstrates an unprecedented mechanism that explains how the large oligomannosyl structures observed for dolichol-linked oligosaccharides can be used to generate the smaller oligomannosyl structures found on glycoproteins. In addition, it suggests that the two major classes of oligosaccharide structures linked to asparagine in glycoproteins may share an intermediate.

MATERIALS AND METHODS

Cells, Virus, Infection, and Radioactive Labeling. HeLa S3 cells (human) were grown in suspension culture (14) and BHK21 cells (baby hamster kidney) were grown in monolayers (11). The Indiana serotype of VSV was grown and purified as previously described (3, 17). VSV radiolabeled with [1-^{14}C]-glucosamine (New England Nuclear, 45–55 mCi/mmol) was prepared from VSV-infected HeLa cells. VSV-infected HeLa and BHK21 cells were pulse labeled with [2-^{3}H]mannose (Amersham/Searle, 2 Ci/mmol) as described previously (14, 15). The pulse–chase labeling of VSV-infected HeLa cells was similar except that following a 30-min incubation with 20 μCi of [2-^{3}H]mannose per ml, the cells were concentrated by centrifugation and resuspended in 10 times the original volume (final concentration of 5×10^5 cells per ml) of medium containing 10 mM unlabeled mannose. Aliquots were removed from the culture and assayed for trichloroacetic acid-insoluble radioactivity during the course of both the pulse and the chase periods.

Preparation of Membrane Glycoprotein and Digestion with Pronase. Membrane pellets were prepared from clarified cell homogenates (14) by centrifugation for 10 min at 20,000 $\times$ *g*. The pellet was resuspended in 1 mM EDTA/10 mM Tris·HCl, pH 7.4, containing 1% NP-40 (Shell Oil Co.), incubated for 30 min at room temperature, and centrifuged again for 10 min at 20,000 $\times$ *g*. The supernatant fraction containing detergent-solubilized membrane protein was used for both polyacrylamide gel electrophoresis and preparation of Pronase-digested glycopeptides after removal of the NP-40 and precipitation of the protein by 1-butanol extraction (15).

Gel Filtration of Glycopeptides. Pronase-digested glycopeptides were analyzed by gel filtration through either a Bio-Gel P-4 column or a Sephadex G-15/G-50 column (14). Neutral

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Abbreviation: VSV, vesicular stomatitis virus.

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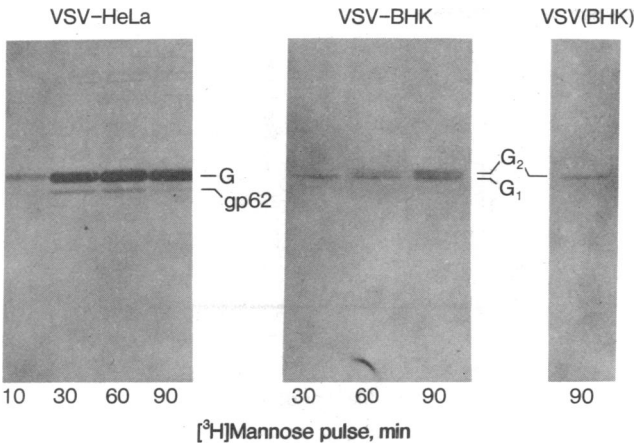

FIG. 1. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis of [^{3}H]mannose-labeled membrane glycoprotein from VSV-infected HeLa and BHK21 cells. Cells were pulse labeled for the indicated times and cell membrane glycoproteins were electrophoresed in 10% polyacrylamide slab gels as previously described (14, 15). Bands corresponding to radiolabeled glycoproteins were detected by fluorography of the dried gel (19). The *Right* panel displays the profile for VSV released by the virus-infected BHK21 cells after a 90-min pulse. G, G_1, and G_2 refer to the major forms of the VSV glycoprotein observed in VSV-infected HeLa and BHK21 cells.

oligomannosyl cores from endo-β-*N*-acetylglucosaminidase digestions were analyzed by gel filtration through a Bio-Gel P-2 column (8). The asparaginyloligosaccharides prepared from Pronase digests of ovalbumin were used as molecular weight markers as described (11) and were radiolabeled by acetylation with either [^{3}H]- or [^{14}C]acetic anhydride (New England Nuclear) in 0.5 M $NaHCO_3$.

Glycosidase Digestion of Glycopeptides. Glycopeptides were digested with exoglycosidases and endo-β-*N*-acetylglucosaminidase D as described (11). Endo-β-*N*-acetylglucosaminidase H was prepared from *Streptomyces plicatus* (American Type Culture Collection no. 27800: *Streptomyces* sp.) (18). Digestions with endo-β-*N*-acetylglucosaminidase H (5–10 milliunits/ml) were carried out in 0.05 M sodium citrate buffer, pH 5.5, for 24 hr at 37°. The oligomannosyl neutral core products ($Man_nGlcNAc$) of the endo-β-*N*-acetylglucosaminidase digestions were isolated by passing the digests through a column of Dowex AG1-X2 (formate) as described elsewhere (8). Digestions with jack bean α-mannosidase (92 international units/mg of protein) were carried out in 0.2 ml of 0.1 M NaOAc, pH 4.2, containing 0.15 M NaCl and 0.1 mM $Zn(OAc)_2$, for 4 days at 37° with daily addition of 2 units of the enzyme. All digestions were incubated under toluene and enzymes were inactivated at 100° for 2–3 min before analysis of the products.

RESULTS

"Precursor" and "Mature" Forms of the Cell-Associated VSV Glycoprotein and Oligosaccharide Chains. The VSV glycoprotein G was the only major species observed after pulse labeling of infected cells with [2-^{3}H]mannose (Fig. 1). A minor glycoprotein, gp62, is detected in HeLa cells in addition to the single major virus glycoprotein (15). It is thought to be a variant of the G protein that may lack some of the G polypeptide chain or possibly one of the oligosaccharide chains; it does not appear to be a precursor of mature G in HeLa cells. In VSV-infected BHK21 cells two major forms of the VSV glycoprotein were observed; the more slowly migrating G_2 form was only detected after longer pulse-labeling periods. The order of appearance

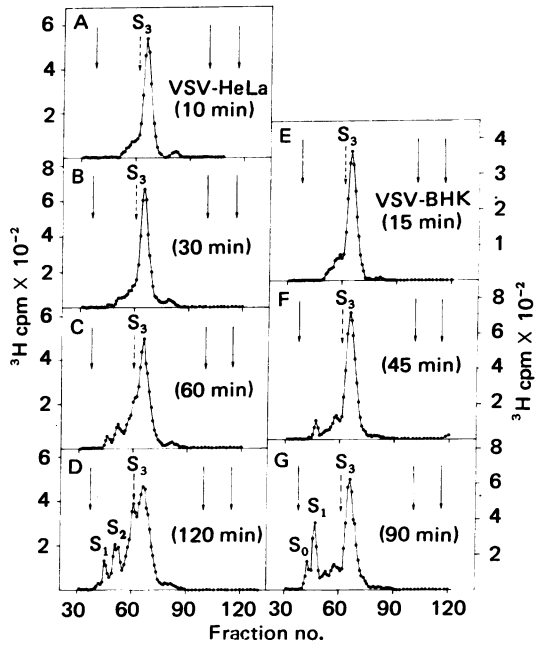

FIG. 2. Bio-Gel P-4 gel filtration of glycopeptides from [^{3}H]-mannose-labeled, VSV-infected cells. The profiles in the left panels (*A–D*) represent glycopeptides derived from the membrane glycoproteins of VSV-infected HeLa cells that were pulse labeled for 10, 30, 60, and 120 min. The right panels show similar samples from VSV-infected BKH21 cells after pulse labeling for 15, 45, or 90 min. The solid arrows indicate, from left to right, the peak elution positions of blue dextran 2000 or bovine serum albumin (void volume; molecular weight approximately 4000), stachyose (molecular weight 666), and mannose (molecular weight 180). S_0, S_1, S_2, and S_3 correspond to virus-size glycopeptides that differ in their amount of sialic acid (3, 2, 1, and 0 residues, respectively). The broken arrows indicate the peak elution position of [^{14}C]glucosamine-labeled asialoglycopeptides (S_3) from purified VSV glycoprotein.

of G_1 and G_2 and the absence of G_1 in released virions from BHK21 cells suggested a precursor–product relationship between the two forms.

The radiolabeled VSV glycoprotein preparations obtained after pulse labeling of VSV-infected cells with [2-^{3}H]mannose for increasing lengths of time were exhaustively digested with Pronase, and the resulting glycopeptides were analyzed by gel filtration through Bio-Gel P-4. With pulse-labeling periods of 30 min or less, a single major peak was observed with VSV-infected HeLa and BHK21 glycopeptides (Fig. 2 *A*, *B*, and *E*). These precursor glycopeptides had a lower apparent molecular weight than the asialoglycopeptides from the virion (S_3 glycopeptides). Virus-size glycopeptides appeared after longer pulse-labeling periods, with the S_3 peak predominating in the mature glycopeptides from HeLa cells (Fig. 2 *C* and *D*) and the S_1 peak predominating in the samples from BHK21 cells (Fig. 2 *F* and *G*). The relatively small amount of S_2 and S_3 glycopeptides in the VSV-infected BHK21 cells after the 90-min labeling period (Fig. 2*G*) confirmed earlier suggestions (15) that terminal sialic acid residues were added almost immediately after the addition of branch *N*-acetylglucosamine and galactose to the oligomannosyl core. The large amount of terminal sialic acid in the virus-size oligosaccharides from BHK21 cells accounts for the increased resolution between precursor and mature glycopeptides and could also account for the resolution of the G_1 and G_2 forms of the virus glycoprotein in VSV-infected BHK21 cells (Fig. 1).

Precursor Oligosaccharides Contain Large Oligomannosyl Cores. To characterize the oligosaccharide structures of this

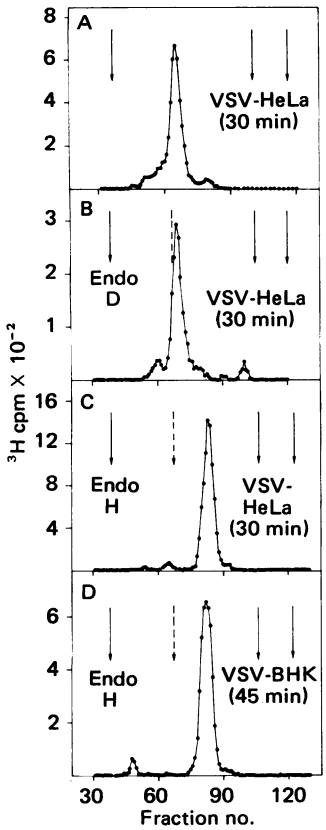

FIG. 3. Bio-Gel P-4 gel filtration of glycosidase-treated precursor glycopeptides from VSV-infected cells. The profile for the untreated glycopeptides from the 30-min pulse-labeled VSV-infected HeLa cells (*A*) is identical to Fig. 2*A*. A portion of this sample was digested prior to gel filtration with either: (*B*) a mixture of exoglycosidases and endo-β-*N*-acetylglucosaminidase D, or (*C*) endo-β-*N*-acetylglucosaminidase H. The glycopeptides from the 45-min pulse-labeled VSV-infected BHK21 cells were similarly treated with endo-β-*N*-acetylglucosaminidase H and subjected to gel filtration (*D*). The gel filtration markers are identical to those in Fig. 2 except that the broken arrow indicates the peak elution position of Asn[*acetyl*-^{14}C](GlcNAc)$_2$Man$_5$ (molecular weight 1391) cochromatographed with the [^{3}H]mannose-labeled glycopeptides.

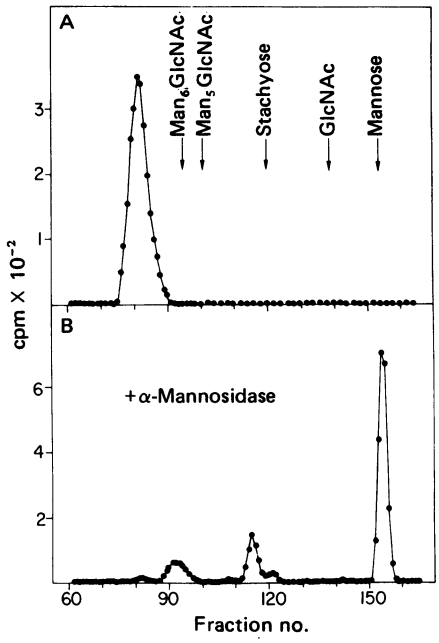

FIG. 4. Bio-Gel P-2 gel filtration analysis of the neutral oligosaccharides from the endo-β-*N*-acetylglucosaminidase digestion of precursor glycopeptides. The [^{3}H]mannose-labeled glycopeptides from the 30-min pulse-labeled VSV-infected HeLa cells were digested with endoglycosidase H for 24 hr. NH_4OH was added to the digest to a final concentration of 0.1 M and the digest was applied immediately to a pasteur pipette column of Dowex AG1-X2 (formate) that had been equilibrated with distilled water. The column was eluted with 3–5 column volumes of distilled water and the effluent containing the neutral oligomannosyl core product was lyophilized, redissolved in 0.2 ml of H_2O, applied to a 0.9- × 175-cm column of Bio-Gel P-2, and eluted with 1 mM NaN_3. (*A*) Neutral oligomannosyl core from precursor glycopeptides; (*B*) same sample as in *A* after treatment with α-mannosidase. The arrows indicate the peak elution positions of markers run in similar analyses.

initial glycosylation product, glycopeptides from samples pulse labeled for short times were subjected to digestion with specific glycosidases and analyzed by gel filtration (Fig. 3). These glycopeptides were resistant to a treatment with a mixture of exoglycosidases and endo-β-*N*-acetylglucosaminidase D (Fig. 3 *A* and *B*) that completely digests virion glycopeptides to a mixture of free branch sugars, the peptide containing the proximal *N*-acetylglucosamine, and a neutral oligosaccharide containing the oligomannosyl core (10, 11). This suggested that these glycopeptides did not contain branch sugars (NeuNAc-Gal-GlcNAc) and that the structure of the oligomannosyl core was different from that in the mature, virus-size glycopeptides. A small amount of mannose-labeled material was digested from glycopeptides eluting ahead of the precursor peak and elutes in the position expected for virus-size neutral cores (Man$_3$GlcNAc; fraction 100 in Fig. 3*B*).

In contrast, the precursor glycopeptides from both HeLa and BHK21 cells infected with VSV were completely digested by the endo-β-*N*-acetylglucosaminidase H from *Streptomyces*, which is capable of cleaving between the two proximal *N*-acetylglucosamine residues in glycopeptides with oligomannosyl cores larger than those that can be cleaved by endo-β-*N*-acetylglucosaminidase D (18) (Fig. 3 *C* and *D*). The endo-β-*N*-acetylglucosaminidase H mannosyl core product from both samples eluted as a single major peak considerably larger than the oligomannosyl core product from mature, virus-size glycopeptides. These products were shown to be neutral oligosaccharides by ion-exchange chromatography (data not shown). These results suggested that the precursor oligosaccharide chains might be similar, if not identical, to the dolichol-bound oligosaccharides postulated as intermediates in the initial glycosylation process (20).

An estimate of the number of mannose residues was obtained by comparative gel filtration of the neutral product from the endo-β-*N*-acetylglucosaminidase H digestion with glycopeptides and oligosaccharides of known size and structure. Comparison of the precursor glycopeptides with Asn[*acetyl*-^{14}C]-(GlcNAc)$_2$Man$_5$ (molecular weight 1391) by gel filtration through a column of Sephadex G-15/G-50 showed that the precursor glycopeptide was several hundred daltons larger than the marker (data not shown). This difference may be due to the oligosaccharide portion, the peptide portion, or both. The neutral oligomannosyl core product was approximately the same size as the marker glycopeptide (data not shown).

Analysis of the precursor neutral oligosaccharide on Bio-Gel P-2 (Fig. 4*A*) indicated that this structure contained at least one and possibly several more mannose residues than the Man$_6$GlcNAc oligosaccharide obtained from the endoglycosidase H digestion of Asn(GlcNAc)$_2$Man$_6$. Digestion of this precursor oligosaccharide product of the endoglycosidase H digestion with α-mannosidase released greater than 85% of the

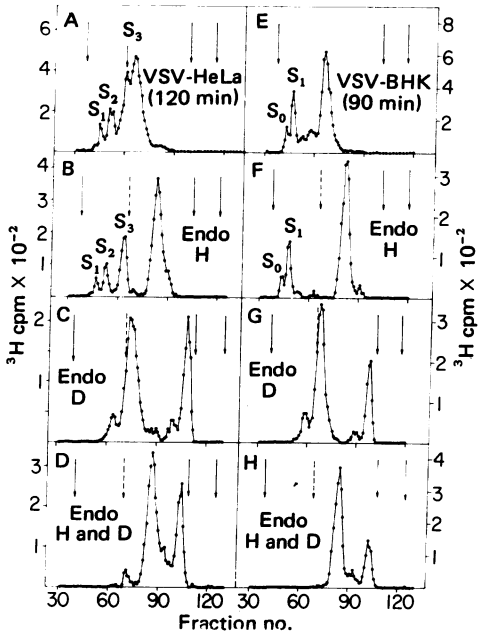

FIG. 5. Bio-Gel P-4 gel filtration of glycosidase-treated glycopeptides from VSV-infected cells. The left panels (*A–D*) correspond to glycopeptides from the 120-min pulse-labeled VSV-infected HeLa cells, and the right panels correspond to glycopeptides from the 90-min pulse-labeled VSV-infected BHK21 cells. The top two panels (*A* and *E*) are identical to Fig. 2 *D* and *G*. Endo D and endo H refer to the particular glycosidase treatment. The column markers (arrows) are identical to those in Fig. 3.

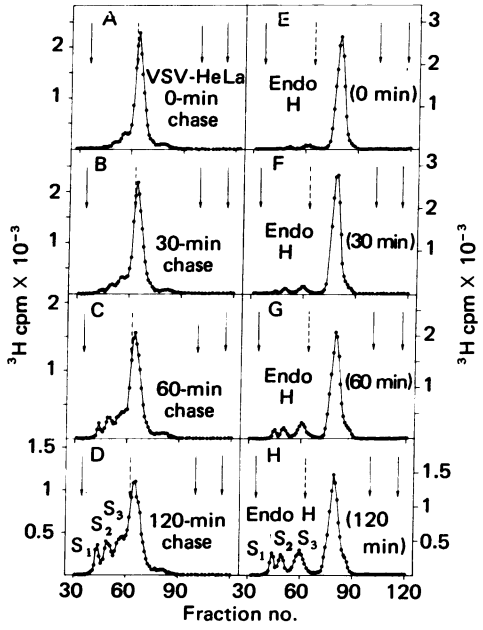

FIG. 6. Bio-Gel P-4 gel filtration of untreated and endo-β-*N*-acetylglucosaminidase H-treated glycopeptides from the pulse–chase labeling of VSV-infected HeLa cells. (*A–D*) Pronase-digested glycopeptides from the 0-, 30-, 60-, and 120-min chase periods. (*E–H*) Corresponding glycopeptide samples after treatment with endoglycosidase H. The gel filtration markers (arrows) are identical to those used in Fig. 3.

[^{3}H]mannose label as free mannose (Fig. 4*B*). Residual partially digested oligosaccharides eluted in the positions corresponding to $Man_2GlcNAc$ (fraction 121), $Man_3GlcNAc$ (fraction 115), and larger oligosaccharides (fractions 80–100). These results indicated that mannose occupied the terminal, nonreducing ends of the oligosaccharide and that most of the [^{3}H]mannose was in the α conformation. The α-mannosidase preparation used in these and previous studies (11) was completely free of α- and β-glucosidase, α- and β-galactosidase, and β-mannosidase activities and contained only trace amounts of β-*N*-acetylglucosaminidase activity (less than 0.005% of the α-mannosidase activity).

Conversion of Precursor to Mature Glycoprotein Involves Trimming of the Oligomannosyl Core. Glycopeptides from longer pulse-labeled samples that contained both precursor and virus-size glycopeptides were treated with the specific glycosidases and analyzed by gel filtration (Fig. 5). These analyses show the presence of both precursor and mature oligomannosyl cores ($Man_{\geq 7}GlcNAc$ and $Man_3GlcNAc$, respectively). Digestion with endoglycosidase H converted the mannose label in precursor glycopeptides to the large neutral oligomannosyl core structure while leaving a residual glycopeptide pattern identical to that obtained from purified virus grown in HeLa (15) or BHK21 (11) cells (Fig. 5 *B* and *F*). The endoglycosidase D and mixture of the exoglycosidases did not affect the precursor glycopeptides, but did digest the virus-size glycopeptides to a major peak and a minor peak corresponding in elution to $Man_3GlcNAc$ and $Man_5GlcNAc$, respectively (Fig. 5 *C* and *G*). Digestion first with endoglycosidase H and then with endoglycosidase D plus exoglycosidases resulted in a neutral oligomannosyl core pattern identical to the sum of the two individual digestions with either HeLa or BHK21 cells infected with VSV (Fig. 5 *D* and *H*). A minor glycopeptide peak eluting ahead of the expected position of S_3 glycopeptides was revealed by the endoglycosidase D and exoglycosidase digestion (Fig. 5 *C* and *G*; also Fig. 3*B*). These glycopeptides apparently had a similar oligosaccharide structure as the major peak of precursor glycopeptides (ref 15; unpublshed data).

To examine the precursor–product relationship of the precursor and mature size oligosaccharide structures, a pulse–chase experiment was performed with VSV-infected HeLa cells, and the size distribution and differential endoglycosidase sensitivity as a function of time of chase were analyzed by the methods demonstrated above. After a 30-min pulse label almost all of labeled glycopeptides eluted in the position of precursor glycopeptides and were sensitive to endoglycosidase H (Fig. 6 *A* and *E*). During the chase period increasing amounts of the virus-size glycopeptides appeared and were resistant to the endoglycosidase H (Fig. 6 *B–D* and *F–H*). Incorporation of [^{3}H]mannose into trichloroacetic acid-precipitable material reached a plateau within 5 min of the initiation of the chase. The radiolabel incorporated during the pulse decreased during the course of the 2-hr chase. This decrease was not unexpected, because label in virus-size, mature structures would be lost as the virus budded from the cell membrane into the growth medium, and because the postulated precursor-to-product processing involved the removal of mannose residues from the oligosaccharide structures. In addition, it is likely that the more distal mannose residues, removed during the trimming process, may have been radiolabeled to a higher specific activity than the mannose residues near the reducing end of the oligosaccharide structures. For these reasons the efficiency of conversion of precursor to mature oligomannosyl core structures could not be quantitated.

DISCUSSION

These studies with VSV-infected HeLa and BHK21 cells have demonstrated that cell-associated glycoprotein pulse labeled with [^{3}H]mannose consisted mainly of the VSV G protein, and that the oligosaccharides initially linked to the virus glyco-

protein contained a large precursor oligomannosyl core structure. During longer pulse or pulse–chase periods, some of these precursor oligosaccharide structures were converted to mature, virus-size oligosaccharide structures containing fewer mannose residues. The two forms of VSV G observed in BHK21 cells presumably represented precursor glycoprotein (G_1) with two large oligomannosyl core structures [$Man_{\geq 7}(GlcNAc)_2$] and mature glycoprotein (G_2) containing oligosaccharide chains composed of the small oligomannosyl core [$Man_3(GlcNAc)_2$] and the branch structures terminating in sialic acid (NeuNAc-Gal-GlcNAc). In VSV-infected HeLa cells a large fraction of the mature G contained asialo-oligosaccharide structures (S_3-size) and comigrated on sodium dodecyl sulfate/polyacrylamide gels with the precursor G. Two forms of the VSV glycoprotein were also observed in virus-infected Chinese hamster ovary cells (21) but were presumed to be identical in oligosaccharide content except for the absence of sialic acid in the G_1 form.

Our results are compatible with a simplified, two-step model for the glycosylation and maturation of VSV G: (*i*) the large oligomannosyl cores are transferred from lipid-linked intermediates (16) to the two major glycosylation sites on the G protein (9) while the polypeptide is being synthesized in the rough endoplasmic reticulum (12–15); and (*ii*) after a 20- to 30-min minimal transit time (7), the glycoprotein appears in smooth internal membranes, where mannose residues are removed by a trimming process and branch sugars and fucose are added from nucleotide-linked monosaccharides. The order in which the trimming and branch sugar addition takes place cannot be determined from the studies presented here, and these processes may occur almost simultaneously. This unique trimming process may be related to the recent observation of a Golgi membrane-enriched form of α-mannosidase that has recently been purified from rat liver and characterized (22).

Although the composition and structure of the mature oligosaccharides from VSV glycoprotein have been well characterized (8, 11), the exact size and composition of the precursor oligosaccharides studied here are somewhat tentative. The glycosidase digestions and gel filtration studies strongly suggest a structure identical to a typical neutral oligosaccharide [$Man_n(GlcNAc)_2$] linked to asparagine with an estimate of 7–9 mannose residues. The possible presence of one or two glucose residues in the precursor oligosaccharide structures, as suggested elsewhere for lipid- and protein-linked oligosaccharides (23–25), was not directly examined and cannot be rigorously excluded. The α-mannosidase digestions, however, indicate that glucose was not present in a terminal, nonreducing position, which would have protected much of the radiolabeled mannose from removal. In addition, the 7–9 hexose units estimated for these precursor oligosaccharides are considerably fewer than the 11–12 hexose units postulated for the glucose-containing, lipid-linked oligosaccharide (23). Glucose may have been present in the oligosaccharide transfered from the lipid-linked intermediate but was rapidly removed from the oligosaccharide chains of the VSV glycoprotein. Furthermore, because the material analyzed was from a 30-min pulse period, some terminal mannose residues may have already been removed. Therefore, the initial glycosylation event may have involved an even larger oligomannosyl structure similar to the dolichol-linked structure observed by Spiro *et al.* (23).

The addition and subsequent trimming of large oligomannosyl cores in the specific case of the VSV glycoprotein is most likely indicative of a similar processing of cellular membrane and/or secreted glycoproteins containing asparagine-linked oligosaccharide chains. Preliminary studies with uninfected HeLa cells indicate that the oligosaccharides initially added to a heterogeneous mixture of membrane glycoproteins have the same size and structure as those observed with the precursor forms of the VSV glycoprotein (L. A. Hunt *et al.*, unpublished data).

Membrane and secreted glycoproteins contain two major classes of asparagine-linked oligosaccharide structures: the complex, acidic structures that contain sialic acid, galactose, *N*-acetylglucosamine, mannose, and fucose; and the mannose-rich, large neutral structures containing only mannose and *N*-acetylglucosamine. The studies presented here suggest a possible relationship in the biosynthesis of these two classes of oligosaccharide structures and suggests that the biosynthetic "decision" as to whether one or the other type of structure will be present on the mature glycoprotein is made subsequent to the initial glycosylation step but prior to the trimming process.

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