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Carbohydrate Research 338 (2003) 887–901
CARBOHYDRATE RESEARCH
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# The binding characteristics and utilization of *Aleuria aurantia*, *Lens culinaris* and few other lectins in the elucidation of fucosyltransferase activities resembling cloned FT VI and apparently unique to colon cancer cells

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Received 10 June 2002; accepted 2 January 2003

**Abstract**

Human colon carcinoma cell fucosyltransferase (FT) in contrast to the FTs of several human cancer cell lines, utilized GlcNAcβ1,4GlcNAcβ-O-Bn as an acceptor, the product being resistant to α1,6-L-Fucosidase and its formation being completely inhibited by LacNAc Type 2 acceptors. Further, this enzyme was twofold active towards the asialo agalacto glycopeptide as compared to the parent asialoglycopeptide. Only 60% of the GlcNAc moieties were released from [$^{14}$C]fucosylated asialo agalacto triantennary glycopeptide by jack bean β-*N*-acetylhexosaminidase. These α1,3-L-fucosylating activities on multiterminal GlcNAc residues and chitobiose were further examined by characterizing the products arising from fetuin triantennary and bovine IgG diantennary glycopeptides and their exoglycosidase-modified derivatives using lectin affinity chromatography. Utilization of [$^{14}$C]fucosylated glycopeptides with cloned FTs indicated that *Lens culinaris* lectin and *Aleuria aurantia* lectin (AAL) required, respectively, the diantennary backbone and the chitobiose core α1,6-fucosyl residue for binding. The outer core α1,3- but not the α-1,2-fucosyl residues decreased the binding affinity of AAL. The AAL-binding fraction from [$^{14}$C]fucosylated asialo fetuin, using colon carcinoma cell extract, contained 60% Endo F/PNGaseF resistant chains. Similarly AAL-binding species from [$^{14}$C]fucosylated TFA-treated bovine IgG using colon carcinoma cell extract showed significant resistance to endo F/PNGaseF. However, no such resistance was found with the corresponding AAL non- and weak-binding species. Thus colon carcinoma cells have the capacity to fucosylate the chitobiose core in glycoproteins, and this α1,3-L-fucosylation is apparently responsible for the AAL binding of glycoproteins. A cloned FT VI was found to be very similar to this enzyme in acceptor substrate specificities. The colon cancer cell FT thus exhibits four catalytic roles, i.e., α1,3-L-fucosylation of: (a) Galβ1,4GlcNAcβ-; (b) multiterminal GlcNAc units in complex type chain; (c) the inner core chitobiose of glycopeptides and glycoproteins; and (d) the nonreducing terminal chiotobiose unit. © 2003 Published by Elsevier Science Ltd.

*Keywords:* *Aleuria aurantia* lectin; Concanavalin A; Fucosyltransferase; Glycopeptides; Glycoproteins; *Griffonia simplicifolia* lectin II; *Lens culinaris* lectin; Wheat germ agglutinin

## 1. Introduction

Many lectins bind to oligosaccharides with different affinities, even though the binding can be inhibited by simple monosaccharides. Debray and co-workers observed that glycoasparagines, glycopeptides and glycoproteins had a higher affinity for lectins than the related oligosaccharides.[1] Their observation could be explained

*Abbreviations:* AA-CP, Acrylamide copolymer; AAL, *Aleuria aurantia* lectin; All, Allyl; Bn, Benzyl; endo F/PNGaseF, Endoglycosidase F/peptide-*N*-glycosidase F; FT, Fucosyltransferase; GP, Glycopeptide; GSLII, *Griffonia simplicifolia* lectin II; LacNAc Type I, Galβ1,3GlcNAcβ-; LacNAc Type II, Galβ1,4GlcNAcβ-; LCL, *Lens culinaris* lectin; Me, Methyl; ONP, *o*-Nitrophenyl; TFA, Trifluoroacetic acid; TLC, Thin-layer chromatography; WGA, Wheat-germ agglutinin.

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0008-6215/03/$ - see front matter © 2003 Published by Elsevier Science Ltd.
doi:10.1016/S0008-6215(03)00021-1

by the fact that the glycan–amino linkage leads to structures more rigid than those of the oligosaccharide themselves. High-affinity interactions between *Lens culinaris* lectin (LCL) and an oligosaccharide have been proposed to depend on the recognition of the chitobiosyl core and the α-(1→6)-linked fucose residue attached to the reducing terminal GlcNAc.[2,3] Further, the binding of LCL coupled to Sepharose was enhanced, if a GlcNAc residue is being exposed at the nonreducing end of the chain,[3] as witnessed with the carbohydrate structure of bovine IgG glycopeptide.[4,5] Fucose is not an effective inhibitor for LCL, suggesting that this high-affinity interaction is not dependent upon the occupation of a subsite which simply binds fucose. Instead, it is possible that this core-dependent interaction involves a composite epitope with contributions from both the fucose and intact GlcNAc residues.[6]

Among the lectins which bind fucosylated glycans, *Ulex europeus* agglutinin I displays high affinity interactions with H-blood group determinants.[7,8] An $Le^x$-containing oligosaccharide was found as a potent inhibitor of *Lotus tetragonolobus* agglutinin (LTA) agglutination.[1] Yan and co-workers noticed that LTA has relatively low affinity for fucosylated oligosaccharides; multiple terminal $Le^x$-containing structures were bound with higher affinity by LTA, indicating the requirement for a high density of immobilized LTA in order to isolate fucose-containing structures.[9] *Aleuria aurantia* lectin (AAL) binds structures with a fucose residue α-(1→6)-linked to the chitobiose core.[10] Yazawa and co-workers demonstrated the utility of immobilized AAL in the isolation of tumor-associated antigens.[11] Mir-Shekari and co-workers used AAL-agarose in the isolation of fucosylated carbohydrate chains from influenza A virus.[12] It appears that the α-(1→6)-linked fucosyl residue plays a crucial role in the binding ability of both LCL and AAL with the carbohydrate structures. Our laboratory was the first to observe, as early as 1997, the enzymatic [$^{14}$C]fucosylation of GlcNAcβ1,4GlcNAcβ-O-Bn by colon carcinoma cells and used that [$^{14}$C]fucosylated product as a positive control in autoradiography TLC.[13] The present paper reports the binding characteristics of LCL and AAL towards [$^{14}$C]fucosylated glycopeptides and glycoproteins, and their usefulness, along with Con A, WGA and GSL II, in the identification of a fucosyltransferase (FT) apparently unique to colon cancer cells and then, demonstrating its probable identity as FT VI.

## 2. Experimental

### 2.1. Cells

The colon carcinoma cell line, Colo 205, the hepatic carcinoma cell line, $HepG_2$, the breast carcinoma cell lines, BT20 and MCF-7, the prostate cancer cell line, DU 145, and the ovarian teratocarcinoma cell line, PA-1, were grown in minimal essential medium; the colon carcinoma cell line, LS 180, the leukemia cell line, HL60, the breast carcinoma cell line, DU4475, and the prostate carcinoma cell line, LNCaP, were grown in RPMI 1640; the colon cell line, SW1116, the ovarian carcinoma cell line, SW626, the breast carcinoma cell lines, MDA-MB-231, MDA-MB-435S, MDA-435/LCC6 and MDA-435/$LCC6^{MDRI}$ and the prostate carcinoma cell line, PC-3 were grown in Leibovitz's L-15 medium. All media were supplemented with 10% fetal bovine serum and the antibiotics, penicillin, streptomycin and amphtoericin B in 250 mL T-flasks under conditions as recommended by American Type Culture Collection, except for DU4475, which was grown as a suspension. MDA-435/LCC6 and MDA-435/$LCC6^{MDRI}$ were kindly provided by Dr Ralph Bernacki of this Institute. The cells were homogenized with 0.1 M Tris–Maleate pH 6.3 containing 2% Triton X-100 using a Dounce all-glass hand-operated homogenizer. The homogenate was centrifuged at 16,000*g* for 1 h at 4 °C. Protein was measured on the supernatants by the BCA micro method (Pierce Chemical Co.) with BSA as the standard. The supernatants were adjusted to 5 mg protein/mL by adding the necessary amount of extraction buffer and then stored frozen at −20 °C until use. Aliquots (10 μL) of the extracts were used in assays run in duplicate.

### 2.2. Synthetic compounds

The chemical synthesis of acceptors used in the present study have already been published.[14,15] GlcNAcβ1,4GlcNAcβ-O-Bn was obtained from Toronto Research Chemicals. GlcNAcβ1,4GlcNAcβ1,4GlcNAc, GlcNAcβ1,4GlcNAcβ1,4GlcNAcβ1,4GlcNAc, Cloned FT V (human) and Cloned FT VI (human, EC 2.4.16.5) were purchased from Calbiochem.

Fetuin triantennary asialo glycopeptide and bovine IgG diantennary glycopeptide were available from our earlier studies.[16,17] α1,2-[$^{14}$C]fucosylated glycopeptides arising from fetuin triantennary asialo glycopeptide and bovine IgG glycopeptide by the action of cloned α1,2-FT and, also, α1,3/4 [$^{14}$C]fucosylated glycopeptides by the action of cloned FTIII were available from previous studies.[18,19]

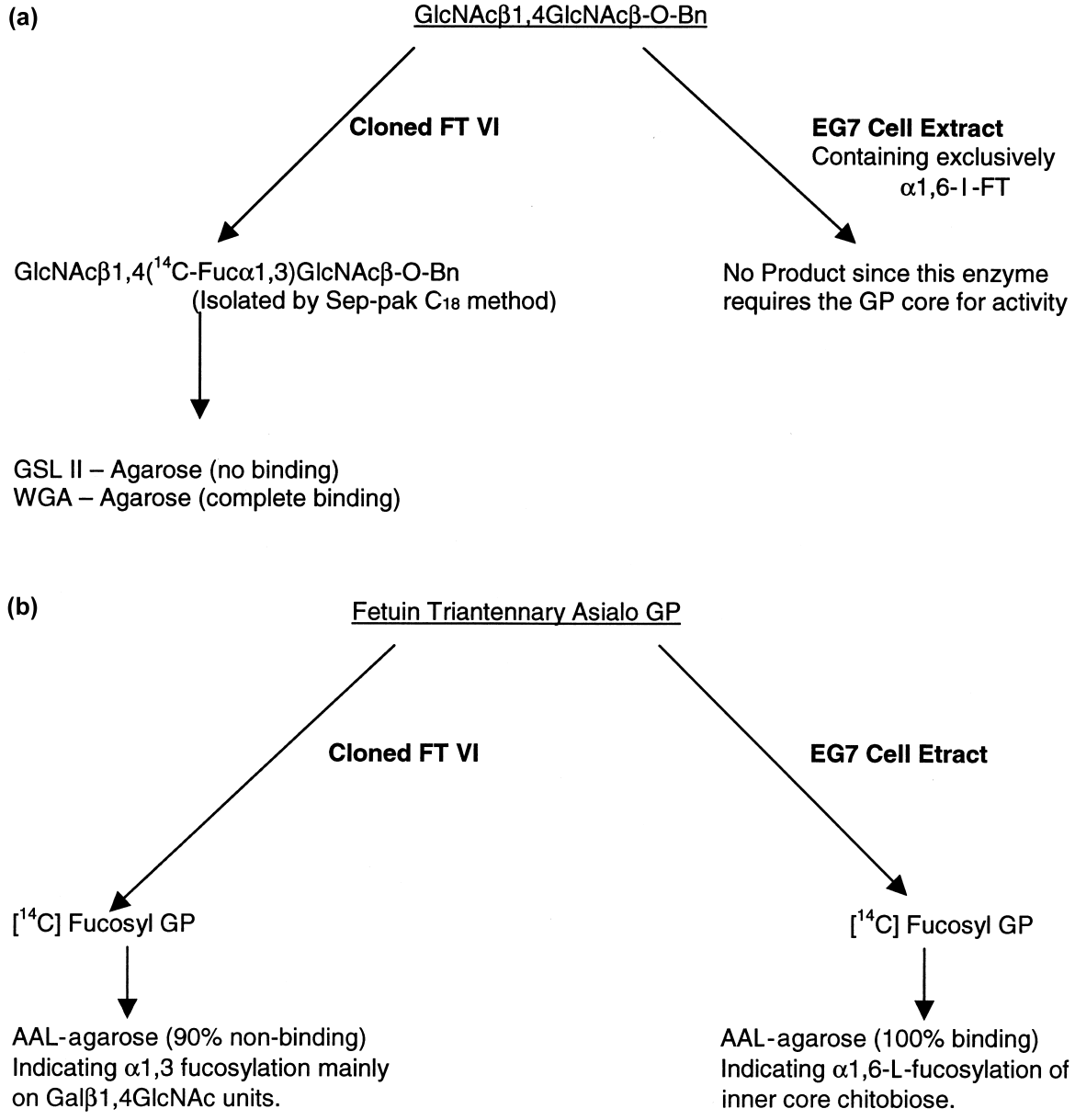
### 2.3. Assay of FTs (EC 2.4.1.65)[19]

The incubation mixtures run in duplicate contained 50 mM Hepes–NaOH, pH 7.5, 5 mM $MnCl_2$, 7 mM ATP, 3 mM $NaN_3$, the acceptor (3.0 mM unless otherwise stated), 0.05 μCi of GDP-[U-$^{14}$C]Fuc (specific activity 290 mCi/mmol) and this enzyme in a total volume of 20 μL; the control incubation mixtures had everything

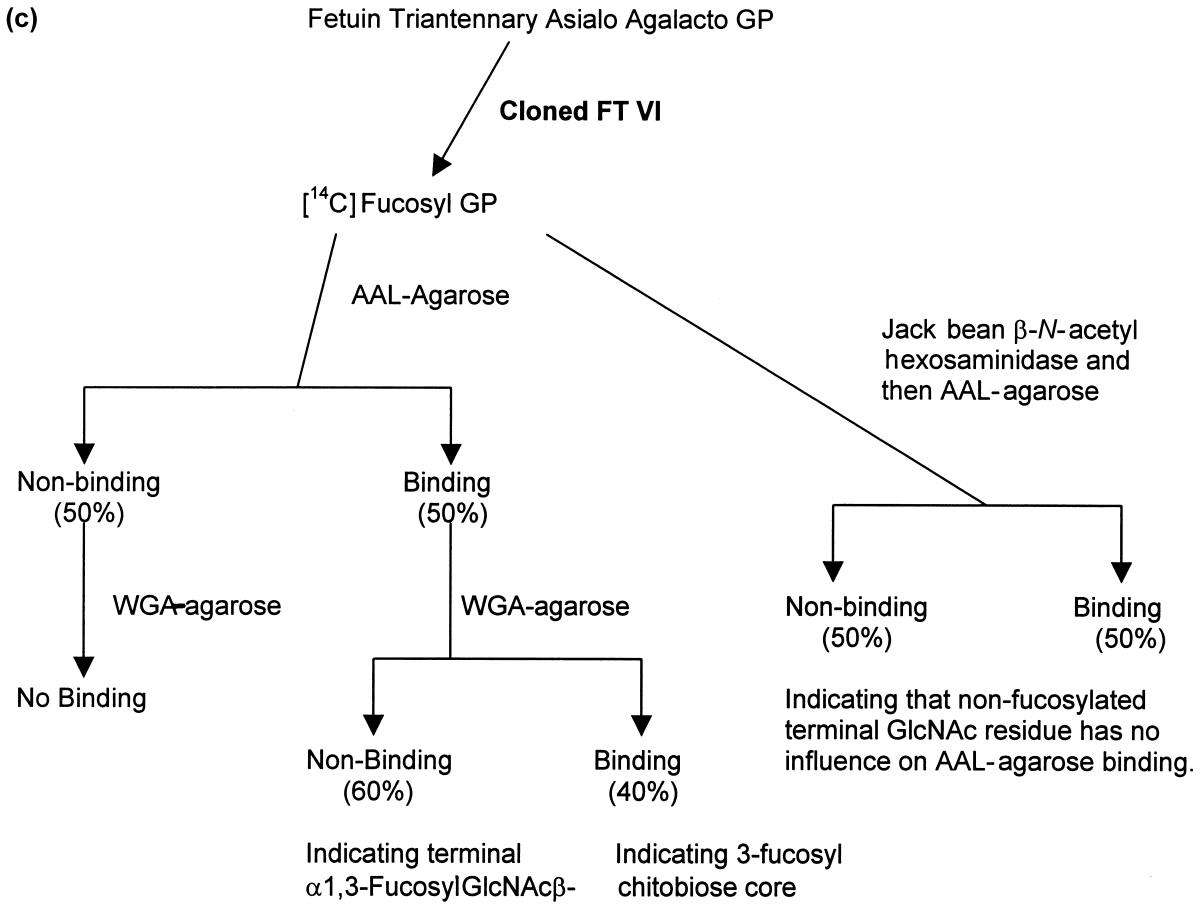
except the acceptor. At the end of incubation for 2 h at 37 °C, the mixture was diluted with 1.0 mL of water and passed through a Dowex-1 [$Cl^-$] column (1 mL in a Pasteur pipet). The column was washed twice with 1 mL of water; the breakthrough and wash that contained the [$^{14}$C]fucosylated neutral acceptor were collected together in a scintillation vial, and the radioactive content was determined using 3a70 scintillation fluid (Research Products International, Mount Prospect, IL) and a Beckman LS9000 instrument. The Dowex column was then eluted with 3.0 mL of 0.2 M NaCl to obtain the [$^{14}$C]fucosylated product from sialylated/sulfated acceptors and then counted for radioactivity as before. Corrections were made by subtracting the radioactivity in the water and NaCl eluates of the control incubation mixtures from the values of the corresponding eluates of the tests. Duplicate sample values did not vary more than 5%.

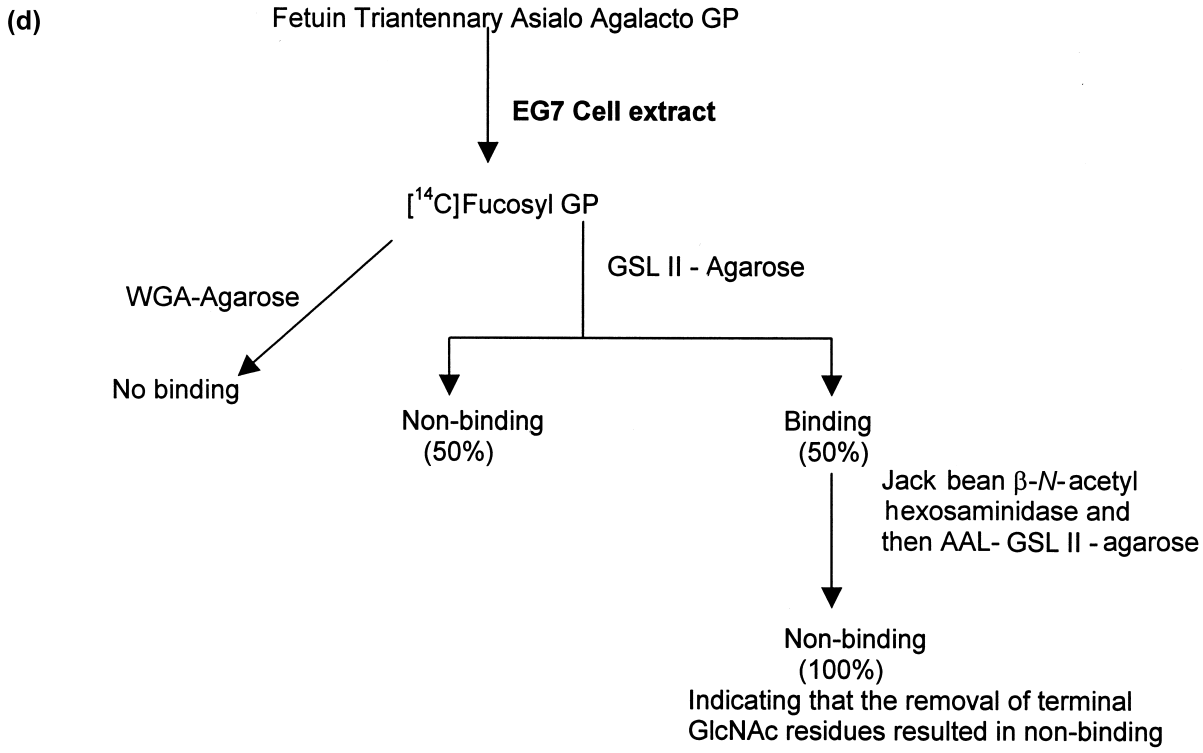
### 2.4. Chemical defucosylation of bovine IgG[20,21]

Bovine IgG (100 mg, I5,506; Sigma) was dissolved in 12 mL of 0.1 M trifluoroacetic acid (TFA) and then heated in a water-bath for 1 h at 100 °C. After cooling to room temperature (rt), the reaction mixture was neutralized with 1.0 N NaOH and then dialyzed exhaustively against water in the cold room for 24 h. The dialyzed solution was lyophilized to dryness (yield, 78.3 mg). The TFA-treated bovine IgG showed fivefold activity as an acceptor for FTs present in the Triton X-100

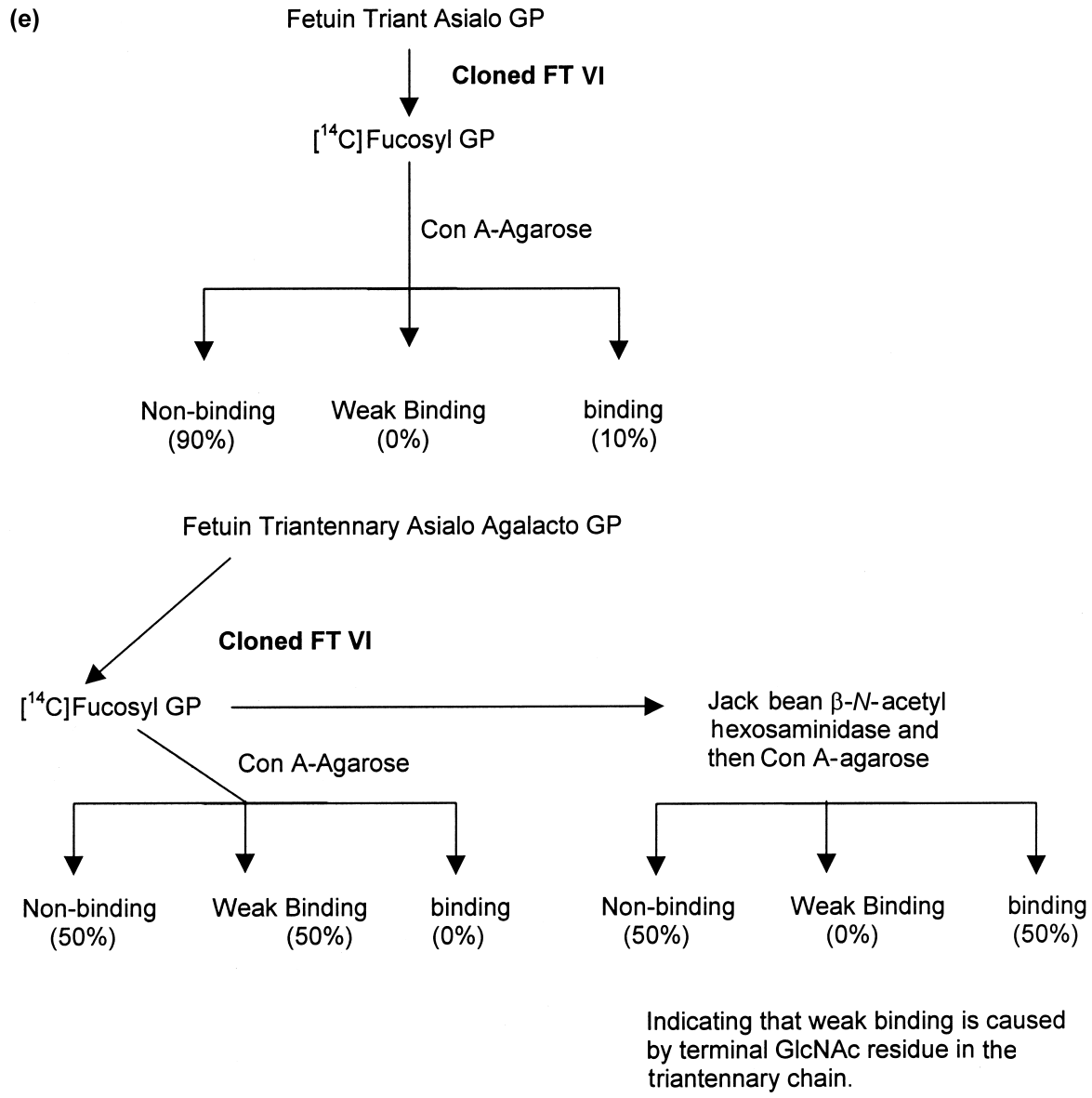



Scheme 1. Lectin affinity chromatography protocol used in characterizing the type of fucosylation.


(c)
Fetuin Triantennary Asialo Agalacto GP
Cloned FT VI
[14C] Fucosyl GP
AAL-Agarose
Jack bean β-N-acetyl hexosaminidase and then AAL-agarose
Non-binding (50%)
Binding (50%)
WGA-agarose
WGA-agarose
Non-binding (50%)
Binding (50%)
No Binding
Indicating that non-fucosylated terminal GlcNAc residue has no influence on AAL-agarose binding.
Non-Binding (60%)
Binding (40%)
Indicating terminal α1,3-FucosylGlcNAcβ-
Indicating 3-fucosyl chitobiose core


(d)
Fetuin Triantennary Asialo Agalacto GP
EG7 Cell extract
[14C] Fucosyl GP
GSL II - Agarose
WGA-Agarose
No binding
Non-binding (50%)
Binding (50%)
Jack bean β-N-acetyl hexosaminidase and then AAL- GSL II - agarose
Non-binding (100%)
Indicating that the removal of terminal GlcNAc residues resulted in non-binding

Scheme 1.




Scheme 1. (*Continued*)

extracts of Colo 205 and LS180, as compared to the acceptor ability of the non-treated bovine IgG on weight basis.

### 2.5. Sequential treatment of the TFA-treated bovine IgG

**2.5.1. Enzymatic [$^{14}$C]fucosylation**. The glycoprotein preparation (4 mg) was incubated with Colo 205 extract (3 mg protein) in a reaction volume of 1.15 mL under the standard incubation conditions for 24 h at 37 °C using Speci-Mix for continuous mixing. The reaction mixture was subjected to chromatography on a Bio-Gel P6 column. The excluded fractions containing the [$^{14}$C]fucosylated glycoprotein were pooled, lyophilized to dryness, dissolved in 1.0 mL water and kept frozen at −20 °C for further experimentation.

**2.5.2. AAL-agarose chromatography**. The AAL-non-, weak- and tight-binding fractions were isolated from [$^{14}$C]fucosyl bovine IgG (TFA-treated), by fractionating on an AAL–agarose column (8.0 mL bed volume); 0.1 mL aliquot from 3-mL fractions was counted for radioactivity. The respective fractions were pooled, dialyzed exhaustively against water in the cold room for 24 h, lyophilized to dryness, and each was dissolved in 600 μL of water and stored frozen at −20 °C.

**2.5.3. Treatment with endoglycosidase F/peptide-*N*-glycosidase F (*Flavobacterium meningosepticum*) (EC 3.2.2.18)**. Each of the three fractions (200 μL) isolated from [$^{14}$C]fucosyl bovine IgG (TFA-treated) by AAL–agarose chromatography and 200 μL of [$^{14}$C]fucosyl bovine IgG (non-treated), were treated separately with 40 U of endo F/PNAGase F (OGS) (40 μL of enzyme

solution plus 60 μL of 5 × buffer) then incubated at 37 °C for 20 h. Each reaction mixture was then diluted with 1.0 mL of water and subjected to chromatography on a Bio-Gel P6 column. Fractions of 1.0 mL were collected and counted for radioactivity.

### 2.6. Sequential treatment of Con A-binding and non-binding fractions of asialo fetuin

**2.6.1. Enzymatic [$^{14}$C]fucosylation**. Each of the Con A–agarose binding and non-binding fractions (4 mg) from asialo fetuin were incubated with LS180 extract (4 mg protein) in a reaction volume of 0.65 mL for 18 h at 37 °C. The reaction mixtures were then subjected to Bio-Gel P6 chromatography, and the [$^{14}$C]fucosyl glycoproteins in the excluded fractions were recovered as described above.

Each of the above [$^{14}$C]fucosyl asialo fetuin preparations (50 μL) were treated with 40 U of endo F/PNGase F and then subjected to Bio-Gel P6 chromatography as described above.

**2.6.2. Chromatography of [$^{14}$C]fucosyl asialo fetuin Con A–agarose-binding fraction on AAL–agarose and then treatment of the separated fractions with endo F/PNA-Gase F**. AAL–agarose binding and non-binding fractions from [$^{14}$C]fucosyl asialo fetuin (Con A-binding) were isolated as described above for bovine IgG. The AAL-non-binding (50 μL) and 100 μL of the AAL binding fractions were treated separately with 40 U of

Table 1
Colon carcinoma cells uniquely express the α1,3-L-fucosylating activity towards chitobiose [a]

| Cell lines | Incorporation of [$^{14}$C]Fuc (CPM × $10^{-3}$) catalyzed by 1 mg of protein of the cell extract | | |
|---|---|---|---|
| | α1,3-L-FT activity Gal2Meβ1,4GlcNAc (3 mM) | α1,4-L-FT activity Gal2Meβ1,3GlcNAc (3 mM) | Fucosylation of chitobiose GlcNAcβ1,4GlcNAcβ-O-Bn (3 mM) |
| *Colon cancer* | | | |
| Colo 205 | 267.7 | 319.0 | 101.3 |
| LS 180 | 257.8 | 210.8 | 180.3 |
| SW 1116 | 223.7 | 89.4 | 11.8 |
| *Ovarian cancer* | | | |
| SW 626 | 88.7 | 24.0 | 2.3 |
| PA-1 | 86.7 | 0.8 | 0.8 |
| *Breast cancer* | | | |
| MDA-MB-231 | 30.5 | 0.5 | 0 |
| MDA-MB-435S | 0 | 0 | 2.5 |
| MDA-435/LCC 6 | 0 | 0 | 0 |
| MDA-435/LCC $6^{MDRI}$ | 0 | 0 | 0 |
| BT 20 | 20.8 | 241.5 | 0 |
| MCF-7 | 48.1 | 1.1 | 0.4 |
| DU4475 | 60.4 | 255.1 | 1.9 |
| *Prostate cancer* | | | |
| LNCaP | 0 | 0 | 0 |
| DU 145 | 26.1 | 0 | 0 |
| PC-3 | 29.9 | 4.9 | 2.0 |
| *Leukemia* | | | |
| HL 60 | 514.6 | 1.0 | 4.7 |
| *Hepatic cancer* | | | |
| HepG2 | 11.8 | 0 | 0 |

[a] Purified preparations of human lung adenocarcinoma α1,3/4-L-FTs[22] and a lymphoma cell line EG7 containing exclusively α1,6-L-FT (Chandrasekaran et al. unpublished results) did not transfer [$^{14}$C]fucose to GlcNAcβ1,4GlcNAcβ-O-Bn.

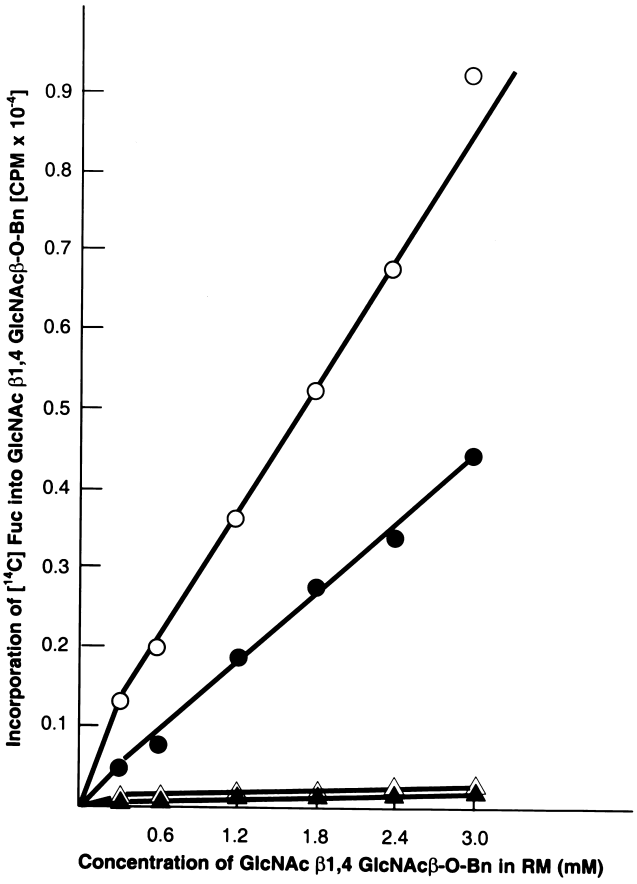



Fig. 1. Inhibition of the enzymatic [$^{14}$C]fucosylation of the acceptor GlcNAcβ1,4GlcNAcβ-O-Bn by sulfated acceptors that measure α1,3- and α1,4-FT activities. (Enzyme source: LS180 cell extract) Acceptor GlcNAcβ1,4GlcNAcβ-O-Bn as such [○-○-○] and in the presence of: 3-O-sulfo Galβ1,4GlcNAcβ1,6(Galβ1,3) GalNAcα-O-Bn [△-△-△], Galβ1,4GlcNAcβ1,6 (3-O-Sulfo Galβ1,3) GalNAcα-O-Bn [▲-▲-▲] and 3-O-sulfo Galβ1,3 GlcNAcβ-O-All [●-●-●].

endo F/PNAGase F and then chromatographed on Bio-Gel P6 columns as described above for bovine IgG.

### 2.7. Isolation of asialo, agalacto fetuin triantennary glycopeptide and therefrom, the dehexosamino glycopeptide

The glycopeptide (18 mg) derived from partial degalactosylation (treatment with β-galactosidase of Aspergillus) of fetuin triantennary asialoglycopeptide was dissolved in 0.5 mL of 1 × buffer (OGS) and mixed with 0.5 mL of 1 × buffer containing 0.5 U of β-galactosidase (bovine testes; OGS) (EC 3.2.1.23) and incubated at 37 °C for 18 h. Then the reaction mixture was subjected to fractionation on a Bio-Gel P2 column to separate the agalacto glycopeptide from the released galactose (yield, 15.2 mg).

About 10 mg of the above preparation was dissolved in 100 μL of water, mixed with 2 U of β-*N*-acetylhexosaminidase (jack bean) (EC 3.2.1.30) in 200 μL of 2 × buffer (OGS) and incubated for 20 h at 37 °C. The resulting dehexosamino glycopeptide was isolated by Bio-Gel P2 fractionation as above (yield, 5.2 mg).

### 2.8. WGA– or GSL II–agarose affinity chromatography

A column of 5-mL bed volume of lectin–agarose (Vector Lab, Burlingame, CA) was employed using 10 mM HEPES, pH 7.5 containing 0.1 mM $Ca^{2+}$, 0.01 mM $Mn^{2+}$, and 0.1% $NaN_3$. The bound material was eluted with 0.5 or 0.2 M GlcNAc in the same buffer as recommended by the manufacturer. The fractionation was done at rt.

### 2.9. Con A–agarose chromatography

A column of 5-mL bed volume of Con A–agarose (Vector Lab, Burlingame, CA) was employed using 50 mM sodium acetate pH 6.5 containing 1 mM each of $CaCl_2$, $MnCl_2$, $MgCl_2$ and 0.1% $NaN_3$. The bound material was eluted with 0.1 M methyl α-D-mannoside in the same buffer. The fractionation was done at rt.

### 2.10. Protocol for affinity chromatography

The protocol used in the characterization of the type of fucosylation is shown in Scheme 1.

## 3. Results and discussion

### 3.1. Novel chitobiose-fucosylating activity of colon carcinoma cells

Several human cancer cell lines were examined for α1,3- and α1,4-L-fucosyltrtansferase activities, as well as for chitobiose-fucosylating activity. The results are presented in Table 1. We found that only colon carcinoma cell lines, especially LS 180 and Colo 205, express chitobiose-fucosylating activity. In this context, it is important to note that the purified preparations of α1,3/4-L-FT, FT A and FT B, from human lung adenocarcinoma[22] were completely inactive towards GlcNAcβ1,4GlcNAcβ-O-Bn. Further, it is interesting to note (see Table 1) that the cancer cell lines, BT 20 and DU 4475, expressing predominantly α1,4-FT activity, and HL 60, PA-1, MCF-7, MDA-MB-231, DU 145 and $HepG_2$ containing almost exclusively α1,3-FT activity, did not show any appreciable activity towards GlcNAcβ1,4GlcNAcβ-O-Bn. The [$^{14}$C]fucosyl product arising from GlcNAcβ1,4GlcNAcβ-O-Bn by the action of LS 180 enzyme extract was isolated by a Sep-Pak C18 fractionation procedure. It moved as a single spot on TLC, as evident from autoradiography,[13] and was

100% resistant to α1,6-L-fucosidase (Bovine epididymis, OGS) (EC 3.2.1.51) (data not shown).

### 3.2. Association of the chitobiose-fucosylating activity with α1,3-L-fucosylating activity

To demonstrate this association the incorporation of [$^{14}$C]fucose into GlcNAcβ1,4GlcNAcβ-O-Bn at varying concentrations was measured both in the presence and absence of 3 mM concentrations of 3-O-sulfo-Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn, Galβ1,4GlcNAcβ1,6(3-O-sulfoGalβ1,3)GalNAcα-O-Bn and 3-O-sulfoGalβ1,3GlcNAcβ-O-All (see Fig. 1), separately, using LS 180 cell extract as the enzyme source. These three sulfated compounds had already been shown to be good acceptors for α1,3/4-L-FT.[19] It was found that LacNAc type II sulfated acceptors completely inhibited the chitobiose-fucosylating activity, whereas, the LacNAc type I acceptor showed ~50% inhibition, only.

### 3.3. Glycopeptides as acceptors for human colon carcinoma cells

A Triton X-100-solubilized extract of Colo 205 cells was used as the enzyme source. Table 2 shows that fetuin triantennary asialo glycopeptide containing three terminal GlcNAc residues is better than the triantennary chain terminating in three Gal residues in serving as acceptor for Colo 205 FTs (activity 213.7 versus 106.0%). The bovine IgG diantennary glycopeptide with an inner core α1,6-L-fucosyl residue became a far better acceptor for Colo 205 FT after the inner core α1,6-fucosyl residue as well as the terminal Gal residues are removed (activity 56.9 versus 170.3%). Even after the complete removal of GlcNAc residues from fetuin triantennary chains, the remaining glycopeptide containing terminal tri-Man core served to some extent as an acceptor for Colo 205 FTs (5.7%). In this context it is important to note that the purified preparations of α1,3/4-L-FT from human lung adenocarcinoma were only 20% active towards Fetuin triantennary asialo agalacto glycopeptide as compared to their activities towards the corresponding asialo glycopeptide.

We carried out separately [$^{14}$C]fucosylation of asialo agalacto fetuin triantennary gp using Colo 205 and B142 (lymphoid) cell extracts as the enzyme sources and isolated the [$^{14}$C]glycopeptides using Bio-Gel P2 chromatography. When these glycopeptides were treated with jack bean β-*N*-acetylhexosaminidase (see Fig. 2, panels A and B), it was found that the glycopeptide resulting from the B142 (lymphoid) enzyme was converted 100% to a smaller [$^{14}$C]glycopeptide, whereas, it was only 60% in the case of the other glycopeptide. The results indicate that [$^{14}$C]glycopeptide from the Colo 205 enzyme contains [$^{14}$C]Fuc residues, both on the outer GlcNAc moieties and on the chitobiose core, whereas, in the other glycopeptide [$^{14}$C]Fuc is present only on the chitobiose core.

Table 2
Human colon carcinoma cell line Colo 205 FT activity towards glycopeptide acceptors

| Glycopeptide (GP) (40 μg) | Colo 205 FT activity: incorporation of [$^{14}$C]Fuc (CPM × $10^{-4}$) into the glycopeptide catalyzed by 1 mg of protein [a,b] |
|---|---|
| Fetuin triantennary sialo GP | 30.4 (31.9) |
| Fetuin triantennary asialo GP | 100.9 (106.0) |
| Fetuin triantennary asialo GP treated with β-galactosidase (*Aspergillus niger*) | 152.7 (160.4) |
| Fetuin triantennary asialo GP treated with β-galactosidases (*A. niger* and bovine testes) | 220.5 (231.7) |
| Fetuin triantennary asialo GP treated with β-galactosidases (*A. niger* and bovine testes) and then β-*N*-acetylhexosaminidase (jack bean) | 5.4 (5.7) |
| Bovine IgG GP | 56.9 (59.8) |
| Bovine IgG GP treated with α-L-fucosidase (bovine epdidymis) | 108.3 (113.8) |
| Bovine IgG GP treated with β-galactosidase (*A. niger*) | 91.5 (96.1) |
| Bovine IgG GP treated with α-L-fucosidase (bovine epdidymis) and then β-galactosidase (*A. niger*) | 162.1 (170.3) |

[a] The values in parentheses are activities expressed as percent of the activity obtained for Galβ1,4GlcNAcβ-O-All (3.0 mM) run simultaneously.

[b] Purified preparations of human lung adenocarcinoma α1,3/4-L-FTs, FTA and FTB[22] were only 20% active towards fetuin triantennary asialo agalacto glycopeptide as compared to fetuin triantennary asialo glycopeptide.

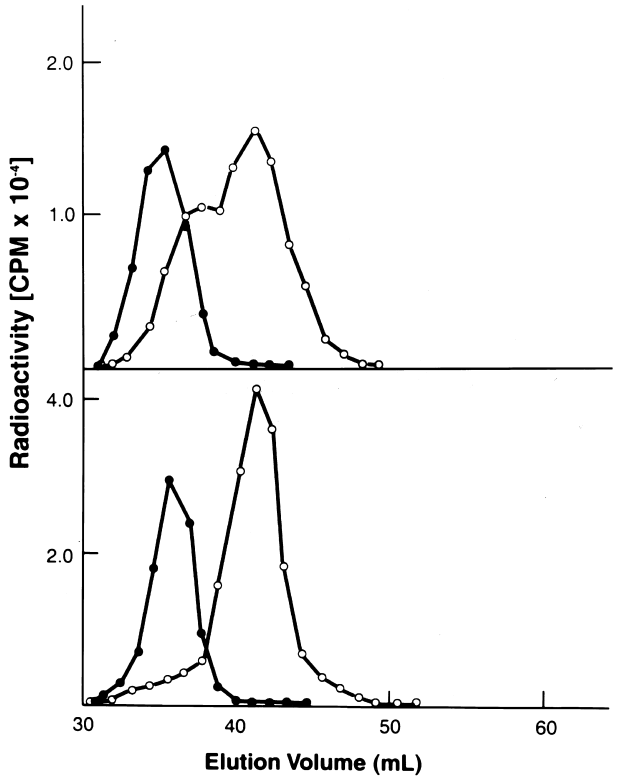



Fig. 2. Bio-Gel P2 column chromatography of [$^{14}$C]fucosylated fetuin triantennary asialo agalacto glycopeptide before (●-●-●) and after (○-○-○) treatment with jack bean β-*N*-acetylhexosaminidase. (A) [$^{14}$C]Fucosylated using Colo 205 cell extract as the enzyme source. (B) [$^{14}$C]Fucosylated using B142 (lymphoid) cell extract as the enzyme source.

### 3.4. Interaction of AAL and LCL with [$^{14}$C]fucosylated fetuin triantennary glycopeptides

α1,2-L-[$^{14}$C]Fucosylated fetuin triantennary asialo GP did not bind to an AAL–agarose column, whereas α1,3/4-L-[$^{14}$C]fucosylated fetuin triantennary sialo GP exhibited some weak binding to this affinity column (see Fig. 3(A)). In contrast to the latter GP the corresponding asialo GP did not exhibit any binding to this affinity column, except for a very small portion (Fig. 3(B)). A considerable portion of α1,3/4-L-[$^{14}$C]fucosylated fetuin triantennary asialo and partially agalacto GP showed weak binding to this affinity column, whereas, the entire portion of the corresponding fully degalactosylated GP showed weak binding to the AAL–agarose column.

A small portion of α1,3/4-L-[$^{14}$C]fucosyl fetuin triantennary asialo agalacto GP and the corresponding dehexosamino GP bound to an LCL–agarose column (Fig. 4(A)). When the LCL-binding and non-binding asialo agalacto glycopeptides were subjected to AAL–agarose chromatography, significant differences between these GPs became evident. The LCL-binding GP contained much larger proportion of AAL tight-binding GP as compared to the LCL non-binding GP. The [$^{14}$C]fucosylated fetuin triantennary asialo agalacto, dehexosamine GP contained, in addition to the AAL non- and weak-binding GPs, a significant amount of the AAL tight-binding GP (Fig. 4(B)).

### 3.5. Interaction of LCL and AAL with [$^{14}$C]fucosylated bovine IgG diantennary GPs (Fig. 5)

All of the GPs showed almost complete binding to the LCL–agarose column (Fig. 5(B)). The α1,2-L-[$^{14}$C]fucosylated GP exhibited nearly complete binding to the AAL–agarose column (Fig. 5(A)), whereas the corresponding α1,3-L-[$^{14}$C]fucosylated GP contained a large proportion of AAL weak-binding GP, and the agalacto GP devoid of the α1,6-fucosyl residue contained only a small amount of AAL tight-binding GP.

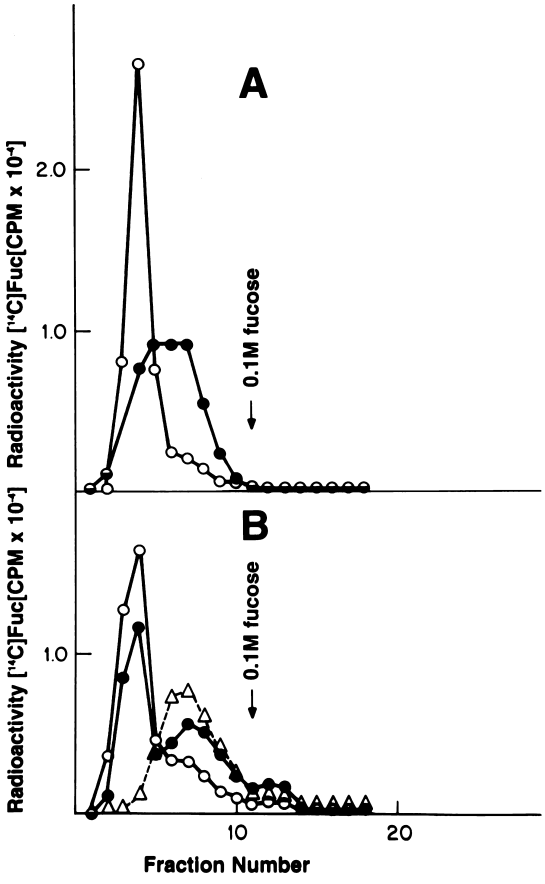



Fig. 3. Interaction of AAL with [$^{14}$C]fucosylated (using cloned α1,2-L-FT or cloned FT III) fetuin triantennary glycopeptides. (A) ○-○-○, α1,2-L-[$^{14}$C]Fucosylated fetuin triantennary asialo GP. ●-●-●, α1,3/4-L-[$^{14}$C]Fucosylated fetuin triantennary sialo GP. (B) ○-○-○, α1,3/4-L-[$^{14}$C]Fucosylated fetuin triantennary asialo GP. ●-●-●, Fetuin triantennary asialo GP, which has been partially degalactosylated by β-galactosidase (A.N.) treatment, and then [$^{14}$C]fucosylated. △--△--△, Fetuin triantennary asialo GP, which has been fully degalactosylated with β-galactosidases (A.N. and B.T.) and then [$^{14}$C]fucosylated.

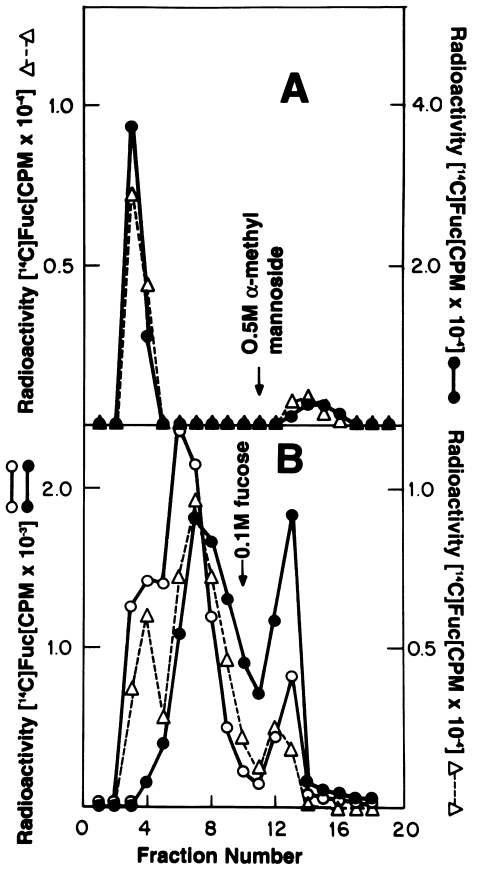



Fig. 4. Fractionation of [$^{14}$C]fucosylated (using cloned FT III) fetuin triantennary GPs on LCL–agarose and then on AAL–agarose columns. (A) Chromatography on LCL–agarose. ●-●-●, [$^{14}$C]Fucosylated fetuin triantennary asialo agalacto GP. △--△--△, [$^{14}$C]Fucosylated fetuin triantennary asialo, agalacto, dehexoasmino GP. (B) Interaction of AAL–agarose with [$^{14}$C]fucosylated fetuin triantennary glycopeptide derivatives. △--△--△, LCL–agarose non-binding fraction from [$^{14}$C]fucosylated fetuin triantennary asialo agalacto GP. ●-●-●, The corresponding LCL–agarose binding fraction. ○-○-○, [$^{14}$C]Fucosylated fetuin triantennary asialo, agalacto, dehexosamino GP.

The results indicate that the α1,3-L-fucosyl residues prevent the tight binding of the diantennary GP containing an α1,6-fucosyl residue to the AAL–agarose column.

### 3.6. Interaction of [$^{14}$C]fucosylated glycoprotein with AAL (Fig. 6)

About 20% of [$^{14}$C]fucosylated Con A-binding asialo fetuin exhibited binding to the AAL–agarose column (Fig. 6(A)). About 50% of [$^{14}$C]fucosylated bovine IgG bound to the column (Fig. 6(B)). On the contrary, in the case of [$^{14}$C]fucosylated TFA-treated bovine IgG, an AAL–agarose weak-binding fraction was identified as a major fraction (Fig. 6(B)).

### 3.7. Characterization of [$^{14}$C]fucosylated glycoproteins by endo F/PNGase F treatment (Fig. 7)

When AAL–agarose non-binding [$^{14}$C]fucosylated asialo fetuin was treated with endo F/PNGase F, ~ 80% of the radioactivity was located in the released carbohydrate chains (Fig. 7(A)). On the other hand, the fraction resistant to endo F/PNGase F was found as a major fraction with AAL–agarose binding [$^{14}$C]fucosylated asialo fetuin (Fig. 7(A)). The [$^{14}$C]fucosylated bovine IgG was mostly resistant to endo F/PNGase F (Fig. 7(B)). The AAL–agarose tight-binding fraction from [$^{14}$C]fucosylated TFA-treated bovine IgG contained both endo F/PNGase F resistant and releasable carbohydrate chains (Fig. 7(B)). The AAL–agarose non- and weak-binding fractions from [$^{14}$C]fucosylated TFA-treated bovine IgG did not contain any endo F/PNGase F resistant carbohydrate chains (Fig. 7(C)). The results thus indicate that either α1,6- or α1,3-L-

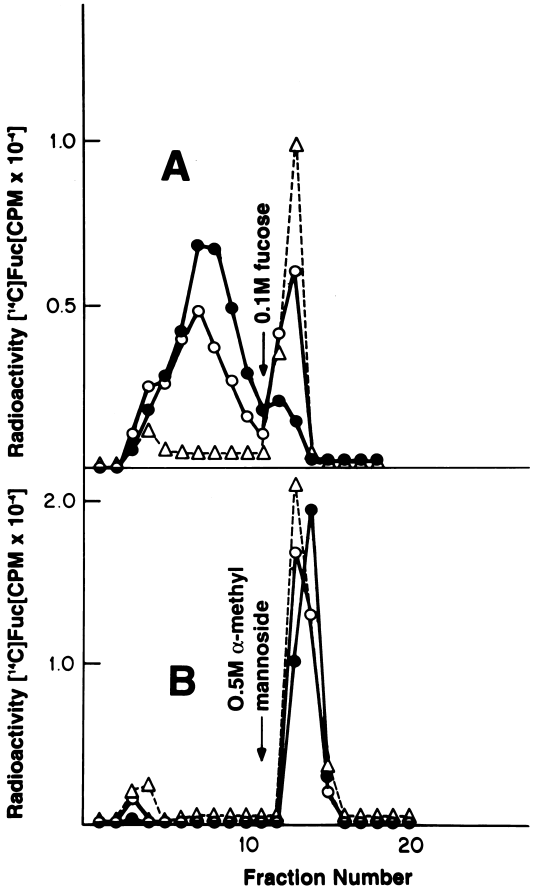



Fig. 5. Fractionation of [$^{14}$C]fucosylated Bovine IgG glycopeptides on LCL– and AAL–agarose columns. (A) Interaction of AAL–agarose with [$^{14}$C]fucosylated IgG GP. ○-○-○, α1,3-L-[$^{14}$C]fucosylated Bovine IgG GP. ●-●-●, Bovine IgG gp treated with α-L-fucosidase (B.E.) and β-galactosidase (A.N.) and then [$^{14}$C]fucosylated. △--△--△, α1,2-L-[$^{14}$C]fucosylated Bovine IgG GP. (b) Affinity chromatography on LCL–agarose. [Symbols and description same as in A].

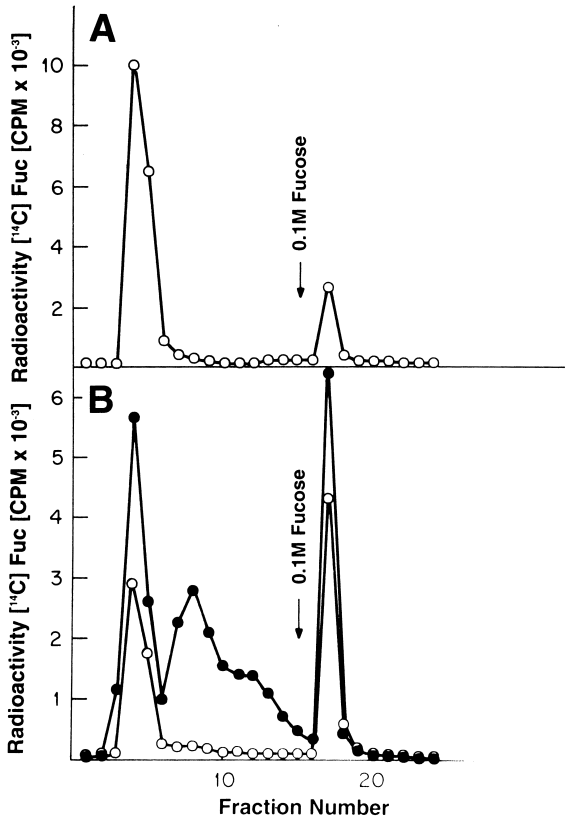



Fig. 6. Affinity chromatography of [$^{14}$C]fucosylated glycoproteins on AAL–agarose column. (A) Asialo fetuin Con A-binding fraction, which has been [$^{14}$C]fucosylated using LS180 extract as the enzyme source. (B) Bovine IgG (TFA treated), which has been [$^{14}$C]fucosylated using Colo 205 extract as the enzyme source [●–●–●]. Bovine IgG, which has been [$^{14}$C]fucosylated using Colo 205 extract as the enzyme source [○–○–○].

fucosylation of the chitobiose core prevents the release of carbohydrate chains by endo F/PNGase F.

### 3.8. Differentiation of cloned FT V and FT VI activities

We examined the acceptor substrate specificities of cloned FT V and FT VI (see Table 3) and found that only FT VI exhibited comparable activities towards LacNAc type I, the chitobiose unit, fetuin triantennary asialo GP and its agalacto derivative.

### 3.9. Usefulness of WGA and GSL II in the differentiation of α1,3- and α1,6-L-fucosylation of chitobiose and GSL II in the identification of terminal GlcNAc residues

The [$^{14}$C]fucosyl product from the acceptor GlcNAcβ1,4GlcNAcβ-O-Bn by the action of cloned FT VI did not bind to GSL II–agarose but showed complete binding to WGA–agarose (Fig. 8). α1,6-L-FT (using EG7 cell extract as the enzyme source) did not act on GlcNAcβ1,4GlcNAcβ-O-Bn.

The AAL–agarose non-binding fraction from [$^{14}$C]fucosyl (using cloned FT VI) fetuin triantennary asialo agalacto glycopeptide (Fig. 9(B)) did not bind to WGA–agarose, indicating the absence of an α1,3[$^{14}$C]fucosyl chitobiose core. The corresponding AAL–agarose-binding fraction (Fig. 9(A)) contained both WGA–agarose-binding and non-binding species, indicating the presence of an α1,3[$^{14}$C]fucosyl chitobiose core and an α1,3-L-[$^{14}$C]fucosyl terminal GlcNAc residue, respectively.

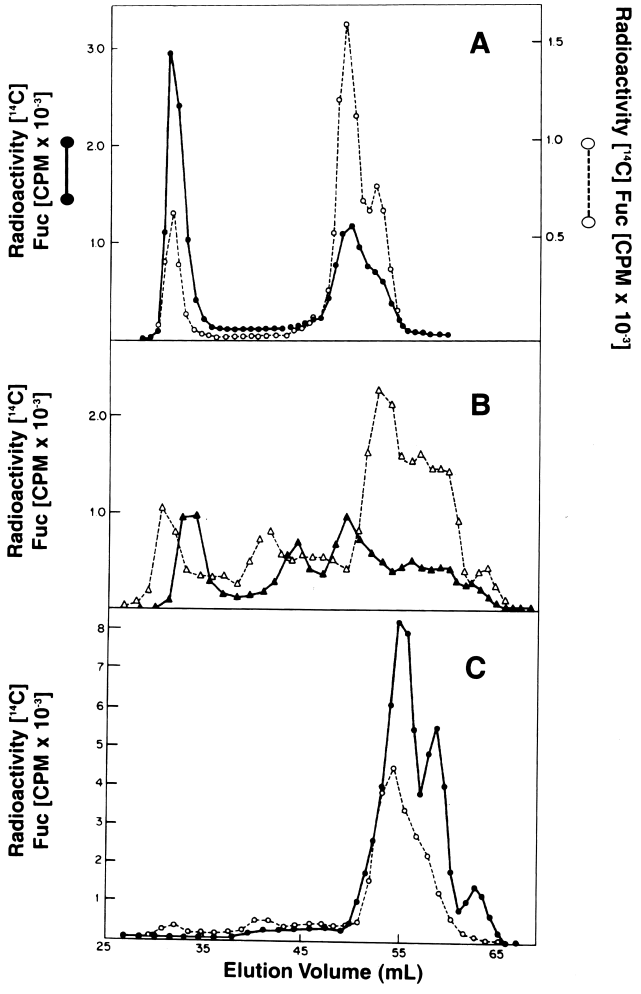



Fig. 7. Fractionation of endo F/PNGaseF treated [$^{14}$C]fucosyl glycoproteins on a Bio-Gel P6 Column. (For details on [$^{14}$C]fucosyl glycoproteins, refer to Fig. 6.) (A) The AAL–agarose binding material isolated from [$^{14}$C]fucosyl asialo fetuin Con A binding fraction [●–●–●]. The corresponding AAL–agarose non-binding material [○–○–○]. (B) The AAL–agarose binding material from [$^{14}$C]fucosyl bovine IgG (TFA treated) [△–△–△]. [$^{14}$C]Fucosylated bovine IgG [▲–▲–▲]. (C) The AAL–agarose non-binding material isolated from [$^{14}$C]fucosyl bovine IgG (TFA treated) [○–○–○]. The AAL–agarose loosely bound material isolated from [$^{14}$C]fucosyl bovine IgG (TFA treated) [●–●–●].

Table 3
Acceptor substrate specificities of the cloned FT V and FT VI activities

| Acceptor (3.0 mM) | FT activity of the cloned enzymes | |
|---|---|---|
| | FT V | FT VI |
| 2-O-MeGalβ1,4GlcNAc | 100.0 | 100.0 |
| 2-O-MeGalβ1,3GlcNAc | 19.4 | 17.6 |
| GalNAcβ1,4GlcNAcβ-O-Bn | 95.0 | 91.9 |
| GlcNAcβ1,4GlcNAcβ-O-Bn | 11.3 | 89.1 |
| GlcNAcβ1,4GlcNAcβ1,4GlcNAc | ND | 63.8 |
| GlcNAcβ1,4GlcNAcβ1,4GlcNAcβ 1,4GlcNAc | ND | 61.5 |
| Fetuin triantennary asialo GP (40 μg) | 36.9 | 73.8 |
| Fetuin triantennary asialo agalacto GP (40 μg) | 5.2 | 42.9 |

ND, not determined.

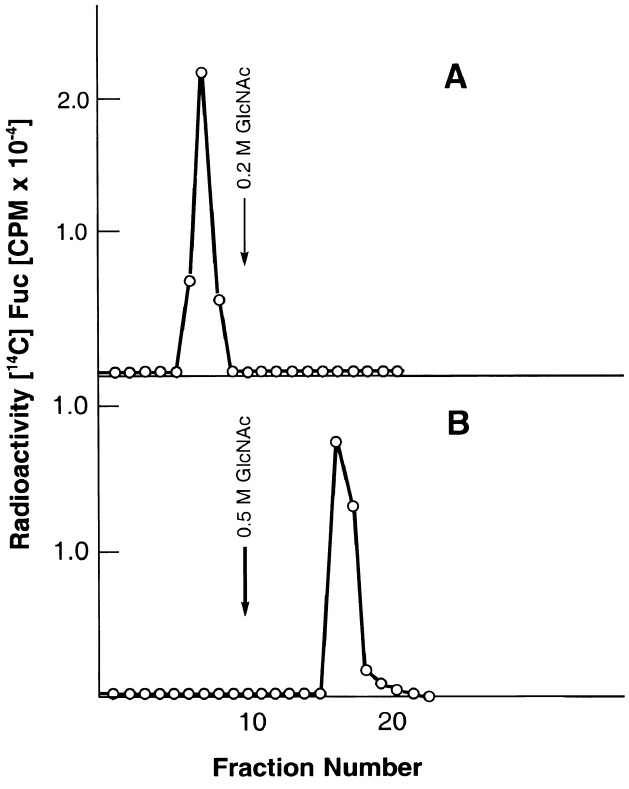



Fig. 8. Affinity chromatography of the [$^{14}$C]fucosylated product arising from GlcNAcβ1,4GlcNAcβ-O-Bn using LS180 cell extract as the enzyme source. (A) GSL II–agarose. (B) WGA–agarose.

On the other hand, [$^{14}$C]fucosyl (using EG7 cell extract) fetuin triantennary asialo agalacto glycopeptide did not bind to a WGA–agarose column (Fig. 9(C)), indicating that α1,6-L-fucosyl chitobiose core does not bind to this lectin. [$^{14}$C]Fucosyl (using FT VI) fetuin triantennary asialo agalacto glycopeptide contained about 50% GSL II–agarose binding species (Fig. 10(A)) that disappeared after treatment with jack bean β-*N*-acetylhexosaminidase (Fig. 10(B)), indicating that the presence of non-fucosylated terminal GlcNAc residues were responsible for its binding to GSL II–agarose.

### 3.10. Usefulness of AAL in the identification of an α1,6-L-fucosyl chitobiose core

The α1,6-L-[$^{14}$C]fucosyl (using EG7 cell extract) fetuin triantennary asialo glycopeptide (Fig. 11(A)) and the corresponding asialo agalacto glycopeptide were bound by an AAL–agarose column (Fig. 11(B)). Almost the entire [$^{14}$C]fucosyl (using FT VI) fetuin triantennary asialo glycopeptide did not bind to AAL–agarose (Fig. 12(A)), indicating that as compared to the inner chitobiose core, the terminal Galβ1,4GlcNAc unit has a

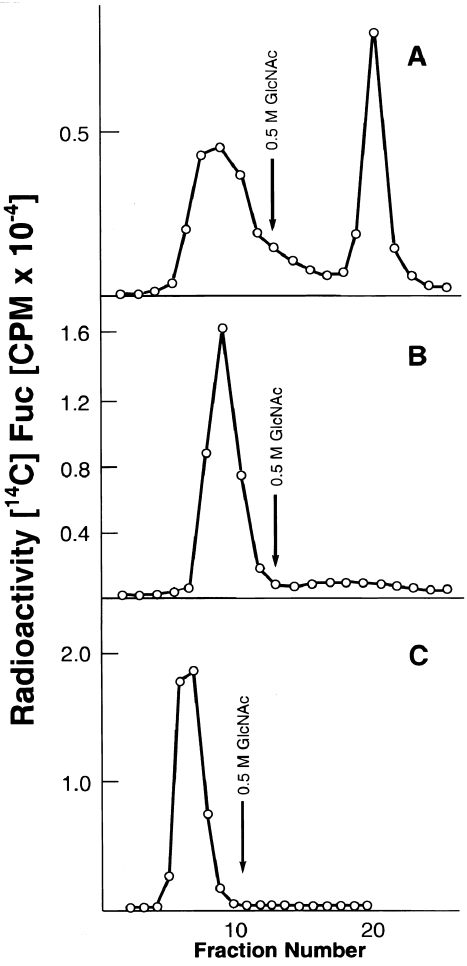



Fig. 9. WGA–agarose affinity chromatography of: (A) the AAL–agarose binding fraction from [$^{14}$C]fucosyl (using cloned FT VI) fetuin triantennary asialo, agalacto glycopeptide; (B) the corresponding AAL–agarose non-binding fraction; (C) [$^{14}$C]fucosyl (using EG7 cell extract) fetuin triantennary asialo agalacto glycopeptide.

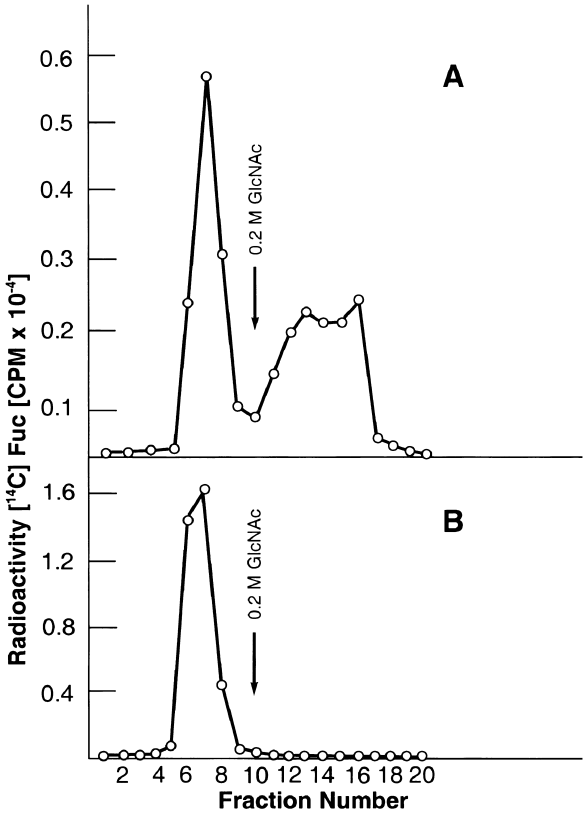



Fig. 10. GSL II–agarose affinity chromatography of: (A) [$^{14}$C]fucosyl (using cloned FT VI) fetuin triantennary asialo agalacto glycopeptide; (B) A after treatment with jack bean β-*N*-acetylhexosaminidase.

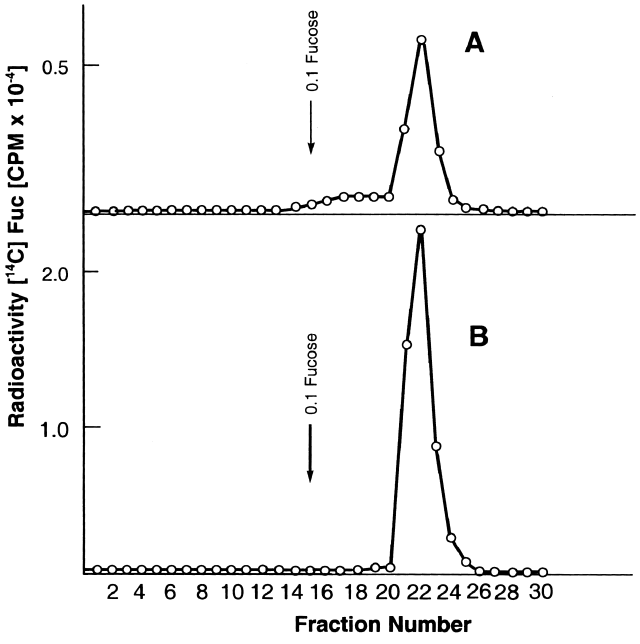



Fig. 11. AAL–agarose affinity chromatography of: (A) [$^{14}$C]fucosyl (using EG7 cell extract) fetuin triantennary asialo glycopeptide; (B) the corresponding asialo agalacto glycopeptide.

much higher affinity for FT VI. About 50% of the [$^{14}$C]fucosyl (using FT VI) fetuin triantennary asialo agalacto glycopeptide (Fig. 12(B)) was bound by the AAL–agarose column. This [$^{14}$C]glycopeptide, even after treatment with jack bean β-*N*-acetylhexosaminidase, showed the same proportion of binding to AAL–agarose (Fig. 12(C)).

The [$^{14}$C]fucosyl (using FT VI) fetuin triantennary asialo glycopeptide contained $>90\%$ Con A–agarose non-binding species (Fig. 13(A)), whereas, [$^{14}$C]fucosyl (using FT VI) fetuin triantennary asialo agalacto glycopeptide contained nearly 50% each of Con A–agarose non- and weak-binding species (Fig. 13(B)). This latter [$^{14}$C]glycopeptide, after treatment with β-*N*-acetylhexosaminidase, gave rise to a Con A–agarose tight-binding species in place of a Con A–agarose weak-binding species (Fig. 13(C)), indicating that the removal of terminal GlcNAc residue by hexosaminidase converted the triantennary chain into a diantennary chain.

Thus, on the basis of all of the above data, the novel FT activity of human colon cancer cells can be attributed to FT VI which can α1,3-L-fucosylate: (a) a Galβ1,4GlcNAcβ unit; (b) chitobiose; (c) chitobiose as the inner core; and (d) multiterminal β-GlcNAc units of complex type chains. A recent study reported the formation of a GlcNAcβ1,4(Fuc α1,3)GlcNAcβ determinant at the non-reducing terminus by recombinant α1,3-FTs, FT V and FT VI.[23]

## 4. Summary

The following findings were made: (i) Sialylation but not α1,2-L-fucosylation of terminal Gal residues in triantennary asialo GP increases the binding affinity of this GP to AAL. (ii) α1,3-L-[$^{14}$C]fucosylated asialo agalacto triantennary GP exhibits a weak-binding affinity towards AAL. (iii) Removal of outer GlcNAc residues from asialo agalacto triantennary GP by β-*N*-acetylhexosaminidase, followed by enzymatic α1,3-L-[$^{14}$C]fucosylation, results in a decrease in the affinity of the above GP towards AAL. (iv) The diantennary backbone, but not the inner-core α1,6-L-fucosyl residue, is an absolute requirement for the LCL binding of a glycopeptide. (v) The α1,3-L-fucosyl residue in the outer core prevents the tight binding of the diantennary GP, containing the inner core α1,6fucosyl residue, to the AAL–agarose. This negative influence on the binding is further augmented after the removal of the terminal Gal as well as the inner core α1,6-L-fucosyl residues from the diantennary GP. (vi) The diantennary GP, which binds tightly to AAL–agarose, is most likely to contain the inner core α1,6-L-fucosyl residue. Further study on the binding properties of AAL with [$^{14}$C]fucosylated glycoproteins shows that: (i) A small

portion ( ~ 15%) of α1,3/4 [$^{14}$C]fucosylated Con A-binding asialo fetuin fraction exhibited tight binding to AAL–agarose. (ii) endo F/PNGase F (*F. meningosepticum*) treatment resulted in the release of ~ 40 and ~ 80% of the [$^{14}$C]fucosyl carbohydrate chains, respectively, from [$^{14}$C]fucosyl AAL-binding and -non-binding species of fetuin, indicating the presence of fucosyl residues in the chitobiose core of AAL-binding species. (iii) α1,3-L-[$^{14}$C]fucosylated bovine IgG contained equal amounts of AAL-binding and -non-binding species. (iv) Removal of core α1,6fucose by TFA increased fivefold the acceptor ability of bovine IgG for α1,3-L-FT. (v) A significant portion of AAL-binding species from [$^{14}$C]fucosyl bovine IgG (TFA treated) was resistant to endo F/PNGase F; no such resistance was seen in the AAL-non- and weak-binding IgG species. (vi) Both weak- and non-binding fractions released more small carbohydrate chains containing [$^{14}$C]Fuc as compared to the AAL-binding IgG species (TFA treated) upon endo F/PNGase treatment. (vii) In contrast to [$^{14}$C]fucosyl TFA treated bovine IgG species, [$^{14}$C]fucosylated bovine IgG released very few of the smaller carbohydrate chains upon endo F/PNGase

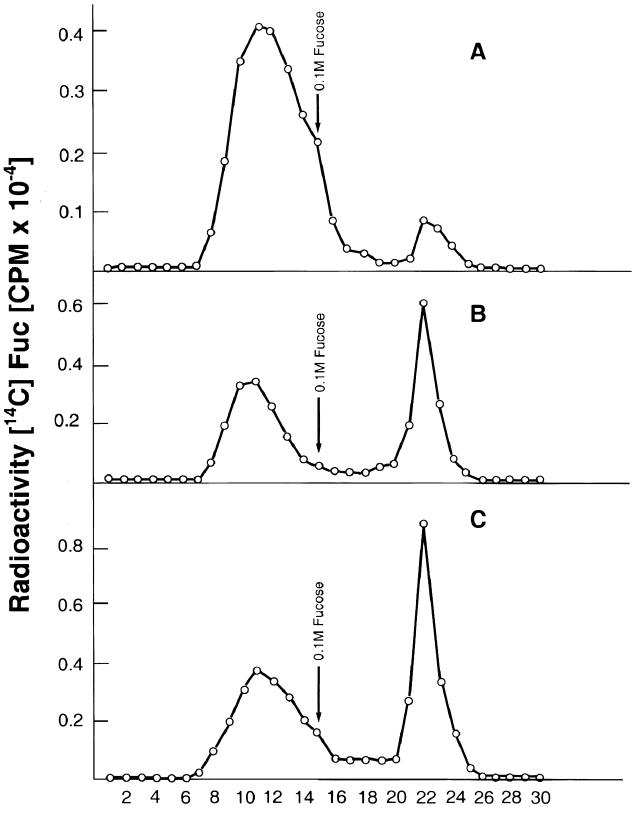



Fig. 12. AAL–agarose affinity chromatography of: (A) [$^{14}$C]fucosyl (using cloned FT VI) fetuin triantennary asialo glycopeptide; (B) the corresponding asialo agalacto glycopeptide; (C) B after treatment with jack bean β-*N*-acetylhexosaminidase.

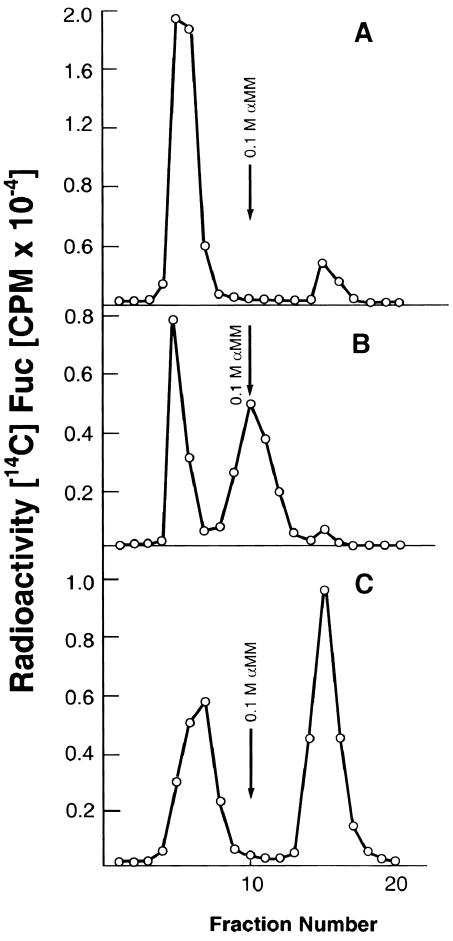



Fig. 13. Con A-agarose affinity chromatography of: (A) [$^{14}$C]fucosyl (using cloned FT VI) fetuin triantennary asialo glycopeptide; (B) the corresponding asialo agalacto glycopeptide; (C) B after treatment with jack bean β-*N*-acetylhexosaminidase.

treatment. These results suggest that FTs of colon cancer cells are capable of catalyzing the inner-core α1,3-L-fucosylation. Additional support for this contention is our finding that the [$^{14}$C]fucosylated product arising from GlcNAcβ1,4GlcNAcβ-O-Bn by the action of LS180 α1,3-FT was found to be completely resistant when treated with α1,6-L-fucosidase from bovine epididymis.

Based on the properties of α1,3-FT of plants and insects, Oriol and co-workers have proposed that a unique ancestor, which could add fucose to the first GlcNAc moiety of chitobiose, might have existed for the FT family.[24] These authors have argued that chitobiose is present in the first polymannose oligosaccharide block added to Asn of the peptide chain, and so, chitobiose must have appeared in evolution before the terminal *N*-acetyllactosamine, which serves as an acceptor for α1,2- and α1,3-L-FTs. Therefore, the latter enzymes might be more recent in evolution than the enzymes working on chitobiose. In parallel to the ob-

servation of Quinto and co-workers that the bacterial nodulation protein, NodZ, is a chitin oligosaccharide α1,6-L-FT, which can also recognize related substrates of animal origin.[25] The present study finds that a colon cancer cell α1,3-L-FT acting on LacNAc type II (Galβ1,4GlcNAcβ-) can recognize chitobiose (GlcNAcβ1,4GlcNAcβ-) as an acceptor substrate and thus has an identity to FT VI. The present study suggests the possibility that FT VI may serve either as a tumor marker or a surrogate enzyme marker for colon cancer. This suggestion is similar to our earlier observation that α1,2-FT may be a surrogate enzyme marker for human prostate cancer.[26]

## Acknowledgements

This study was supported by NIH grant No. CA 35329.

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