Journal of Supramolecular Structure 12:209–226 (1979)
Tumor Cell Surfaces and Malignancy 541–558

Biosynthesis and Maturation of Cellular Membrane Glycoproteins

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The biosynthesis and the processing of asparagine-linked oligosaccharides of cellular membrane glycoproteins were examined in monolayer cultures of BHK21 cells and human diploid fibroblasts after pulse- and pulse-chase labeling with [2-^{3}H] mannose. After pronase digestion, radiolabeled glycopeptides were characterized by high-resolution gel filtration, with or without additional digestion with various exoglycosidases and endoglycosidases. Pulse-labeled glycoproteins contained a relatively homogenous population of neutral oligosaccharides (major species: $Man_9GlcNAc_2ASN$). The vast majority of these asparagine-linked oligosaccharides was smaller than the major fraction of lipid-linked oligosaccharides from the cell and was apparently devoid of terminal glucose. After pulse-chase or long labeling periods, a significant fraction of the large oligomannosyl cores was processed by removal of mannose units and addition of branch sugars (NeuNAc-Gal-GlcNAc), resulting in complex acidic structures containing three and possibly five mannoses. In addition, some of the large oligomannosyl cores were processed by the removal of only several mannoses, resulting in a mixture of neutral structures with 5–9 mannoses. This oligomannosyl core heterogeneity in both neutral and acidic oligosaccharides linked to asparagine in cellular membrane glycoproteins was analogous to the heterogeneity reported for the oligosaccharides of avian RNA tumor virus glycoproteins (Hunt LA, Wright SE, Etchison JR, Summers DF: J Virol 29:336, 1979).

Key words: membrane glycoproteins, human diploid fibroblasts, BHK21 cells, exoglycosidases and endoglycosidases, asparaginyl-oligosaccharides, gel filtration, processing of oligomannosyl core

Biochemical studies of cellular membrane glycoproteins of animal cells in tissue culture [1, 2] have suggested that these polypeptides have asparagine-linked oligosaccharides that are similar to the carbohydrate chains of a number of well-characterized serum glycoproteins [3] with respect to both heterogeneity in oligomannosyl core size and the presence of both acidic and neutral structures. The common structural feature of

Abbreviations: ASN – asparagine; Fuc – fucose; Gal – galactose; Glc – glucose; GlcNAc – N-acetylglucosamine; Man – mannose; NeuNAc – sialic acid, N-acetylneuraminic acid.

Received April 23, 1979; accepted August 16, 1979.

0091-7419/79/1202-0209$03.20

these carbohydrate chains is a cluster of mannose units linked to the asparaginase residue in the polypeptide by a N,N-diacetylchitobiose structure [$(Man)_nGlcNAc$-GlcNAc-ASN], with complex, acidic chains differing from high-mannose neutral chains by the size of the oligomannosyl core and the presence of branch structures terminating in sialic acid (NeuNAc±Gal-GlcNAc–) [3]. The specific function of these oligosaccharides in cellular membrane glycoproteins is not clear, but growth-dependent changes in both the size distribution of oligomannosyl cores and the ratio of acidic to neutral chains have been detected in studies of [^{3}H] mannose-labeled human diploid fibroblasts [2]. These differences may have represented either changes in the carbohydrate composition of individual glycoproteins or changes which were secondary to a new population of polypeptides.

Recent studies with vesicular stomatitits and Sindbis virus-infected cells strongly suggested that viral membrane glycoproteins acquired large oligomannosyl core structures by the en bloc transfer of a preformed oligosaccharide from lipid-linked intermediates (dolichol-phosphate-oligosaccharide), and these asparagine-linked oligosaccharides were subsequently processed to complex acidic structures by the trimming of mannose and sequential addition of branch sugars [4–6]. Because these viral glycoproteins were being synthesized and processed by cellular enzymatic machinery and because the lipid-linked oligosaccharides from a number of virus-infected and uninfected tissue culture cell lines have been shown to have a fairly common structure [4, 7, 8], the heterogeneous array of asparagine-linked oligosaccharides of cellular membrane glycoproteins must be synthesized and matured by similar, if not identical, intracellular processes.

The aim of these studies was to confirm this prediction using [2-^{3}H] mannose pulse- and pulse-chase labeling of the asparagine-linked oligosaccharides of membrane glycoproteins of BHK21 cells and human diploid fibroblasts. The specific objectives were threefold: 1) to determine the size and structure of the asparagine-linked oligosaccharides of pulse-labeled glycoprotein and compare these "precursor" structures with the lipid-linked oligosaccharides; 2) to demonstrate the processing of "precursor" glycoproteins into glycoproteins with "mature" carbohydrate structures containing smaller oligomannosyl cores; and 3) to determine the size of the oligomannosyl cores of these mature asparagine-linked oligosaccharides and their distribution between the complex acidic structures and high-mannose neutral structures.

METHODS

HeLa S3 cells were grown in suspension culture, and the BHK21 cells and human embryonic lung fibroblasts were grown in monolayer culture [5]. The human diploid fibroblasts were provided by Noël Jarnevic of the Department of Pathology and Oncology. Suspension cultures of HeLa cells or monolayer cultures of the human diploid fibroblasts and BHK21 cells were pulse-labeled with 100 μCi/ml [2-^{3}H] mannose (2 Ci/ml, Amersham) in glucose-deficient medium [5, 9]. Pulse-chase labeling was similar except that the radioactive medium was removed at the end of the pulse-labeling and replaced with medium containing 2 mM unlabeled mannose for the duration of the "chase" period. Previous studies with virus-infected cells in culture [5] and uninfected cells in culture (unpublished observations) indicated that the incorporation of [^{3}H] mannose into trichloroacetic acid-precipitable material reached a plateau within approximately 5 min of the initiation of the "chase" after "pulse" labeling for 30–60 min, and then decreased with increasing chase periods. The medium for the overnight labeling of BHK21 cells contained 60 μCi/ml [2-^{3}H] mannose and one-third the normal amount of glucose.

The preparation of membrane glycoproteins and the digestion with pronase (grade B, Calbiochem) were identical to procedures previously employed for vesicular stomatitis virus-infected tissue culture cells [5, 9]. Lipid-linked oligosaccharides (dolichol-phosphate-oligosaccharides) were isolated by selective organic extraction of whole cells with chloroform/methanol (2:1) and chloroform/methanol/water (10:10:3), and the oligosaccharides were released from the lipid by mild acid hydrolysis [7, 10].

Glycopeptides and oligosaccharides were digested with exoglycosidases and endo-β-N-acetylglucosaminidases as previously described [5, 9, 11]. Endo-β-N-acetylglucosaminidase H from Streptomyces plicatus, endo-β-N-acetylglucosaminidase D and exoglycosidases from Streptococcus pnemoniae, and jack bean α-mannosidase were provided by Dr. James Etchison, University of Utah. Clostridium perfringens neuraminidase (type IX) and yeast α-glucosidase (type III) were purchased from Sigma Chemical Company.

Glycopeptides and oligosaccharides were analyzed before and after glycosidase digestions by gel filtration through a BioGel P-4 column [5, 9]. The elution positions of various neutral oligomannosyl cores ($Man_nGlcNAc_1$) of known composition were used to calibrate the BioGel P-4 column in the gel filtration of endo-β-N-acetylglucosaminidase digestion products from [2-^{3}H] mannose-labeled cellular membrane glycopeptides [5, 11, 12].

RESULTS

Lipid-Linked and Protein-Linked "Precursor" Oligosaccharides

The newly synthesized asparagine-linked oligosaccharides of cellular glycoproteins were analyzed and compared with the corresponding lipid-linked oligosaccharides after pulse-labeling of BHK21 cells with [2-^{3}H] mannose and isolation of lipid-linked and peptide-linked oligosaccharide fractions from whole cells. [2-^{3}H] mannose was used in the labeling experiments because this sugar was incorporated into the lipid-linked oligosaccharides along with glucosamine and glucose, and was added to glycoprotein only at the initial stage of en bloc glycosylation in the rough endoplasmic reticulum [9]. Additional glucosamine and other radiolabeled sugars (galactose, fucose) would have been immediately added to pre-existing oligosaccharides at the second stage of glycosylation in Golgi-like intracellular membranes [9], and could therefore not have been used to examine the initial glycosylation and processing.

Pronase-digested glycopeptides and oligosaccharides released from the lipid fraction by mild acid hydrolysis were analyzed by gel filtration on BioGel P-4, with or without prior digestion with endo-β-N-acetylglucosaminidase H (Fig. 1B,D). The gel filtration profile of the radiolabeled BHK21 glycopeptides (Fig. 1B) contained a single major peak eluting after the [^{14}C] acetylated asparaginyl-oligosaccharide and a second minor peak eluting before the [^{14}C] marker, and was almost identical to the gel filtration profile for pulse-labeled glycopeptides from HeLa cells (Fig. 1A). After digestion with endo-β-N-acetyl-glucosaminidase H, radiolabel in both major and minor peaks of HeLa and BHK21 glycopeptides was shifted to a single peak with lower apparent molecular weight, consistent with the release of the radiolabeled oligomannosyl core from the N-acetylglucosaminyl-peptide. As illustrated in Figure 2, the site of hydrolysis of this enzyme was between the two N-acetylglucosamine residues proximal to the peptide moiety of particular oligomannosyl di-N-acetylchitobiose structures [13–15] and between the two N-acetylglucosamine residues of lipid-derived oligosaccharides [4]. The radiolabeled products from the

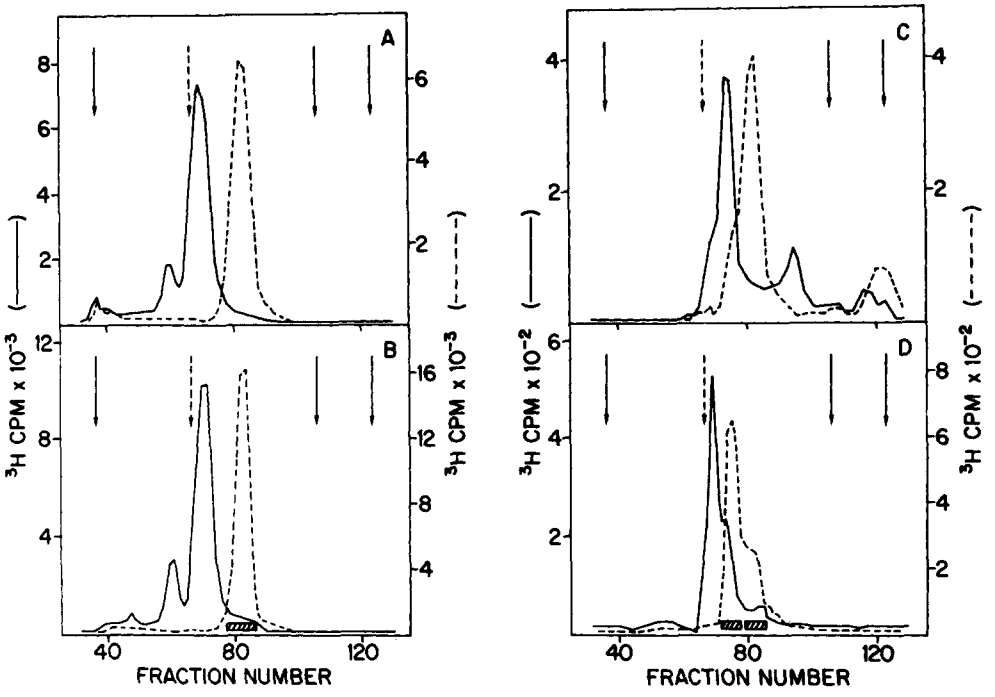


Fig. 1. BioGel P-4 gel filtration of oligosaccharides from [^{3}H]mannose-labeled lipid and protein fractions of BHK21 and HeLa cells. The pulse-labeling times were 60 min for the HeLa cells and 40 min for the BHK21 cells. Pronase-digested glycopeptides from HeLa (A) and BHK21 cells (B) were analyzed before (———) and after (- - - - -) endo-β-N-acetylglucosaminidase H treatment, and the profiles of radioactivity were superimposed by alignment of the peak elution positions of four column markers (from left to right: blue dextran (void volume), [^{14}C]-acetylated-asparaginyl oligosaccharide from ovalbumin, stachyose, and mannose). Radiolabeled products from the mild acid hydrolysis of lipid-linked oligosaccharides of HeLa (C) and BHK21 cells (D) were analyzed before (————) and after (- - - - - - -) endo-β-N-acetylglucosaminidase H treatment, and the profiles were also superimposed. The boxes with diagonal lines at the bottom of panels B and D indicate the representative fractions pooled from similar preparative BioGel P-4 chromatography of equivalent endoglycosidase-treated samples for further structural analysis (Fig. 3).

endoglycosidase-digested BHK21 and HeLa cell glycopeptides were demonstrated by Dowex ion-exchange chromatography [12] to be neutral oligosaccharides, and the elution positions were indicative of an oligomannosyl core with 8–9 mannoses and one N-acetylglucosamine [5, 16]. These results suggested that essentially all of the radiolabel in these glycopeptides was in the form of mannose, since [^{3}H] label incorporated into glycoproteins in the form of fucose, galactose, sialic acid, or N-acetylglucosamine would have been incorporated directly into higher-molecular-weight, endo-β-N-acetylglucosamine-H-resistant glycopeptides [9]. In addition, if significant radiolabel had been incorporated into N-acetylglucosamine, radiolabel would have been detected in later eluting fractions corresponding to the GlcNAc-peptide product of endoglycosidase digestion in Figure 1 A,B.

More recent analysis of the peptide-derived neutral oligosaccharides on a different BioGel P-4 column that was capable of resolving a mixture of high-mannose structures differing by one or more hexoses confirmed that the vast majority of radiolabel eluted with $Man_9GlcNAc_1$. Very small amounts of radiolabel eluted in the positions expected

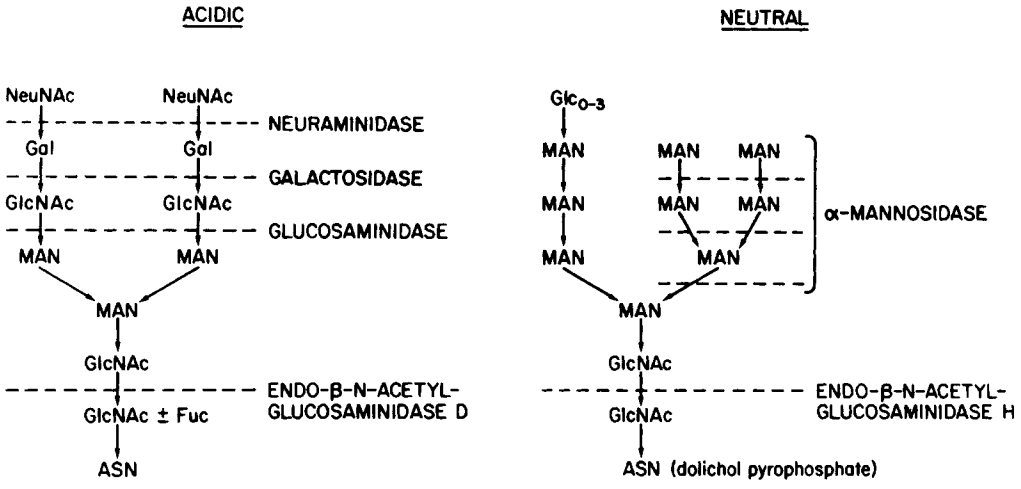


Fig. 2. Typical neutral and acidic asparaginyl oligosaccharide structures and their susceptibility to glycosidases. The acidic structure is identical to that reported for the complex asparaginyl oligosaccharides of Sindbis virus glycoproteins [19], and the neutral structure is identical to that reported for the lipid-linked "precursor" oligosaccharide from vesicular stomatitis virus-infected cells [8], with specific sugar linkages omitted. Horizontal dashed lines show the positions of hydrolysis by enzymes. Neuraminidase, galactosidase, and glucosaminidase from Streptococcus pneumoniae [17] and jack bean α-mannosidase are exolgycosidases that remove the appropriate sugars only when they are at the nonreducing termini of the oligosaccharides. The two forms of endo-β-N-acetylglucosaminidase, D and H, differ in their substrate requirements [13–15]. Both peptide-linked and lipid-derived oligosaccharides (dolichol-phosphate-oligosaccharides) are susceptible to the appropriate glycosidases. With mild acid hydrolysis, the oligosaccharide is released from the dolichol-phosphate.

for oligosaccharides with one less hexose ($Man_8GlcNAc_1$) and one additional hexose (data not shown).

The lipid-derived, [2-^{3}H] mannose-labeled oligosaccharides from BHK21 cells eluted in a single major peak and in an apparent unresolved minor peak on the lower-molecular-weight "shoulder" of the major peak (Fig. 1D). After endo-β-N-acetylglucosaminidase H digestion the oligosaccharides eluted in higher-numbered fractions, as expected from the removal of one of the two N-acetylglucosamines from the radiolabeled oligomannosyl structure (Fig. 2). The major species of lipid-linked oligosaccharide from BHK21 cells was apparently several monosaccharide residues larger than the major species of [2-^{3}H] mannose-labeled oligosaccharide linked to protein, based upon the relative elution positions of the endo-β-N-acetylglucosamine H-released neutral oligosaccharides (Fig. 1B,D). In addition, the lipid-derived oligosaccharides from HeLa cells were smaller before and after endoglycosidase treatment than the corresponding BHK21 oligosaccharides (Fig. 1C,D), the major radiolabeled products of endoglycosidase digestion of both protein and lipid-derived material from the HeLa cells eluting in the same relative position from the BioGel P-4 column (Fig. 1A,C).

Several investigators have presented evidence of terminal glucose residues in radiolabeled asparaginyl-oligosaccahrides and corresponding lipid-linked oligosaccharides extracted from various tissue culture cells [4, 6, 8]. In order to test whether the presence or absence of terminal glucose residues might have accounted for the differences in gel filtration of the major lipid- and protein-derived oligosaccharides, the sensitivity of

radiolabel in these structures to jack bean α-mannosidase was examined. If glucose or some monosaccharide other than mannose was present at one or more of the nonreducing termini of the oligomannosyl cores, less of the [^{3}H] label would be released as free mannose, and the decrease in size between the original undigested oligosaccharides and the residual oligosaccharides after exoglycosidase digestion would not be as great (Fig. 2). A more direct method for determining the presence or absence of terminal glucose in both the peptide- and lipid-derived oligosaccharides from the same labeled BHK21 cell culture was not readily available because 1) there was no specific way to radiolabel the glucose (in contrast to the cell-free glycosylation studies using GDP-[^{3}H/^{14}C]glucose [4] or the studies utilizing virus-infected, lectin-resistant cell lines that incorporate [^{3}H]- or [^{14}C] galactose into glycoproteins as [^{3}H]- or [^{14}C]glucose rather than directly as galactose into acidic asparagine-linked oligosaccharides and serine/threonine-linked oligosaccharides [8]); and 2) no well-characterized glucosidases were available to remove terminal glucose residues. In contrast to a previous report of yeast α-glucosidase-mediated removal of terminal glucose from lipid-derived oligosaccharides [18], yeast α-glucosidase had no effect on the gel filtration profiles of the lipid- and peptide-derived oligosaccharides from BHK21 cells (data not shown) or lipid-derived oligosaccharides from Chinese hamster ovary cells in studies by other workers [6].

Neutral oligomannosyl cores corresponding to the major peak of peptide-derived material and the major and minor peaks of lipid-derived material from BHK21 cells were isolated by preparative gel filtration and reanalyzed before and after α-mannosidase digestion (Fig. 3). Prior to α-mannosidase treatment the peptide-derived oligosaccharides and the lipid "shoulder"-derived oligosaccharides eluted in similar positions, distinct from the earlier elution position of the major lipid-derived oligosaccharides. After α-mannosidase digestion a large fraction of the [^{3}H] label of the peptide-derived material and the minor lipid-derived material ("shoulder") eluted in the position of free mannose (Fig. 3, middle and bottom panels). The residual peptide-derived oligosaccharides eluted in a broad peak centered at the psition expected for $Man_3GlcNAc_1$ and in a minor peak just after the position of undigested oligosaccharide (Fig. 3, bottom panel). The α-mannosidase digestion apparently did not go to completion, since the expected products for complete digestion would have been free mannose and the dissacharide, mannose-N-acetylglucosamine ($Man\underline{\beta}GlcNAc$, reference [8]). This apparent incomplete α-mannosidase digestion of the oligomannosyl core (with $Man_3GlcNAc$ as a major residual product instead of $Man_1GlcNAc_1$) has been previously observed for similar peptide-derived oligosaccharides from vesicular stomatitis virus-infected cells [5, 6]. Similarly, the residual lipid "shoulder"-derived material eluted in the position of $Man_{3-5}GlcNAc_1$ and in a major peak just after the position of undigested material (Fig. 3, middle panel).

In contrast, only a small fraction of radiolabel was released as free mannose by α-mannosidase from the peak of lipid-derived oligosaccharide, and the remaining oligosaccharides eluted in a sharp peak with a slightly decreased apparent molecular weight (Fig. 3, top panel). The significant differences in both the relative amount of free mannose released and size of residual oligosaccharides between the major lipid- and peptide-derived samples after α-mannosidase digestion were most likely due to substrate differences rather than incomplete digestion because of limiting enzymes. The small amounts of apparent high-molecular-weight residual oligosaccharide observed in the digestion products of the lipid-derived "shoulder" material and the peptide-derived material (fractions 80–90 in Figure 3, middle and bottom panels) suggested that a fraction of these samples was also partially protected from α-mannosidase digestion.

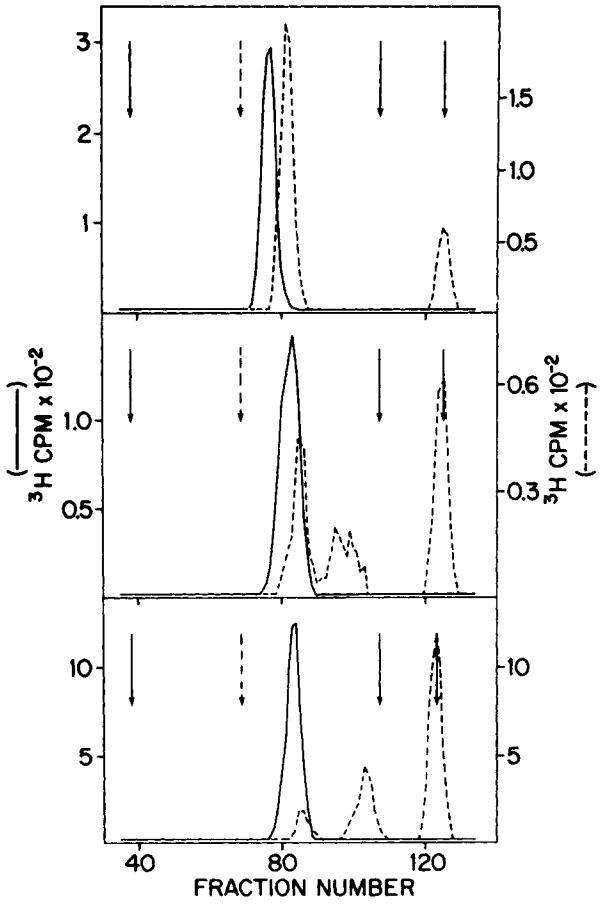


Fig. 3. BioGel P-4 gel filtration of endo-β-N-acetylglucosaminidase H-digested products of [^{3}H] mannose-labeled oligosaccharides from lipid and protein fractions of BHK21 cells. Neutral oligosaccharides were isolated by preparative BioGel P-4 gel filtration, with the representative pooled fractions indicated in Figure 1B and 1D. Aliquots of the radiolabeled oligosaccharides were rechromatographed on the column either before (————) or after (- - - - - -) further treatment with jack bean α-mannosidase, and the profiles were superimposed. Upper panel, peak of lipid-derived oligosaccharides; middle panel, lower-molecular-weight "shoulder" of lipid-derived oligosaccharides; lower panel, peak of peptide-derived oligosaccharides.

The combined results of α-mannosidase digestion and gel filtration were similar to those of other investigators [4, 6], and indicated that the major radiolabeled species of the lipid-derived oligosaccharides 1) contained several more monosaccharide residues than the major radiolabeled species of peptide-derived oligosaccharide ($Man_9GlcNAc$), and 2) was significantly more resistant to α-mannosidase digestion. A logical explanation was that these lipid-derived oligosaccharides contained several residues of glucose at a nonreducing terminus as reported by other investigators [4, 6, 8], whereas most of the peptide-derived "precursor" oligosaccharides from the same radiolabeled BHK21 cell cultures were deficient in terminal glucose. Much of the lower-molecular weight "shoulder" fraction of the lipid-derived oligosaccharides was also apparently deficient in terminal glucose, whereas

a small fraction of the peptide-derived oligosaccharides may have contained a single terminal glucose residue (Fig. 3, middle and bottom panels). The proposed "precursor" oligosaccharide structures shown in Figure 4 were consistent with the experimental results presented here and previously published studies on the detailed structure of lipid-linked oligosaccharides from virus-infected chinese hamster ovary cells [8].

Processing of "Precursor" Asparagine-Linked Oligosaccharides

The question of whether a relatively homogenous population of asparagine-linked "precursor" oligosaccharides ($Glc_{0-1}Man_{8-9}GlcNAc_2ASN$) could be processed into a number of distinct oligosaccharide structures was addressed experimentally by pulse-chase labeling of BHK21 cells (45-min pulse, 0,2,4,24-h chase) with [2-^{3}H] mannose. Total cell membrane glycoprotein was isolated from the cell cultures, digested with pronase, and analyzed by gel filtration on a BioGel P-4 column before and after digestion with endo-β-N-acetylglucosaminidase H (Fig. 5). The gel filtration profile of the 0-h "chase" material before and after endoglycosidase digestion (Fig. 5A,E) was similar to that previously observed for oligosaccharides from the protein fraction of pulse-labeled BHK21 and HeLa cells in Figure 1A and 1B, with essentially all of the radiolabeled glycopeptide converted to neutral oligosaccharides eluting from the column in higher-numbered fractions.

With increasing "chase" periods, a significant fraction of the radiolabeled oligosac-

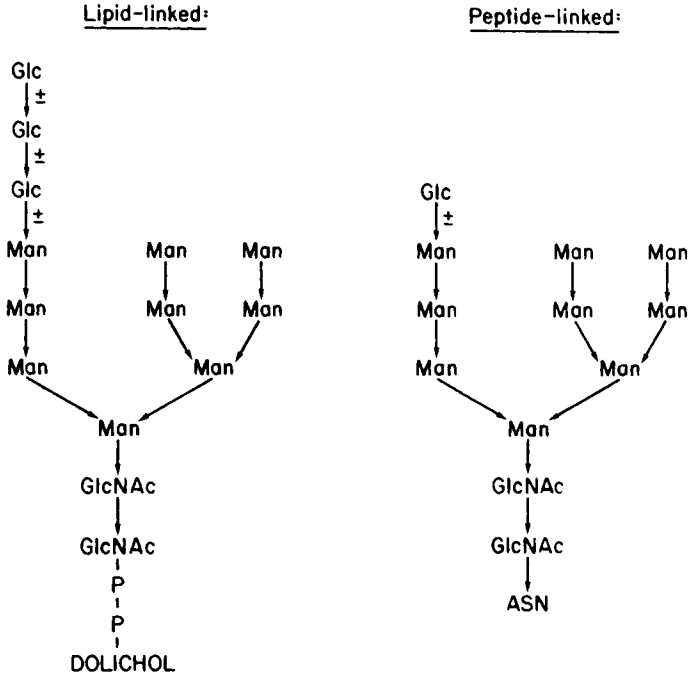


Fig. 4. Structures proposed for the lipid-linked and peptide-linked "precursor" oligosaccharides. The number and arrangement of mannose and glucose units were derived from previously proposed structures for the oligomannosyl core of unit A asparagine-linked carbohydrates of calf thyroglobulin [14] and the lipid-linked oligosaccharides from vesicular stomatitis virus-infected chinese hamster ovary cells in tissue culture [8], and were consistent with the combined gel filtration and glycosidase studies with the BHK21 cellular membrane glycopeptides.

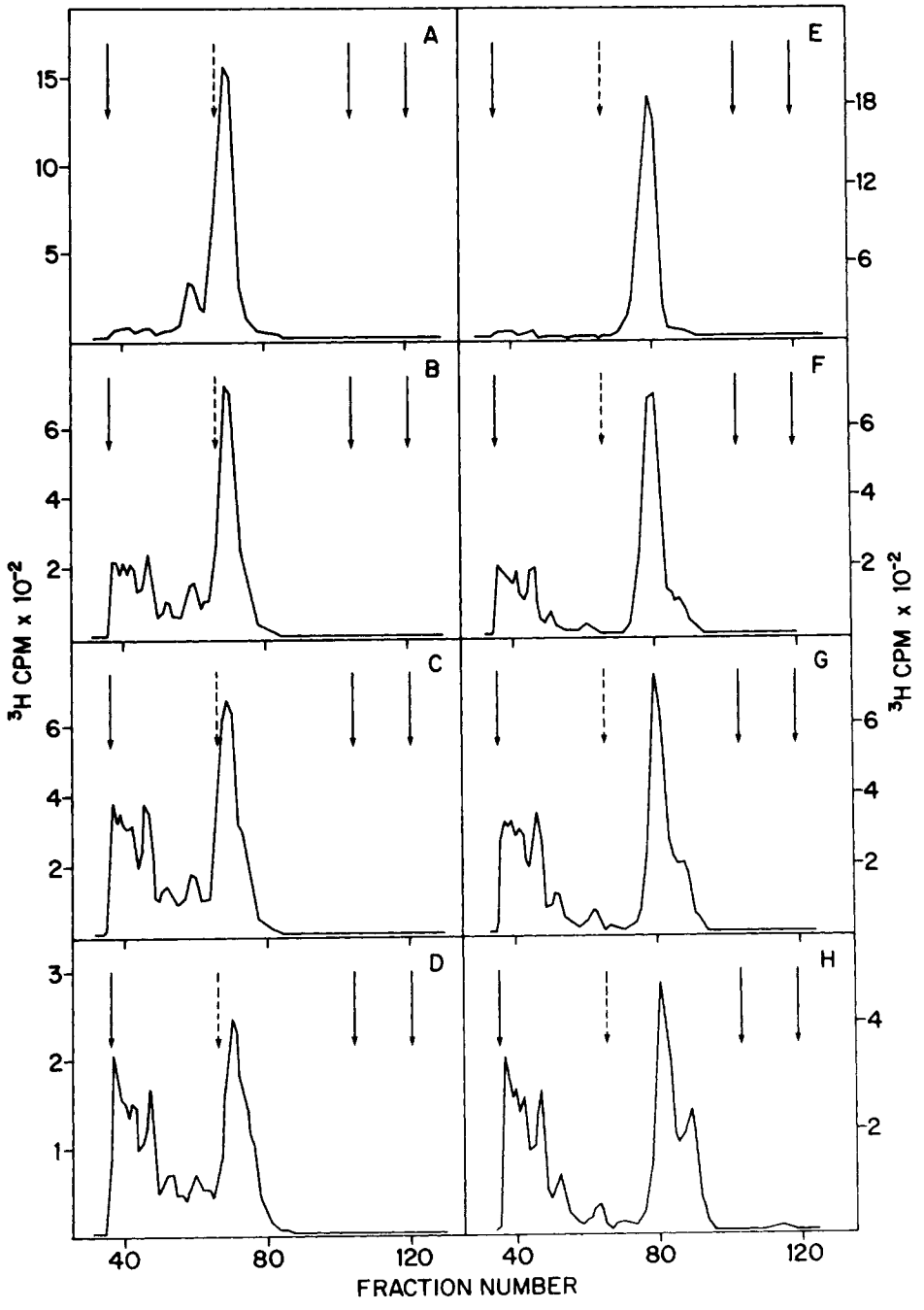
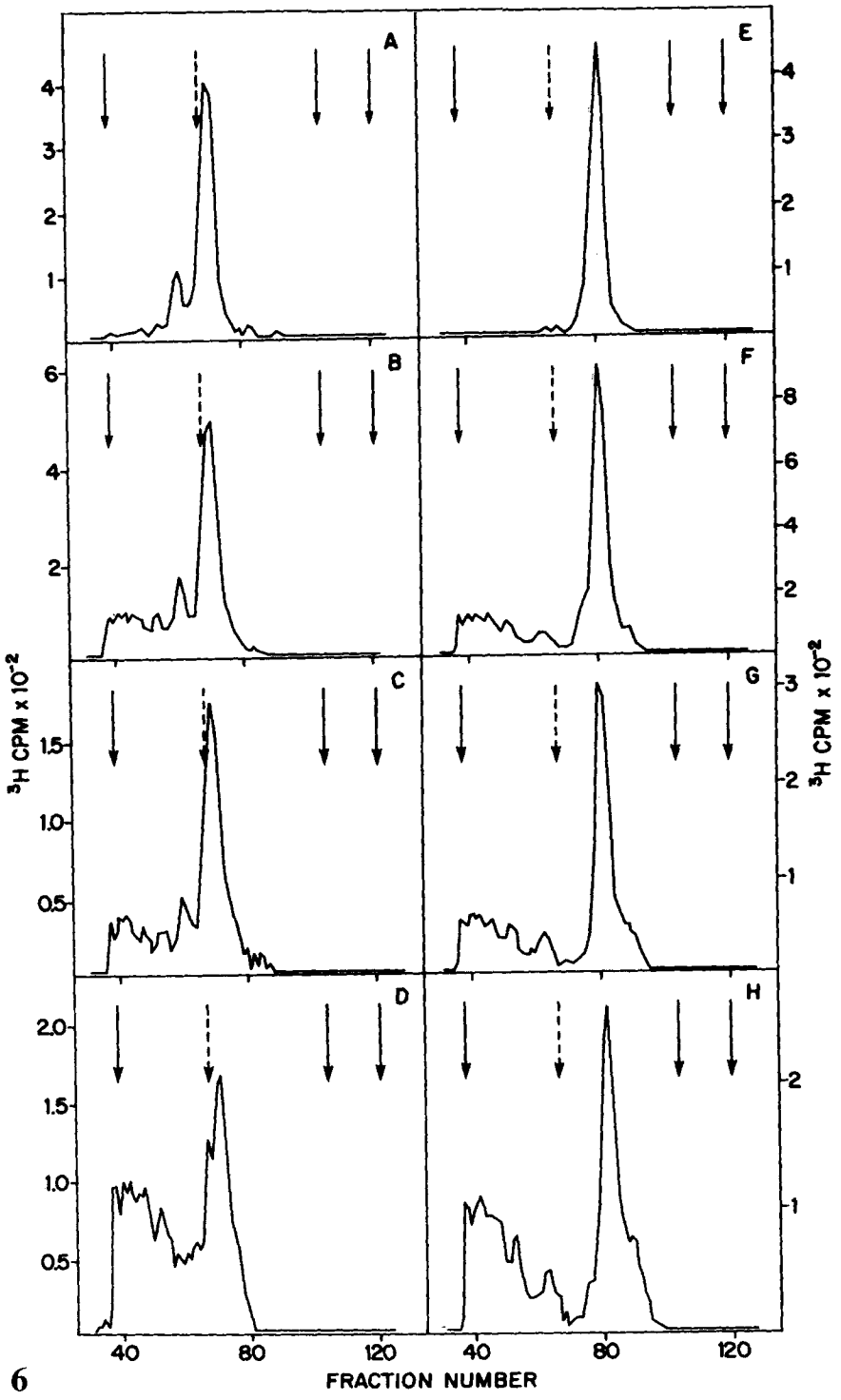


Fig. 5. BioGel P-4 gel filtration of membrane glycopeptides from pulse-chase labeling of BHK21 cells. Monolayer cultures were pulse-labeled with [2-^{3}H] mannose for 45 min and then "chased" in unlabeled medium for 0, 2, 4, and 24 h. Radiolabeled glycopeptides were analyzed either before (A–D) or after (E–H) treatment with endo-β-N-acetylglucosaminidase H. A, E, O-h chase; B, F, 2-h chase; C, G, 4-h chase; D, H, 24-h chase.



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charides was apparently converted to endo-β-N-acetylglucosaminidase H-resistant structures and appeared in a more heterogenous distribution of glycopeptides with apparently higher molecular weights. In addition, some of the asparagine-linked "precursor" oligosaccharides were apparently converted during the incubation in unlabeled medium to smaller oligomannosyl structures ($Man_{5-7}GlcNAc_2ASN$) that were also sensitive to digestion by endo-β-N-acetylglucosaminidase H (fractions 85–95 in Fig. 5H).

An analogous experiment with human diploid fibroblasts gave very similar results (Fig. 6): With increasing "chase" periods radiolabel appeared in a heterogenous population of apparently larger-molecular-weight, endo-β-N-acetylglucosaminidase H-resistant glycopeptides and smaller, endo-β-N-acetylglucosaminidase H-sensitive oligomannosyl core structures.

Structure of "Mature" Asparagine-Linked Oligosaccharides

The resistance to endo-β-N-acetylglucosaminidase H of the apparent high-molecular-weight glycopeptides that appeared after increasing "chase" periods in the labeling of both BHK21 and human diploid fibroblast cells suggested, but did not prove, that these glycopeptides contained complex, acidic oligosaccharides with smaller oligomannosyl core structures. Because more detailed analysis of these oligosaccharide structures required significantly greater amounts of radioactivity than were present in the 24-h chase samples, [2-^{3}H] mannose-labeled glycopeptides were isolated from the membranes of BHK21 cells after a continuous labeling period of 24-h. BioGel P-4 analysis of these radiolabeled glycopeptides before (Fig. 7A) and after (Fig. 7C) digestion with endo-β-N-acetylglucosaminidase H resulted in gel filtration profiles that were virtually indistinguishable from the corresponding profiles of the 45-min pulse–24-h chase sample (Fig. 5D,H). The results of oligosaccharide structural analysis of the 24-h labeled cellular membrane glycoproteins were therefore presumed to be applicable to the "mature" asparagine-linked oligosaccharides derived from the high-mannose "precursor" structure during the 45-min pulse–24-h chase labeling (Fig. 5).

The major peak of radiolabeled glycopeptides eluting after the [^{14}C] column marker in Figure 7A was demonstrated to be a mixture of high-mannose, neutral glycopeptides on the basis of sensitivity to both endo-β-N-acetylglucosamindase H (Fig. 7C) and jack bean α-mannosidase (Fig. 7B). The partial digestion with α-mannosidase released a large amount of radiolabel that coeluted with free mannose, and the residual neutral glycopeptide peak was in the position expected for glycopeptides with smaller oligomannosyl core structures, consistent with the original neutral glycopeptides containing terminal mannose residues (Fig. 2). In contrast, the glycopeptides of higher apparent molecular weight were unaffected by these exoglycosidase and endoglycosidase digestions, but were affected by digestion with Clostridrium perfringens neuraminidase (Fig. 7D). This indicated that these glycopeptides contained terminal sialic acid (Fig. 2), but still exhibited considerable heterogenity upon gel filtration in the asialo form.

Fig. 6. BioGel P-4 gel filtration of membrane glycoproteins from pulse-chase labeling of human diploid fibroblasts. Monolayer cultures were pulse-labeled for 30 min with [2-^{3}H] mannose and then "chased" for 0, 2, 4, and 24 h. A–D, not treated with glycosidase; E–H, treated with endo-β-N-acetylglucosaminidase H; A, E, O-h chase; B, F, 2-h chase; C, G, 4-h chase; D, H, 24-h chase.

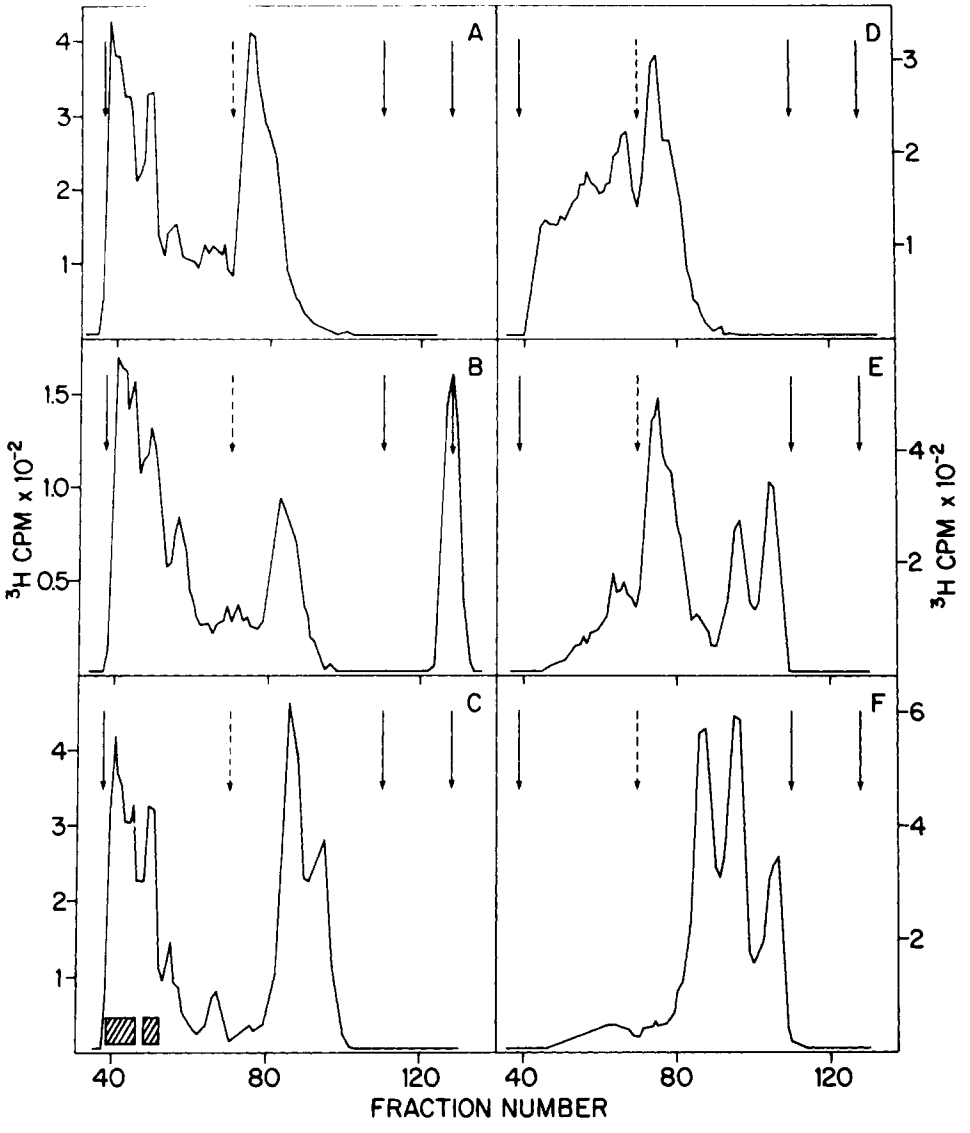

Fig. 7. BioGel P-4 gel filtration of glycosidase-treated membrane glycopeptides from a BHK21 monolayer culture labeled for 24 h with [2-^{3}H]mannose. Aliquots of a sample of pronase-digested glycopeptides were analyzed by gel filtration, either without further treatment (A) or after treatment with various glycosidases (B–F). B, α-mannosidase-treated glycopeptides; C, endo-β-N-acetylglucosaminidase H-treated glycopeptides; D, neuraminidase-treated glycopeptides; E, exoglycosidase and endo-β-N-acetylglucosaminidase D-treated glycopeptides; F, glycopeptides after treatment first with endo-β-N-acetylglucosaminidase H and then with the mixture of exoglycosidases and endo-β-N-acetylglucosaminidase D. The two small boxes with diagonal lines at the bottom of panel C indicate the regions of a similar preparative gel filtration profile of endoglycosidase-treated glycopeptides from which radiolabeled acidic glycopeptides were isolated and analyzed in more detail (Fig. 8).

Digestion with the mixture of exoglycosidases and endo-β-N-acetylglucosaminidase D from Streptococcus pneumoniae should have converted the [2-^{3}H] mannose label in the complex, acidic glycopeptides to neutral oligomannosyl cores ($Man_nGlcNAc_1$) while not affecting the neutral glycopeptides with large oligomannosyl cores (Fig. 2) [13, 17]. Digestion with these glycosidases did indeed convert the radiolabel in the large, sialic acid-enriched glycopeptides (but not the major and minor peaks of "precursor"-like neutral glycopeptides) to two new peaks that eluted in the positions expected [5, 12, 16] for $Man_5GlcNAc_1$ and $Man_3GlcNAc_1$ neutral oligosaccharides (Fig. 7E). Digestion first with endo-β-N-acetylglucosaminidase H and then with the mixture of glycosidases containing endo-β-N-acetylglucosaminidase D (Fig. 7F) converted almost all of the radioactivity in glycopeptides to neutral oligosaccharides with a gel filtration profile identical to the sum of the neutral oligomannosyl core products from the individual endoglycosidase digestions (Fig. 7C and 7E), as expected if essentially all of the radiolabel was in mannose. This indicated that the majority of radiolabeled product eluting in the position of $Man_5GlcNAc_1$ after the combined exoglycosidase and endo-β-N-acetylglucosaminidase D digestion (Fig. 7E) was derived from the large, sialic acid-enriched glycopeptides rather than from the smaller neutral glycopeptides with intermediate-size oligomannosyl core structures. In addition, the absence of radiolabel eluting in the positions of free N-acetylglucosamine (between stachyose and mannose gel filtration markers) and galactose (same position as free mannose) or small-molecular-weight glycopeptides (GlcNAc(±fucose)-asparaginyl peptide) expected after the combined endo- and exoglycosidase digestions (Fig. 7E,F) indicated that essentially all of the radiolabel incorporated into membrane glycoproteins during the 24-h labeling period was in the oligomannosyl core of asparagine-linked oligosaccharides.

The apparent heterogeniety in oligomannosyl core size for complex acidic oligosaccharides linked to asparagine in cellular membrane glycoproteins was a rather unusual result when compared to the commonly reported 3-mannose core for asparagine-linked acidic oligosaccharides [3], and was therefore examined in more detail by isolation and analysis of subclasses of these apparent high-molecular-weight acidic glycopeptides. Two individual regions of the preparative gel filtration profile of endo-β-N-acetylglucosaminidase H-resistant glycopeptides were isolated, as indicated by the diagonally lined boxes at the bottom of the analogous analytical profile in Figure 7C, and rechromatographed on the BioGel P-4 column either before or after additional glycosidase treatments (Fig. 8). Neuraminidase digestion shifted the elution positions of both the largest (Fig. 8, top) and next-to-largest (Fig. 8, bottom) acidic glycopeptides to higher-numbered fractions, indicating that both individual subclasses were enriched for terminal sialic acid. The gel filtration profiles after the combined exoglycosidase and endo-β-N-acetylglucosaminidase D digestion of each subclass indicated that the largest glycopeptides were enriched for the larger core structure(s) (Fig. 8, top), whereas the next-to-largest glycopeptides were enriched for the $Man_3GlcNAc_1$-size core (Fig. 8, bottom).

In summary, the "mature" glycopeptides from the 24-h labeling of BHK21 membrane glycoproteins contained two major classes of asparagine-linked oligosaccharides (with proposed structures shown in diagrammatic form in Fig. 9): 1) a mixture of neutral oligosaccharides, the major fraction containing 8–9 mannoses and a lesser fraction containing 5–7 mannoses, represented by the broad peak of glycopeptides eluting after the [^{14}C] column marker in Figure 7A,E; and 2) a mixture of acidic oligosaccharides with three and possibly five mannoses, represented by the large apparent molecular weight peaks eluting after the void volume in Figure 7A,C.

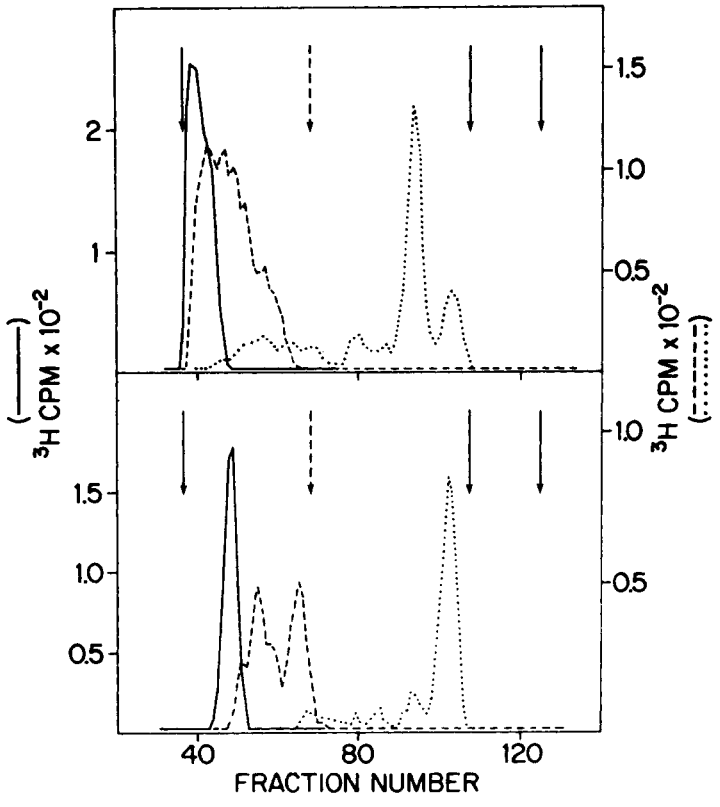

Fig. 8. BioGel P-4 gel filtration of acidic glycopeptides from a BHK21 monolayer culture labeled for 24 h with [2-^{3}H] mannose. Glycopeptides were pooled from two regions of apparent high-molecular-weight, endo-β-N-acetylglucosaminidase H-resistant glycopeptides in a preparative gel filtration profile, as indicated at the bottom of Figure 7C. Aliquots of the first, largest glycopeptide peak (upper panel) and the second glycopeptide peak (lower panel) were analyzed by gel filtration before or after glycosidase treatments, and the profiles of radioactivity superimposed: untreated (————); neuraminidase-treated (- - - - - - -); exoglycosidase- and endo-β-N-acetylglucosaminidase D-treated (. . . . . .).

DISCUSSION

These studies with [2-^{3}H] mannose-labeled BHK21 cells and human diploid fibroblasts have demonstrated that the processes involved in the biosynthesis and maturation of the asparagine-linked oligosaccharides of cellular membrane glycoproteins are analogous to those processes previously described for vesicular stomatitis and Sindbis virus membrane glycoproteins in virus-infected cells [4–6] and the heavy chain of IgG in murine plasmacytoma cells [6]. Although the pulse-labeled glycoproteins from the membranes of the BHK21 and human cells were a mixture of multiple polypeptide species, the newly synthesized asparagine-linked oligosaccharides were as homogenous in structure ($Man_9GlcNAc_2$-ASN, with lesser amounts of a possible glucose-containing oligosaccharide, $Glc_1Man_9GlcNAc_2$-ASN, and a slightly smaller oligosaccharide, $Man_8GlcNAc_2ASN$) as those observed in virus-infected cells with only a single major species of pulse-labeled membrane glycoprotein [5, 21]. The "precursor" forms of the cellular membrane glycoproteins subsequently underwent maturation of their asparagine-linked oligosaccharides as they were processed through intracellular

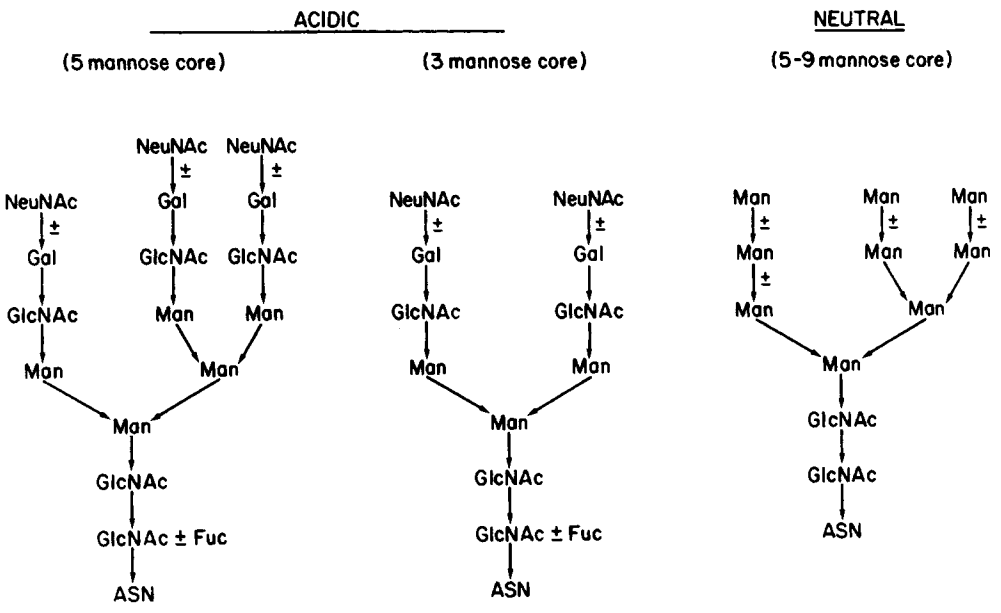

Fig. 9. Structures proposed for the "mature" asparagine-linked oligosaccharides of cellular membrane glycoproteins. The mannose-chitobiose-asparaginyl structure and the number of mannose units for the neutral oligosaccharides were based upon the gel filtration results and the sensitivity to endo-β-N-acetylglucosaminidases H or D. The proposed three-mannose acidic structure was derived from the glycosidase and gel filtration studies and previously proposed three-mannose core structures for immunoglobulin [3] and Sindbis virus glycoproteins [19]. The actual number of branch structures (NeuNAc-Gal-GlcNAc-) may have been either two or three, based upon analysis of the asparagine-linked oligosaccharides of the vesicular stomatitis virus glycoprotein [11, 20], the Sindbis virus glycoproteins [19] and several serum glycoproteins [3, 26]. The proposed five-mannose acidic oligosaccharide structure was consistent with the gel filtration and glycosidase studies of the high-molecular-weight BHK21 glycopeptides and the five-mannose core structure previously reported to be an intermediate in the biosynthesis of the three-mannose acidic oligosaccharides [27].

membranes, with two alternative pathways: 1) removal of mannoses and sequential addition of branch sugars (NeuNAc-Gal-GlcNAc-), to give acidic structures with three, and possibly five, mannoses; or 2) limited removal of mannoses, resulting in a mixture of neutral structures with 5–9 mannoses. This observed heterogenity in oligomannosyl core size and the presence of both acidic and neutral structures in the "mature" membrane glycoproteins from BHK21 and human cells confirmed the results of previous long-term (24- to 48-h) labeling studies with rat and human fibroblasts [1,2]. This heterogenity was also surprisingly similar to that reported recently for the oligosaccharides of avian RNA tumor virus glycoproteins [16].

These present studies further demonstrated that the relatively common high-mannose structure observed for the lipid-linked oligosaccharides in virus-infected and uninfected cells [4, 6–8] could indeed serve as the "precursor" for essentially all asparagine-linked oligosaccharides of cellular and viral membrane glycoproteins. Since the neutral oligomannosyl cores of most of the pulse-labeled "precursor" glycopeptides from BHK21 cells were apparently deficient in terminal glucose on the basis of size and α-mannosidase sensitivity relative to the major species of lipid-linked oligosaccharides from the same cells, the initial glycosylation products on membrane glycoproteins may have contained additional

glucose that was rapidly removed following the en bloc transfer from lipid to protein [4–6]. Although lipid-linked oligosaccharides containing glucose have been shown to be transferred more rapidly and to a greater extent than their corresponding nonglucosylated oligosaccharides to endogenous protein acceptors in a cell-free system [22], it was difficult to rule out the possibilities that 1) some of the glycoprotein may have acquired glucose-deficient oligosaccharides in the en bloc transfer; or 2) some of the glucose may have been removed immediately prior to or during the en bloc transfer in intact cells, so that not all of the asparagine-linked oligosaccharides initially contained two to three terminal glucoses.

The pulse-chase labeling studies (Figs. 5 and 6) indicated that whatever amount of terminal glucose that may have been present in the initial protein linked structure was removed from most of the membrane glycoproteins before significant trimming of mannose and conversion of "precursor" neutral structures to "mature" acidic and neutral structures. This processing of "precursor" to "mature" oligosaccharides proceeded over a period of several hours or more, as demonstrated by the increasing relative amounts of radiolabel in acidic glycopeptides and smaller neutral glycopeptides in the BHK21 and human cells after the 2-, 4-, and 24-h "chase" periods. The necessity of transporting newly synthesized glycoportein through the intracellular membrane system (from the site of en bloc addition in the rough endoplasmic reticulum [9] to the Golgi-membranes prior to trimming of mannose and addition of branch sugars [5, 9]) could have accounted for both the minimal lag of approximately 30 min between the initial glycosylation and final processing of oligosaccharides, and the asynchronous processing of these asparagine-linked oligosaccharides after pulse labeling. These conclusions were consistent with the relative enrichment of several specific α-glucosidases in rough endoplasmic reticulum-like membrane fractions [23] and a specific α-mannosidase (capable of removing four of nine mannoses shown in the neutral structure in Fig. 9 as ±) in Golgi-like membrane fractions [24].

More quantitative analysis of the time course of processing or the ratio of acidic to neutral asparagine-linked oligosaccharides in the "mature" glycopeptides from the pulse-chase labeling experiments was not possible for several reasons 1) the specific activity of labeling of the different mannoses may not have been identical; 2) some of the residual large neutral cores ($Man_{8-9}GlcNac_1$) in the 4- and 24-h chase samples (Figs. 5 and 6) may have represented unprocessed glycoproteins instead of glycoproteins that were supposed to contain large mannose core structures as their "mature" oligosaccharides; 3) radiolabeled mannose was lost as part of the maturation process; and 4) radiolabel may also have been lost because of protein turnover during the longer "chase" periods. Studies of the turnover of plasma membrane proteins in hepatoma cells [25] suggested that the turnover rate was similar for essentially all membrane proteins (half-life of approximately 100 h), so that one might not expect that neutral or acidic oligosaccharides would be lost preferentially by membrane glycoprotein turnover.

Although a number of acidic oligosaccharides with three mannose cores (Fig. 9, middle structure) have been well characterized for serum and membrane glycoproteins [3, 12, 19, 20], acidic oligosaccharides with five mannose cores have not been previously reported for cellular membrane glycoproteins. The asparaginyl-oligosaccharide with five mannoses shown in Figure 9 (left panel) was the most logical structure for the high-molecular-weight glycopeptides from the [2-^{3}H]mannose-labeled BHK21 cells if one assumed that the mixture of exoglycosidases had trimmed all of the complex acidic oligosaccharides to side chain-free structures ($Man_nGlcNAc_2$(± fucose)-peptide) prior to endo-β-N-acetylglucosaminidase D digestion. An alternative structure for the Man_5-$GlcNAc_1$-

size neutral oligosaccharide was a three-mannose core with a residual galactose and N-acetylglucosamine, or just N-acetylglucosamine, attached to one of the mannoses, as has been recently reported in studies of the complex acidic oligosaccharides of fetuin [26]. The envelope glycoprotein of vesicular stomatitis virus has oligosaccharides almost identical to those of fetuin [11, 20, 26], but $Man_3GlcNAc_1$ was the only major neutral oligosaccharide obtained from the combined exoglycosidase and endo-β-N-acetylglucosaminidase D digestion of radiolabeled glycopeptides from purified virus or virus-infected BHK21 cells [5, 11]. Because the identity of these larger core structures from cellular membrane glycoproteins and previously reported five-mannose acidic structures for avian RNA tumor virus glycoproteins [16] was based primarily on high-resolution gel filtration and specific glycosidase studies, the actual structure(s) must be considered tentative until more detailed studies are completed using more extensive exoglycosidase digestions (galactosidase, glucosaminidase, α-mannosidase [12]), periodate oxidation, and/or acetolysis [8].

Recent cell-free glycosylation studies have suggested that the three mannose acidic oligosaccharides are derived from five mannose neutral structures by the following ordered steps: 1) addition of the first branch N-acetylglucosamine to a specific α1,3-linked mannose in the five-mannose core; 2) removal of the final two mannoses; and 3) further addition of 1–2 branch N-acetylglucosamines to the three-mannose core [27]. If acidic oligosaccharides with five mannoses did exist, they would presumably occur by the same initial N-acetylglucosamine addition to the specific mannose, followed by further addition of N-acetylglucosamines without mannose removal.

Another uncertainty in the possible acidic structures proposed in Figure 9 was the actual number of branch structures (NeuNAc-Gal-GlcNAc-) linked to the different-size mannose cores. The apparent molecular weight of glycopeptides derived from gel filtration on BioGel P resins may not be especially meaningful in detailed structural determinations because of peptide heterogeneity and the negative charge exclusion property [5, 9, 11]. However, the good correlation between the larger apparent size of acidic glycopeptides and the larger neutral core after combined exoglycosidase and endoglycosidase treatment (Fig. 8) suggested that the acidic oligosaccharides with larger apparent core size may have contained a larger average number of branch sugars than the three mannose acidic oligosaccharides. Alternatively, the largest glycopeptides may have contained more than one oligosaccharide, such as an additional unlabeled serine- or threonine-linked oligosaccharide, rather than larger individual oligosaccharide structures.

The significance of this extensive heterogeneity for asparagine-linked oligosaccharides in cellular membrane glycoproteins is not clear. Changes in the size distribution of the oligomannosyl cores of membrane glycopeptides have been observed between growing and nongrowing human diploid fibroblasts [2]. In addition, differences in gel filtration profiles of fucose- and glucosamine-labeled glycopeptides from whole-cell membrane protein or individual cellular membrane glycoprotein species have been reported between normal and virus-transformed cells [28–30]. These studies, however, did not analyze the actual oligosaccharide structures nor did they rule out possible contributions of serine/threonine-linked oligosaccharides to the differences in gel filtration profiles. More detailed studies of a number of individual membrane glycoproteins and the oligosaccharide structures at specific glycosylation sites (both asparagine- and serine/threonine-linked) within these proteins will be necessary in order to make a significant correlation of growth- and transformation-dependent changes with possible changes in membrane glycoprotein biosynthesis, structure, and function.

ACKNOWLEDGMENTS

These studies were supported in part by Public Health Service grant AI 14757-01 from the National Institute of Allergy and Infectious Diseases and by a Biomedical Research Support grant from the National Institutes of Health to the University of Kansas Medical Center.

The author thanks Dr. James Etchison for various glycosidases and helpful discussions and comments, Alex Yem for technical assistance in part of these studies, and Brenda Smith for typing of the manuscript.

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