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Fucosylation of Membrane Proteins in Soybean Cultured Cells[1]

EFFECTS OF TUNICAMYCIN AND SWAINSONINE

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HIDETAKA HORI, GUR P. KAUSHAL, AND ALAN D. ELBEIN*
Department of Biochemistry, University of Texas Health Science Center, San Antonio, Texas 78284

ABSTRACT

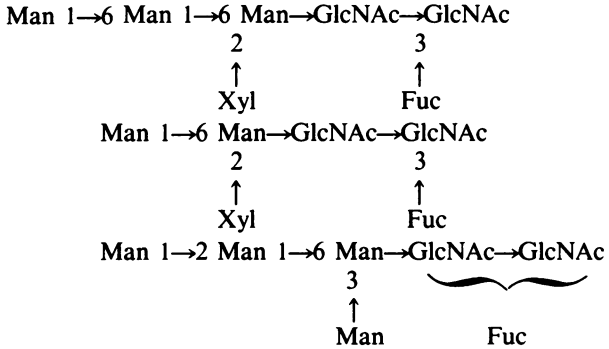
Cultures of soybean cells incorporate [5,6-^{3}H]-L-fucose into various cellular components including lipids and proteins. The membrane glycoproteins were digested with pronase to produce glycopeptides, and the glycopeptides were isolated on columns of Biogel P-4. The major fucose-labeled glycopeptide sized as a Hexose$_{15-17}$-*N*-acetylglucosamine$_2$ (GlcNAc$_2$) on columns of Biogel P-4. Fucose incorporation was also examined in the presence of the processing inhibitor swainsonine, and the glycosylation inhibitor tunicamycin. In the presence of swainsonine, the incorporation of fucose was not reduced but the glycopeptides were smaller in size and migrated like Hexose$_{12-13}$-GlcNAc$_2$ structures. On the other hand, tunicamycin inhibited the incorporation of fucose into the glycopeptides by 70 to 80%, indicating that the L-fucose was present in N-linked oligosaccharides.

The membrane glycoproteins were doubly-labeled by incubating soybean cells in [^{3}H]fucose and [^{14}C]mannose. By repeated separation on Biogel P-4, six glycopeptides were purified that ranged in size from Hexose$_8$GlcNAc$_2$ to Hexose$_{15-17}$-GlcNAc$_2$. The three larger glycopeptides (I, II, III) were highly labeled with [^{3}H]fucose and also contained [^{14}C] mannose. Evidence that both isotopes were in the same glycopeptide was obtained by the finding that the mannose-labeled glycopeptides were shifted to smaller-sized structures when the [^{3}H]fucose was removed by mild acid hydrolysis. Glycopeptide IV also contained [^{3}H]fucose and [^{14}C] mannose but only part of the [^{3}H]fucose was released by mild hydrolysis. Glycopeptides V and VI contained only small amounts of tritium, but were labeled with [^{14}C]mannose. None of the six glycopeptides was susceptible to the action of endo-β-*N*-acetylglucosaminidase H, and none of these glycopeptides was bound to columns of concanavalin A-sepharose. The smaller glycopeptides (IV, V, VI) were partially susceptible to α-mannosidase digestion, but this enzyme did not release any radioactive mannose from the larger-sized glycopeptides. These data indicate that the fucosylated glycopeptides are N-linked structures containing fucose, mannose, GlcNAc, and probably other sugars, and that the mannose units are blocked by other sugars. Thus, these results indicate that plant membrane glycoproteins contain a significant amount of modified oligosaccharide side chains.

Many membrane glycoproteins play important roles in recognition phenomena in both plant and animal cells (6). A number of these glycoproteins have been classified as *N*-linked glycoproteins since their oligosaccharide chains are linked to the amide nitrogen of asparagine (15). For example, the sugar side chain of soybean lectin has been shown to be a high-mannose type of structure that is linked to the protein via a GlcNAc[2] → asparagine bond (4). It is now clear from a variety of studies in animal and plant systems, that the oligosaccharide portion of these N-linked glycoproteins is biosynthesized by means of lipid-linked saccharide intermediates (12). In this pathway, the sugars GlcNAc, mannose, and glucose are added sequentially to dolichyl-phosphate to form the lipid-linked oligosaccharide, Glc$_3$Man$_9$ GlcNAc$_2$-pyrophosphoryl-dolichol (16, 19). The oligosaccharide portion of this intermediate is then transferred to protein.

In animal cells, once the oligosaccharide is transferred to protein, it is rapidly processed by the removal of all three glucose residues (27) and a number of mannose residues (21, 22) via a number of specific processing glycosidases. In addition, a number of sugars such as GlcNAc, galactose, sialic acid, and fucose may be added to give rise to the high-mannose, hybrid and complex types of oligosaccharides that are typically found in these cells (8). For example, the addition of L-fucose to animal glycoproteins occurs after the removal of three glucoses and four mannoses and the addition of one GlcNAc (17).

The reactions involved in processing of plant glycoproteins have not been studied to any great extent, but in these systems also, the initial reactions involve the removal of all three glucose residues (11). However, it is not clear how many mannose residues are ultimately removed nor what types of 'complex' chains are present in plants. Lima bean lectin and pineapple bromelain also contain asparagine-linked oligosaccharides (13, 18), but the oligosaccharide structures of these proteins are quite different from the typical high-mannose oligosaccharides. That is, these oligosaccharides do not contain the typical Manα1,3-[Manα1,6]ManβGlcNAc-GlcNAc-core, but instead have structures such as those shown below:



Since relatively little information is available on the incorporation of xylose or fucose into plant glycoproteins, the present study was undertaken to examine the fucosylation of plant

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[2] Abbreviations: GlcNAc, *N*-acetylglucosamine; Glc, glucose; Man, mannose; Endo H, endoglucosaminidase H.

glycoproteins. Some studies on fucose incorporation into phytohemagglutinin have recently been reported by Chrispeels (1).

MATERIALS AND METHODS

Materials. [2-^{3}H]Mannose (16 Ci/mmol) was purchased from Amersham Co., [U-^{14}C]Man (210 mCi/mmol) from ICN Chemicals and [5,6-^{3}H]L-fucose (67 mCi/mmol) from Pathfinders Labs. Jack bean α-mannosidase was obtained from Sigma and α-L-fucosidase was from Boehringer-Mannheim Co. Endo-β-*N*-acetylglucosaminidase H was purchased from Health Research Inc., Albany, NY. Tunicamycin was kindly supplied by Dr. Robert Hamill, Eli Lilly Co., and swainsonine by Dr. Peter Dorling, Murdoch University, Murdoch, Australia. Biogel P-4 (200–400 mesh) was purchased from Biorad Labs.

Growth of Cells and Incorporation of Isotopes. Soybean cells were grown in suspension culture for 7 to 10 d in Erlenmeyer flasks as previously described (9). The cultured cells were harvested by filtration on a Büchner funnel and washed with freshly prepared sucrose-free medium. About 15 g of cells were placed in 30 ml of sucrose-free medium in a 125-ml Erlenmeyer flask and tunicamycin (30 μg/ml) or swainsonine (30 μg/ml) were added where indicated. Some flasks served as controls without inhibitors. After an incubation of 30 min at 28°C to allow the inhibitors to take effect, 100 μCi of [^{3}H]fucose, [^{3}H]mannose, or [^{14}C]mannose were added. The cultures were allowed to incubate with the isotopes for varying periods of time and the cells were harvested by filtration and washed well with fresh medium to remove unincorporated radioactive sugars. The washed cells were fractionated as described below.

Cell Fractionation. The cells were suspended in 50 mM Tris-HCl buffer (pH 7.0) and homogenized with a glass Teflon homogenizer. The homogenate was centrifuged at 5000*g* for 10 min to pellet the cell debris. The supernatant liquid was saved and the cell debris was homogenized again and centrifuged. The combined supernatant liquids from this homogenization were subjected to ultracentrifugation at 105,000*g* for 60 min to isolate the 'membrane fraction.'

The membrane fraction was suspended in $CHCl_3$:CH_3OH:H_2O (1:1:1) to extract the lipid-linked monosaccharides as previously described (3, 10). After phase separation and removal of the lower $CHCl_3$ layer (which contains the lipid-linked monosaccharides and perhaps other glycolipids), the particulate material was reisolated by centrifugation and extracted with $CHCl_3$:CH_3OH:H_2O (10:10:3) to extract the lipid-linked oligosaccharides (10). After this extraction, the particulate material was reisolated by centrifugation, washed several times with methanol and then water, and digested exhaustively with pronase to release soluble glycopeptides. The insoluble residue remaining after pronase was considered pronase-resistant.

The supernatant fluid from the first high-speed centrifugation was adjusted to 70% with respect to ethanol and allowed to remain overnight in the cold. The ethanol-insoluble material was isolated by centrifugation and labeled 'cytosol/alcohol-insoluble fraction.' This material was also subjected to exhaustive pronase digestion to liberate glycopeptides. The supernatant liquid from this ethanol treatment was concentrated to dryness, taken up in a small volume of H_2O, and labeled 'cytosol/alcohol-soluble fraction.'

The cellular debris resulting from the original homogenization was homogenized in 1% SDS in a glass/Teflon homogenizer. The homogenate was centrifuged and the supernatant liquid was discarded. The pellet was again homogenized in 1% SDS and centrifuged. This SDS treatment was repeated three times. The remaining pellet was washed three times with water to remove SDS and labeled 'cell walls.' The radioactivity associated with each of these fractions from control cells, tunicamycin-treated cells, and swainsonine-treated cells is shown in Table I (see "Results").

Enzymic Digestions. The various cell fractions obtained as indicated above were dried under a stream of nitrogen to remove any organic solvents, and the residues were suspended in 1 ml of 20 mM Tris-HCl buffer (pH 7.5) containing 10 mM $CaCl_2$. One mg of pronase in 0.5 ml of the above buffer was then added and the mixtures were incubated for 24 h under a toluene atmosphere. At the end of this time, another aliquot of pronase solution was added, and a third addition at 48 h. Samples were analyzed after 72 h of digestion.

Purified glycopeptides obtained from Biogel columns were concentrated to dryness, taken up in 0.1 ml of 50 mM sodium citrate buffer (pH 6.0), and incubated with 5 munits of Endo H for 24 h. Another 5 munits enzyme were added for a second 24 h incubation. Products were analyzed by gel filtration on Biogel P-4.

Glycopeptides were dissolved in 0.1 ml of 0.1 M sodium acetate buffer (pH 5.0) containing 0.4 mM $ZnCl_2$ and digested with 1 unit of α-mannosidase for 24 h under a toluene atmosphere. Another 1 unit of enzyme was added after 24 h and incubations continued for an additional 24 h.

Finally, glycopeptides were dissolved in 0.1 ml of 100 mM sodium citrate buffer (pH 4.4) and digested with 20 munits of α-L-fucosidase for 24 h under a toluene atmosphere. A second addition of fucosidase was made at that time for an additional incubation of 24 h.

Chromatographic Procedures. Glycopeptides and oligosaccharides were dissolved in 1 ml of 0.1% acetic acid and separated on a 1.5 × 150 cm column of Biogel P-4 (200–400 mesh). One-ml fractions were collected and an aliquot of every fraction was removed for the determination of radioactivity.

Lipids were purified on DEAE-cellulose (acetate) columns as previously described (3). DEAE-cellulose was activated and equilibrated with acetic acid. The resin was packed into 2 × 22 cm columns and washed well with 99% methanol and $CHCl_3$:CH_3OH (2:1). The lipids were suspended in 99% CH_3OH and applied to the column. The column was washed with 99% CH_3OH and then eluted with 400 ml of a linear gradient of 0 to 0.4 M ammonium acetate in 99% CH_3OH. Aliquots of each fraction were analyzed for radioactivity.

Glycopeptides were hydrolyzed in 0.1 N HCl in an evacuated tube at 80°C for 1 h. The hydrolysate was evaporated to dryness, taken up in 1 ml of 0.5% acetic acid solution, and applied to a Biogel P-4 column. The released sugar monomer which was eluted at the position of authentic fucose was applied to Whatman 3MM paper. The papers were chromatographed in isobutanol:pyridine:0.1 N HCl (5:3:2) for 24 h by ascending chromatography. Standard sugars were visualized with the silver nitrate dip (29).

RESULTS

Distribution of Radioactivity among Various Fractions. Suspension-cultured soybean cells incorporated [^{3}H]fucose into various fractions as shown in Table I. In general, the amount of radioactivity found in the different fractions was the same in control cells or cells incubated in tunicamycin or swainsonine. Thus, about 64 to 68% of the radioactivity was found in the 70% ethanol supernatant liquid (Cytosol/Alcohol Soluble), and about 22% in the cell wall fraction. Some 4.5 to 8% of the radioactivity was in the lipid fraction and most of this radioactivity was extracted with $CHCl_3$:CH_3OH:H_2O (1:1:1). The amount of radioactivity in this lipid material did appear to be increased by swainsonine. Approximately 2.0 to 3.5% of the total fucose incorporated was found in the membrane fraction and most of this material was digestible by pronase. The amount of radioactivity found in the pronase-digestible membrane fraction was inhibited significantly by tunicamycin and stimulated somewhat

Table I. *Localization of Radioactivity Derived from L-5,6-^{3}H-Fucose in Various Subcellular Fractions*

Cell Fractionation	Radioactivity Incorporated					
	Control		Swainsonine		Tunicamycin	
	dpm/g cell fresh wt (%)					
Cell walls	79,303	(22)	85,125	(22)	89,475	(21)
Cytosol/alcohol-soluble fraction	246,935	(68)	242,210	(64)	285,120	(68)
Cytosol/alcohol-insoluble fraction						
Pronase digested	8,342	(2.3)	8,878	(2.3)	10,880	(2.6)
Pronase resistant	2,212	(0.6)	2,484	(0.7)	2,889	(0.7)
Membrane						
Pronase digested	8,833	(2.4)	10,190	(2.7)	5,893	(1.4)
Pronase resistant	2,064	(0.5)	2,920	(0.8)	3,089	(0.7)
Lipid						
1:1:1 Extracted	16,063	(4.4)	27,674	(7.3)	23,408	(5.5)
10:10:3 Extracted	641	(0.2)	1,248	(0.3)	993	(0.2)
Total recovered radioactivity	364393		380729		421747	

by swainsonine. Finally, about 3% of the total radioactivity was found in the Cytosol/Alcohol-Insoluble Fraction and about 65 to 75% of this material was digestible with pronase.

Characterization of Labeled Lipids. To determine whether the fucose-containing lipids were charged, they were chromatographed on DEAE cellulose as previously described (3). When the lipid that was extracted with $CHCl_3$:CH_3OH:H_2O (1:1:1) was applied to DEAE-cellulose, about 83% of the radioactivity emerged in the wash indicating that it was in neutral glycolipids, while 17% was eluted in a broad peak with the ammonium acetate gradient. Also with the lipids extracted with $CHCl_3$:CH_3OH:H_2O (10:10:3), 77% of the radioactivity emerged in the wash, while 23% was eluted with 0.1 M ammonium acetate. The acidic lipids from DEAE-cellulose were pooled, washed with water to remove ammonium acetate, and subjected to hydrolysis in 0.02 N HCl in 20% CH_3OH for 30′ at 100°C. About 10% of the radioactivity in these lipids was released into the aqueous phase by this mild acid hydrolysis. Thus, much of the radioactivity is not released by mild acid hydrolysis. However, the sugar that was released cochromatographed with authentic fucose upon paper chromatography. These lipids were not further analyzed at this time.

Effect of Tunicamycin and Swainsonine on Membrane and Cytoplasmic Glycopeptides. The glycopeptides released from the membrane fraction by pronase digestion were chromatographed on columns of Biogel P-4 in order to compare the profiles of inhibited cells to those of normal cells. Figure 1 compares the glycopeptide patterns of untreated soybean cells (top profile) to that of swainsonine-treated (middle) and tunicamycin-treated (lower) cells. In the control, the major fucose-labeled peak emerged at fractions 97 to 107 corresponding to oligosaccharides of $Hexose_{15\text{-}17}GlcNAc_2$ sizes. In addition, a shoulder of radioactivity was also seen in the $Hexose_{11}GlcNAc_2$ area, and several smaller sized peaks in the tri- or tetrasaccharide areas. It should be noted that the major, fucose-labeled glycopeptide is larger in size by at least several hexose residues than the previously described mannose-labeled glycopeptides (12). However, since the size of the peptide portions of these glycopeptides is not known, an accurate size determination of the oligosaccharide cannot be made.

The middle profile shows that when cells were labeled with L-fucose in the presence of swainsonine, the major glycopeptide was considerably smaller in size than that of control cells. In this case, the peak emerged at fractions 105 to 115 which corresponded roughly to a $Hexose_{13}GlcNAc_2$ structure. This peak was in the same general position as the shoulder of the control cells. The smaller sized peaks were still present in the swainsonine-treated cells. Swainsonine is a plant alkaloid (1,7,8-trihydroxyoctahydroindolizine) that inhibits α-mannosidase (5) and also specifically inhibits the glycoprotein processing mannosidase II (26) in animal cells. This inhibition prevents normal glycoprotein processing to complex chains and results in the formation of hybrid types of oligosaccharides (7). Although the details of glycoprotein processing in soybean cells are not known, it is possible that swainsonine prevents mannose trimming which might be necessary for the formation of the large-sized glycopeptides.

The lower profile shows the effect of tunicamycin on the fucosylation of membrane glycoproteins in soybean cells. Tunicamycin is a glucosamine-containing antibiotic (23) that prevents the formation of the first lipid intermediate, GlcNAc-pyrophosphoryl-dolichol (25). Since the inhibitor prevents the formation of lipid intermediates, glycoproteins that have asparagine-linked oligosaccharides cannot be glycosylated (20). It can be seen that tunicamycin caused a dramatic inhibition in the amount of [^{3}H]fucose found in the membrane glycopeptides. Thus, the radioactivity associated with the major peak was decreased by more than 70%. On the other hand, the smaller peaks emerging at fractions 160 to 180 were not affected by tunicamycin. This data provides preliminary evidence that the fucose in the major glycopeptides is in N-linked oligosaccharides.

Similar experiments were done with the Cytosol/Alcohol-Insoluble Fraction isolated by ethanol precipitation of the supernatant fraction. The profiles of these glycopeptides were very similar to those of the membrane glycopeptides. That is, the major fucose-labeled glycopeptide of control cells emerged in fractions 97 to 105 like a $Hexose_{15\text{-}17}GlcNAc_2$, whereas in the presence of swainsonine, this peak was smaller in size and migrated in the $Hexose_{13}GlcNAc_2$ area of the column. And in the presence of tunicamycin, the amount of [^{3}H]fucose incorporated into the major peak was inhibited more than 80% (data not shown).

Labeling Membrane Glycopeptides with [^{3}H]Fucose and [^{14}C] Mannose. Since the major glycopeptides labeled with [^{3}H]fucose were larger in size than the [^{3}H]mannose-labeled glycopeptides previously described (11), it was of interest to simultaneously label the glycopeptides with fucose and mannose in order to compare the properties with respect to these two labels. Thus, soybean cells were incubated with [^{3}H]fucose and [^{14}C]mannose to doubly label the glycoproteins. The membrane fraction was isolated as described in "Materials and Methods" and digested with pronase. The resulting glycopeptides were then separated on Biogel P-4 columns as shown in Figure 2. It can be seen that the major peak of fucose-labeled material eluted in fractions 97

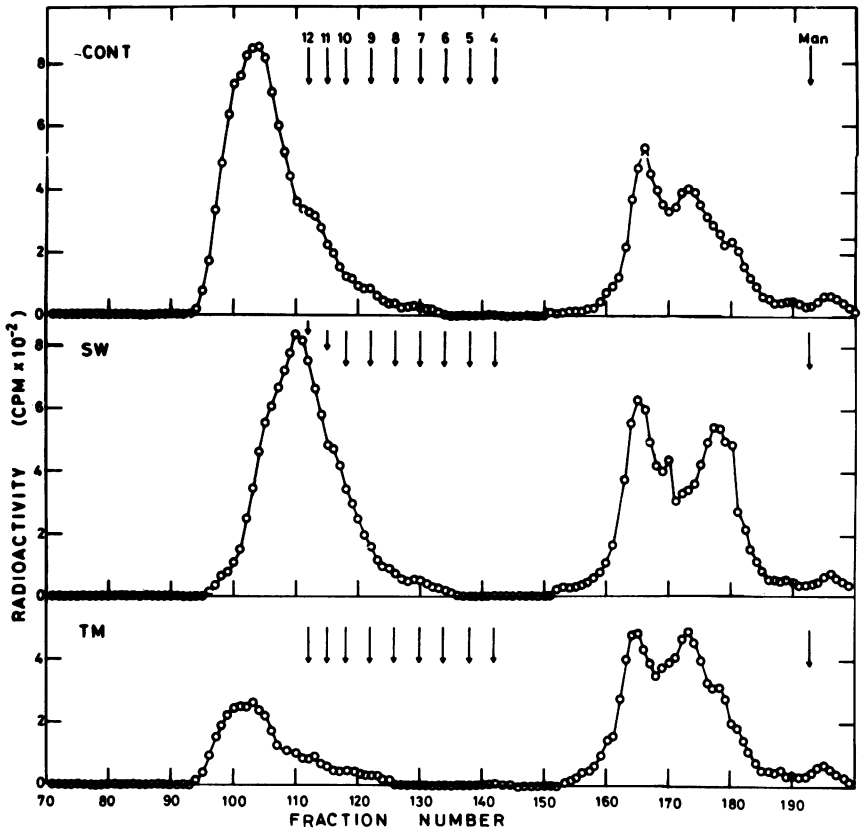

FIG. 1. Isolation of fucose-labeled glycopeptides by gel filtration. Soybean cells were incubated in the presence of tunicamycin (TM) or swainsonine (SW) for several hours. A control flask was incubated without inhibitor. Cultures were then labeled by the addition of [5,6-^{3}H]fucose. The membrane glycoproteins were isolated as described in "Materials and Methods" and digested exhaustively with pronase. The liberated glycopeptides were separated on a 1.5 × 150 cm column of Biogel P-4. One-ml fractions were collected and an aliquot of each fraction was removed for the determination of radioactivity. Arrows and numbers represent the elution position of various standards as follows: 12, $Hexose_{12}GlcNAc_2$; 11, $Hexose_{11}GlcNAc_2$; 10, $Hexose_{10}GlcNAc_2$, etc.

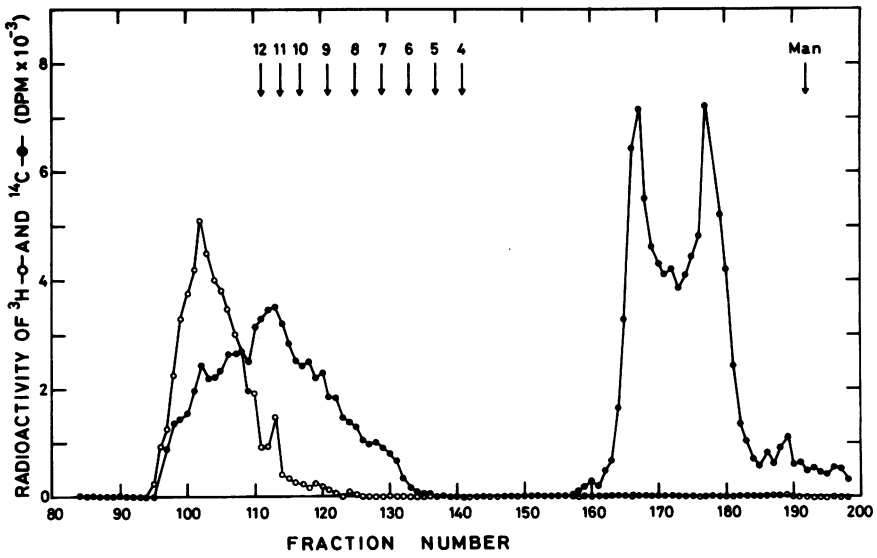

FIG. 2. Isolation of doubly labeled glycopeptides by Biogel P-4 chromatography. Membrane glycoproteins were prepared doubly labeled by incubating soybean cells in [5,6-^{3}C]fucose and [U-^{14}C]mannose. After pronase digestion, the glycopeptides were isolated on a Biogel P-4 column. Aliquots of each fraction were removed for the determination of ^{3}H and ^{14}C. Standards are the same as in Figure 1.

to 104, while the smaller glycopeptides eluting at fractions 110 or later did not appear to contain radioactive fucose. On the other hand, the major peak of [^{14}C]mannose-labeled material was in the smaller-sized glycopeptides that eluted near the $Hexose_{12}GlcNAc_2$ standard, but the larger-sized fucose-labeled glycopeptides also contained [^{14}C]mannose.

Characterization of Doubly Labeled Glycopeptides. The membrane glycopeptides from cells labeled with [^{3}H]fucose and [^{14}C] mannose were separated on a column of Biogel P-4 as seen in Figure 2. Beginning with fraction 95, every six fractions were pooled and rechromatographed on the Biogel column. After several such runs, six glycopeptide peaks were isolated as shown in Figure 3. The first three peaks (I, II, III) contained [^{3}H]fucose as well as [^{14}C]mannose, while the last three peaks (IV, V, VI) contained [^{14}C]mannose but little or no [^{3}H]fucose. In these cases, the radioactivity plotted as [^{3}H]fucose probably represents spillover of the [^{14}C]mannose. The sizes of the six glycopeptides corresponded approximately to the following oligosaccharides: I, $Hexose_{17}GlcNAc$; II, $Hexose_{15}GlcNAc_2$; III, $Hexose_{13}GlcNAc_2$; IV, $Hexose_{11}GlcNAc_2$; V, $Hexose_{10}GlcNAc_2$; and VI, $Hexose_8$ $GlcNAc_2$.

To show that fucose and mannose were in the same glycopeptides and to examine the effect of fucose release, each glycopeptide was subjected to mild acid hydrolysis (0.1 N HCl, 80°C, 60 min) to cleave the labile fucose linkage. After hydrolysis, the samples were rechromatographed on the Biogel P-4 columns as shown in Figure 4. It can be seen that this treatment released a large peak of ^{3}H from glycopeptides I, II, and III, and that the

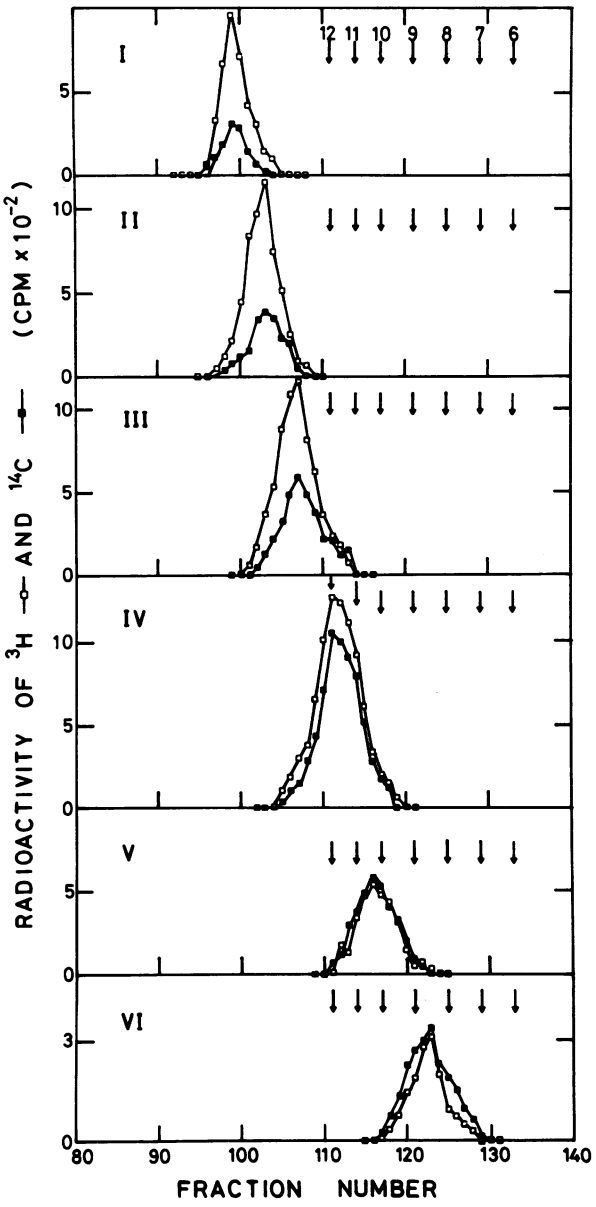

FIG. 3. Isolation of individual glycopeptides by gel filtration. In order to isolate individual glycopeptides, the peak shown in Figure 2 (fractions 95-135) was divided into six equal fractions (*i.e.* every 6 tubes were pooled). Each of these fractions was rechromatographed on the Biogel P-4 column. Then each peak was rerun on the Biogel column a second time as shown here. Aliquots were removed for the determination of ^3H and ^{14}C. Standards are as shown in Figure 1.

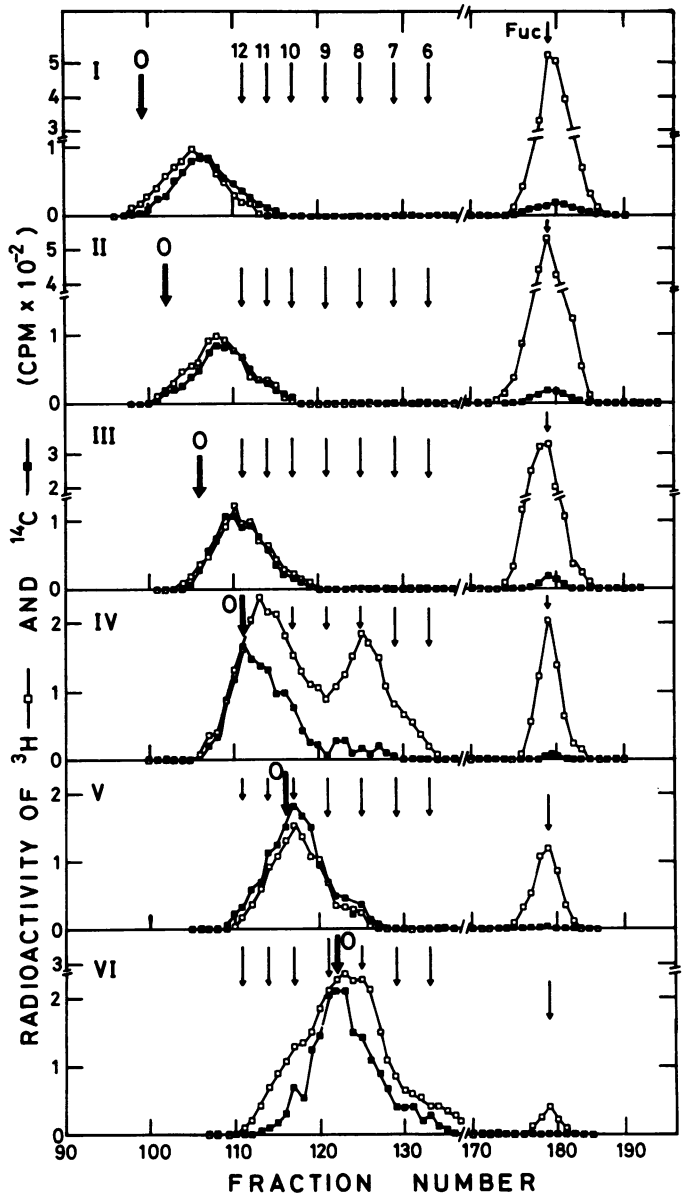

FIG. 4. Effect of mild acid hydrolysis on the doubly labeled glycopeptides. Each of the glycopeptides shown in Figure 3 was subjected to mild acid hydrolysis (0.1 N HCl, 80°C, 60 min) and then the hydrolysate was rechromatographed on the Biogel column. Aliquots of each fraction were analyzed for ^3H and ^{14}C. Standards are shown in Figure 1. The arrow marked 0 represents the original migration position of each glycopeptide.

released radioactivity now migrated in the hexose area of the column. Furthermore, after this treatment, glycopeptides I, II, and III were shifted in their migration properties. That is, all of the [^{14}C]mannose label now migrated slower on the column and this change in migration was equivalent to the loss of 2 or 3 hexose residues. These data indicate that the fucose and mannose are in the same glycopeptides. Glycopeptide IV also liberated a large peak of tritium in the monosaccharide area, but two other peaks of ^3H were detected that migrated in the $Hexose_{11}GlcNAc_2$ and $Hexose_8$ $GlcNAc_2$ areas. The larger sized peak (*i.e.* $Hexose_{11}GlcNAc_2$) also contained [^{14}C]mannose but the smaller one appeared to be free of ^{14}C. Finally, glycopeptide peaks V and VI were not greatly affected by the mild acid hydrolysis. Only a small peak of ^3H was released from glycopeptide V, but no shift in the migration of V or VI was observed. Again, it should be pointed out that the ^3H counts that show up in the same areas as the ^{14}C radioactivity and show the same level of radioactivity are probably due to spillover of ^{14}C in the ^3H channel.

The radioactive sugars that were released by mild acid hydrolysis of the glycopeptides were identified by paper chromatography as shown in Figure 5. The majority of the radioactivity released from glycopeptides II, III, and IV migrated with authentic fucose on paper chromatograms. In each case, a small peak of radioactivity moved more slowly and was observed in the Glc-Man areas of the paper. Whether this slower-migrating peak of radioactivity represents metabolites of [^3H]fucose that have also been incorporated into macromolecules, or whether it represents acid-labile hexoses derived from the [^{14}C]mannose is not known. It is also possible that this radioactivity represents degradation

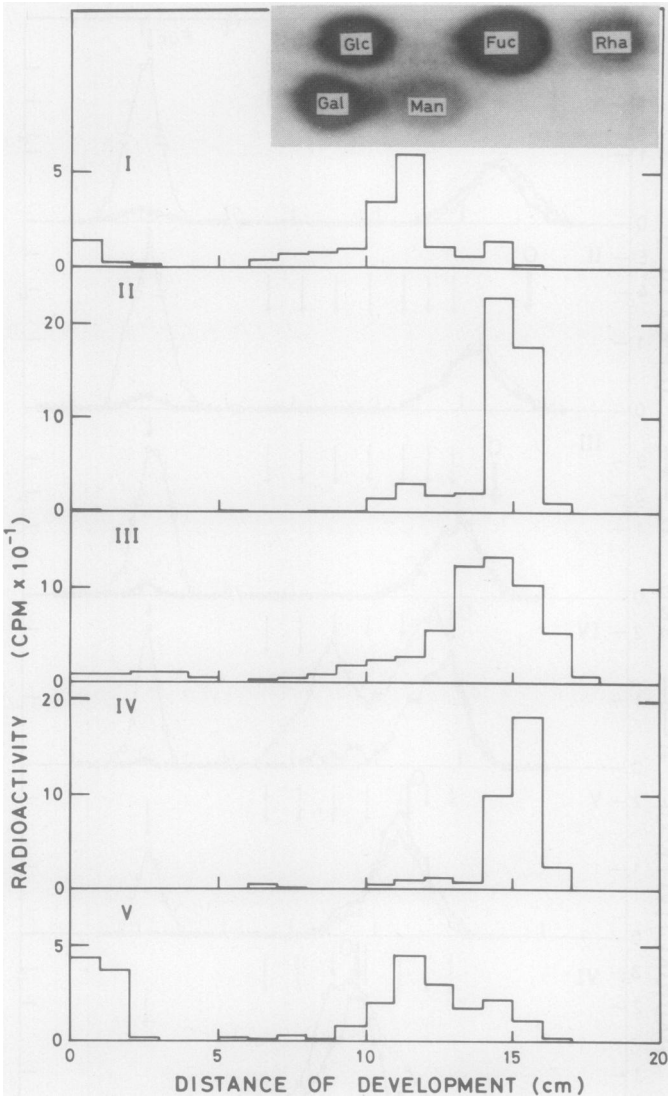

FIG. 5. Paper chromatographic identification of sugars released by mild acid hydrolysis. After mild acid hydrolysis of each glycopeptide, the hydrolysates were separated on Biogel P-4 columns as shown in Figure 4. The monosaccharide areas were pooled, concentrated, and streaked on Whatman 3MM paper. The papers were chromatographed in isobutanol:pyridine:0.1 N HCl (5:3:2) and radioactive areas were detected by cutting the papers into 1-cm strips and counting them in the scintillation counter. Standard sugars were visualized with silver nitrate.

products of fucose arising during the hydrolysis, since [^{3}H]fucose itself was found to be somewhat unstable to acid hydrolysis. On the other hand, the radioactivity liberated by mild acid hydrolysis from glycopeptides I and V migrated mostly in the Glc-Man areas of the paper and only a small peak of radioactive fucose was observed.

The glycopeptides, labeled only with [^{3}H]fucose, were separated into six fractions (Fig. 3). Each of these glycopeptides was then treated with endo-β-*N*-acetylglucosaminidase H to determine whether they were susceptible to this enzyme. Endo H cleaves high-mannose and hybrid chains between the two internal GlcNAc residues (24), but this enzyme requires that the mannose residue linked α1,6 to the β-linked mannose be substituted with an α1,3-linked mannose (14). None of the six glycopeptide peaks (I through VI) were susceptible to the action of this enzyme (data not shown). On the other hand, the enzyme did cleave typical high-mannose structures from soybean cells

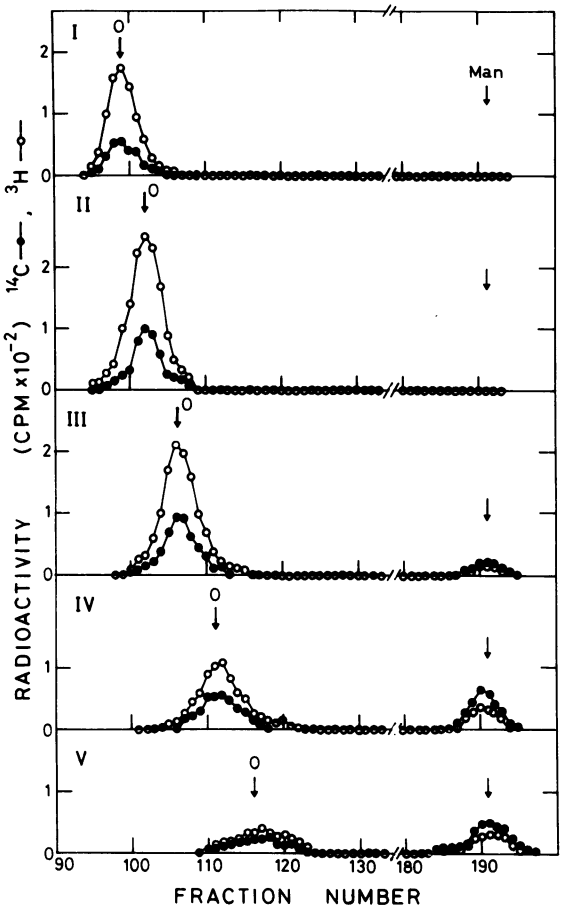

FIG. 6. Effect of α-mannosidase digestion on glycopeptides. Glycopeptides I through V were digested with α-mannosidase and the products were identified by chromatography on Biogel P-4. Aliquots of every fraction were analyzed for ^{3}H and ^{14}C.

(11) and animal cells (7).

The various glycopeptides, labeled with [^{3}H]fucose and [^{14}C] mannose, were isolated as indicated in Figure 3. Each of the purified glycopeptides was then treated with jack bean α-mannosidase as shown in Figure 6. Glycopeptides I and II were completely resistant to this treatment and no [^{14}C]mannose was released even with exhaustive digestion. Furthermore, no [^{14}C] mannose was released by α-mannosodase even when these glycopeptides were treated with mild acid to remove any blocking fucose residues. Thus, these glycopeptides must have other sugars, besides fucose, that prevent the mannosyl residues from being hydrolyzed by the α-mannosidase. A small peak of [^{14}C] mannose was released from glycopeptides III and IV by α-mannosidase but this treatment did not greatly change the mobility of the original oligosaccharide (Fig. 6). Thus, this treatment probably released no more than one mannose residue from these structures. Finally, glycopeptide V was the most susceptible to α-mannosidase digestion which liberated more than 50% of the ^{14}C as free mannose. In addition, the original peak was shifted in migration and became more heterogeneous. Since peak V contains only small amounts of [^{3}H]fucose, these data suggest that this glycopeptide may be an intermediate in the synthesis of the large glycopeptides and that some of the mannose residues are capped with other sugars.

The [^{3}H]fucose-labeled glycopeptides, purified by Biogel P-4 chromatography, were applied to columns of concanavalin A-Sepharose to examine their binding properties (2). Complex types of oligosaccharides do not bind to this column, whereas hybrid

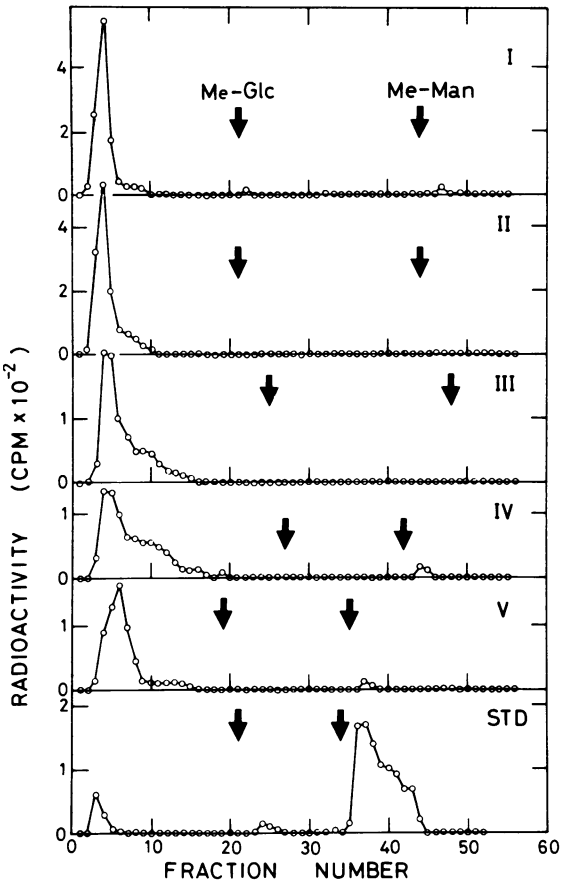

FIG. 7. Binding of glycopeptides to concanavalin A-Sepharose. The column was washed with buffer to remove unbound material and then eluted with 10 mM α-methylglucoside and then 200 mM α-methylmannoside. Fractions (0.5 ml) were collected and the radioactivity determined.

and high-mannose oligosaccharides bind with different affinities and can be eluted with various concentrations of α-methylmannoside (28) (Fig. 7). It can be seen that none of the glycopeptides bound to the concanavalin A and in each case, essentially all of the [^{3}H]fucose-labeled material emerged in the wash. On the other hand, a standard oligosaccharide, $Man_{7-8}GlcNAc_2$, did bind to the column and required 200 mM α-methylmannoside for removal (lower profile). These data lend further support to the assertion that these N-linked glycopeptides do not contain terminal α-linked mannose residues and also do not have any exposed 2-linked mannose units. Such structures are believed to be necessary for binding to concanavalin A.

DISCUSSION

Some plant glycoproteins contain L-fucose as part of the N-linked oligosaccharide chains, but relatively little information is available on the biosynthetic steps involved in fucosylation of these proteins. Chrispeels (1) has examined the fucosylation of phytohemagglutinin in *Phaseolus vulgaris* cotyledons. He found that fucose incorporation into phytohemagglutinin was inhibited by tunicamycin, suggesting that fucose was incorporated into N-linked oligosaccharides. The glycopeptides produced by proteinase treatment were labeled with both [^{3}H]fucose and [^{3}H]glucosamine and appeared to be fairly large based on gel filtration. These structures were mostly resistant to digestion by endo-β-*N*-acetylglucosaminidase H indicating that they were not the typical high-mannose structures.

In the present study, we examined the fucosylation of membrane glycoproteins in cultured soybean cells. These cells incorporated [^{3}H]fucose into lipids as well as cytoplasmic and membrane glycoproteins. The incorporation of fucose into the cytoplasmic and membrane glycoproteins was strongly inhibited by tunicamycin, also suggesting that fucose was incorporated into N-linked oligosaccharides. Interestingly enough, swainsonine, an inhibitor of glycoprotein processing in animal cells (7), did not inhibit the incorporation of fucose into glycoproteins but it did significantly alter the size of the fucosylated glycopeptides. Thus, in the presence of this inhibitor, the glycopeptides were smaller in size than those of control cells. Swainsonine is a plant alkaloid that inhibits α-mannosidase activity (5) and specifically inhibits the glycoprotein processing enzyme, mannosidase II (26). It is possible that the fucosylated glycoproteins in soybean cells have to be processed by the removal of mannose residues before other sugars can be added to elongate the oligosaccharide chains. Thus, if removal of mannose is inhibited, other sugars may not be added. Perhaps fucose is added before trimming occurs, or fucose can still be added in the absence of trimming.

In the present study, we doubly labeled the membrane glycoproteins by incubating soybean cells in [^{3}H]fucose and [^{14}C] mannose. After pronase digestion, we were able to separate and purify six glycopeptides from the membrane glycoproteins. The three largest glycopeptides were highly labeled with both [^{3}H] fucose and [^{14}C]mannose. That both of these isotopes were present in the same structures was indicated by the fact that the position of the [^{14}C]mannose-labeled glycopeptides was shifted to smaller-sized structures when fucose was removed by mild acid hydrolysis. As previously demonstrated by Chrispeels (1) and by Ishihara *et al.* (13), these fucose-labeled glycopeptides were resistant to the action of endoglucosaminidase H indicating that they were not typical high mannose chains. However, other experiments reported here and in those other studies do indicate that the fucose is in an oligosaccharide that has the mannose and GlcNAc core structure. In the case of stem bromelain (13), the fucose-containing oligosacchride is of low mol wt and contains only 5 to 7 monosaccharide units. On the other hand, in the phytohemagglutinin (1), the oligosaccharides are considerably larger in size although an exact calibration was not done in that case. The fucose-containing glycopeptides described in the present study are considerably larger (*i.e.* $Hexose_{15-17}GlcNAc_2$) than the usual high-mannose structures (*i.e.* $Man_9GlcNAc_2$). Although the size of the peptide portion of the glycopeptides is not known, it seems likely that only one or a few amino acids are present since the glycopeptides were obtained by exhaustive pronase digestion. Thus, it appears that a number of sugar residues are added to the glycoproteins during the processing steps. It is possible that the fucose is present in some type of repeating unit that is attached to the Man-GlcNAc core structure. Future studies are being designed to obtain sufficient amounts of these glycopeptides to be able to determine sugar composition as well as detailed oligosaccharide structure.

All of the fucose-labeled glycopeptides described here were resistant to endoglucosaminidase H, indicating that they were not typical high-mannose structures. Similar results were reported by Chrispeels (1) and by Ishihara *et al.* (13) with their fucosylated glycoproteins. In addition, we found that those glycopeptides did not bind to concanavalin A-Sepharose and were largely resistant to α-mannosidase digestion. These data further support the idea that the Man-GlcNAc core structure is blocked by fucose and perhaps by other sugars. In this regard, a recent paper by Vitale and Chrispeels (28) has nicely shown that the phytohemagglutinin located in the Golgi complex of developing cotyledons contains terminal GlcNAc residues on its N-linked oligosaccharides. However, these terminal GlcNAc residues are slowly removed once the protein has been transported to the

protein bodies. It seems possible that some of the proteins described here could have terminal GlcNAc which are not removed.

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