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Purification and properties of the α-3/4-L-fucosyltransferase released into the culture medium during the growth of the human A431 epidermoid carcinoma cell line

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A soluble α-3/4-fucosyltransferase secreted into the growth medium of the human A431 epidermoid carcinoma cell line has been purified 700 000 fold by a series of steps involving chromatography on Phenyl Sepharose 4B, CM-Sephadex C-50 and GDP-hexanolamine Sepharose 4B. The untreated spent culture medium transferred almost ten times more fucose to the subterminal *N*-acetylglicosamine residue in the Type 1 (Gal β1-3GlcNAc) disaccharide than to the subterminal sugar in the Type 2 (Gal β1-4GlcNAc) disaccharide; the relative activity with these two substrates remained virtually unchanged throughout the purification procedure. At no stage was any α-3-fucosyltransferase species acting solely on *N*-acetylglucosamine residues in Type 2 chains separated from the bulk of the α-3/4-fucosyltransferase activity. The purified enzyme preparation showed insignificant activity with glycoprotein substrates having N-linked oligosaccharide chains with terminal Type 2 sequences but transferred fucose to a mucin-type glycoprotein with O-linked oligosaccharide chains with terminal Type 1 structures. Lactose was a poor substrate but the activity of the enzyme was influenced by the presence of substituents on the terminal β-galactosyl residue and 2′-fucosyllactose was almost as good an acceptor as the Type 1 disaccharide. The properties of the purified enzyme with regard to specificity, divalent cation requirements, pH optimum, and M_r, closely resembled those of the Lewis-blood-group gene associated α-3/4-fucosyltransferase isolated from human milk.

Keywords: A431 cells; α-3/4-fucosyltransferase; Lewis blood-group-gene encoded enzyme; Le^a determinant; Le^b determinant; X determinant; Y determinant; sialyl-Le^a determinant; sialyl-X determinant

Introduction

The tissue distribution of the Lewis-blood-group associated antigens Le^a (Galβ1-3[Fucα1-4]GlcNAc), Le^b [Fucα1-2Galβ1-3[Fucα1-4]GlcNAc) and sialyl-Le^a (NeuAcα2-3Galβ1-3[Fucα1-4]GlcNAc) differs from that of the closely related X (Galβ1-4[Fucα1-3]GlcNAc), Y (Fucα1-2Galβ1-4[Fucα1-3]GlcNAc) and sialyl-X (NeuAcα2-3Galβ1-4[Fucα1-3]GlcNAc) antigens inasmuch as virtually all tissues that express the Type 1 (Galβ1-3GlcNAc) chain-based Lewis antigens also express the Type 2 (Galβ1-4GlcNAc) chain-based X-related antigens, but not all tissues that express the X antigens also have Lewis antigens [1]. The classification of the Lewis group of antigens as a 'blood-group' system arose from the fact that the Le^a and Le^b antigens were first detected on erythrocytes [2], but the molecules carrying the Le^a and Le^b determinants were subsequently shown to be glycosphingolipids secondarily acquired from the plasma in which the cells circulate [3, 4] and it is still not clear from which tissues these glycolipids originate. Normal haemopoietic tissues lack both Type 1 structures and detectable levels of the α-4-fucosyltransferase necessary for the completion of the Lewis determinants [1], and cellular expression of the Type 1 antigens is confined largely to endodermally derived tissues such as lining epithelia and glandular epithelia [5, 6].

The co-presence of the α-3- and α-4-fucosyltransferase

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activities in the tissues expressing Lewis antigens makes it difficult to assess, in extracts of whole tissues or cells, the extent to which the Lewis-gene-encoded enzyme contributes to the synthesis of X-related structures, that is, the extent to which the enzyme functions as an α-3-fucosyltransferase acting on the subterminal *N*-acetylglucosamine residues in Type 2 sequences as well as an α-4-fucosyltransferase acting on the subterminal *N*-acetylglucosamine residues in Type 1 chains. The human epidermoid carcinoma A431 cell line was earlier shown to express blood group Le^a and Le^b structures on the carbohydrate chains of the epidermal growth factor receptor which occurs on its cell surface [7], and Kukowska-Latallo *et al.* [8] used a cDNA library constructed from mRNA derived from this cell line in order to isolate DNA believed to be encoding the Lewis-gene associated glycosyltransferase. Transfection of the library into COS-1 cells, and screening for expression of X antigen and α-3-fucosyltransferase activity, led to the isolation of a DNA species that engendered the expression of Le^a as well as the strong expression of X structures on the surface of the COS-1 cells. However, the soluble α-3/4-fucosyltransferase purified from human milk donated by blood group Le(a−b+) women has been found to transfer fucose very poorly *in vitro* on to *N*-acetylglucosamine in unsubstituted Type 2 structures [9, 10] and therefore would not be expected to give strong expression of X antigen. In a search for a further potential source of α-4-fucosyltransferases that could be used for specificity studies, a number of human cell lines were examined; of those tested, the A431 cell line was found to be the most potent source [11]. Moreover, the enzyme experiments revealed that α-4-fucosyltransferase activity was detectable not only in the cellular extracts but also in a soluble form in the culture medium from which the A431 cells had been harvested [11].

In this paper the purification of the soluble α-3/4-fucosyltransferase that is secreted into the growth medium of A431 cells is described and the properties of the enzyme are compared with those of the extensively purified *Le* blood-group-gene associated α-3/4-fucosyltransferase isolated from human milk.

Materials and Methods

Substrates

Nucleotide sugars. GDP-L-[^{14}C]fucose (270 mCi mmol^{-1}) was purchased from Amersham UK, and unlabelled GDP-fucose was synthesized by the method of Nunez *et al.* [12].

Low molecular weight acceptors. N-Acetyllactosamine (Galβ1-4GlcNAc) and lacto-*N*-biose 1 (Galβ1-3GlcNAc) were synthesized by the methods of Alais and Veyrières [13] and Flowers [14], respectively. Galβ1-3Glc was synthesized by the method of Kuhn and Baer [15]. 2′-Fucosyllactose (Fucα1-2Galβ1-4Glc), lacto-*N*-tetraose (Galβ1-3GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-neotetraose (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), lacto-*N*-fucopentaose I (Fucα1-2Galβ1-3GlcNAcβ1 - 3Galβ1 - 4Glc), lacto - *N* - fucopentaose II (Galβ1 - 3[Fucα1 - 4]GlcNAcβ1 - 3Galβ1 - 4Glc), lacto - *N* - fucopentaose III (Galβ1 - 4[Fucα1 - 3]GlcNAcβ1 - 3Galβ1-4Glc), lacto-*N*-difucohexaose I (Fucα1-2Galβ1-3[Fucα1-4]GlcNAcβ1 - 3Galβ1 - 4Glc), lacto - *N* - difucohexaose II (Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4[Fucα1-3]Glc) were recovered from human milk as described by Donald and Feeney [16], and the purity and structure of all these oligosaccharides were confirmed by ^{1}H NMR analysis. Lactose was purchased from BDH Biochemicals (UK) and recrystallized before use. Synthetic 2′-fucosyl-*N*-acetyllactosamine (Fucα1-2Galβ1-4GlcNAc) [17] was a gift of Professor P. Sinaÿ (Ecole Normale Superieure, Paris, France) and the Types 1 and 2 disaccharides with hydrophobic spacer arms, Galβ1-3GlcNAc-*O*-$(CH_2)_8$COOMe and Galβ1-4GlcNAc-*O*-$(CH_2)_8$COOMe were kindly supplied by Professor R. U. Lemieux (University of Alberta, Edmonton, Canada). The blood group A active trisaccharide, GalNAcα1-3Galβ1-4GlcNAc, was isolated from an A-active ovarian cyst glycoprotein as described [18], and the A-active tetrasaccharide GalNAcα1-3[Fucα1-2]Galβ1-4Glc was purchased from BioCarb AB, Sweden. The sialic acid containing oligosaccharides, 3′-sialyllactose (NeuAcα2-3Galβ1-4Glc), 6′-sialyllactose (NeuAcα2-6Galβ1-4Glc), 3′-sialyl-*N*-acetyllactosamine (NeuAcα2-3Galβ1-4GlcNAc) and 6′-sialyl-*N*-acetyllactosamine (NeuAcα2 - 6Galβ1 - 4GlcNAc), isolated from human urine by the method of Parkkinen and Finne [19], were kindly provided by Dr Cecilia C. P. Soh (MRC Clinical Research Centre). Sialyllacto-*N*-tetraose (NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc) was a gift from Dr D. Smith (University of Virginia, USA).

Macromolecular acceptors. The glycoproteins fetuin, human transferrin and α_1-acid glycoprotein were purchased from Sigma Chemical Company Ltd, UK. A blood group precursor glycoprotein (No. 484) was isolated as described [20] from a patient who was a nonsecretor of ABH or Lewis-blood-group substances [21]. The glycoprotein therefore lacked the terminal sugars normally transferred by the glycosyltransferases encoded by the *A*, *B*, *H* or *Le* genes and the only possible substituents on the terminal oligosaccharide sequences would be α-3′ or α-6′ linked sialic acid on the Type 1 chains and α-3′ or α-6′ linked sialic acid or α-3 linked fucose on the Type 2 chains. Asialoglycoproteins were prepared from both the serum and ovarian cyst glycoproteins by treatment of a 1% solution with 0.05 M H_2SO_4 for 1 h at 80 °C. The products were thoroughly dialysed against distilled water and freeze-dried. Type XIV pneumococcal polysaccharide was a gift from the late Dr John Humphrey (National Institue for Medical Research, UK).

Enzyme inhibitors

N-Ethylmaleimide and *p*-hydroxymercuribenzoate were purchased from Sigma Chemical Company Ltd, UK.

Paper and thin layer chromatography

Chromatography papers No. 40, DE 81 and 3MM were purchased from Whatman Ltd, UK and HPTLC silica gel 60 plates were supplied by BDH Chemicals Ltd, UK. Unlabelled reference sugars were located on paper chromatograms with alkaline silver nitrate reagent [22] and on TLC plates with 0.5% (w/v) orcinol in ethanol containing 0.5% H_2SO_4 (v/v).

The following solvent systems were used: solvent **1**, ethyl acetate:pyridine:water (10:4:3 by vol); solvent **2**, ethyl acetate:pyridine:water (2:1:2 by vol); solvent **3**, ethyl acetate:pyridine:water (12:5:4 by vol); solvent **4**, propan-1-ol:ethyl acetate:pyridine:water (5:1:1:3 by vol); solvent **5**, phenol:propan-2-ol:formic acid:water (185:5:10:100 by vol); solvent **6**, ethanol:butanol:pyridine:water:acetic acid (500:50:50:150:15 by vol); solvent **7**, butanol:ethanol:water (5:3:2 by vol).

Column chromatography media

Phenyl Sepharose CL-4B, CM-Sephadex C-50, Sephadex G-150, Sephacryl S-200 and CNBr-activated Sepharose 4B were obtained from Pharmacia Fine Chemicals Ltd, UK. Concanavalin A, Blue Dextran and molecular weight markers for gel filtration were purchased from Sigma Chemical Company Ltd, UK. GDP-hexanolamine was synthesized by the method of Beyer *et al.* [23]. Concanavalin A and GDP-hexanolamine were coupled to CNBr activated Sepharose 4B by the procedure of Cuatrecasas [24].

A431 cells and spent growth medium

Human epidermoid carcinoma A431 cells, kindly provided by Dr M. J. Crumpton (ICRF Laboratories, London) were grown at 37 °C in Falcon tissue culture flasks in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% heat-inactivated foetal calf serum, 2 mM glutamine, 100 units/l penicillin and 100 $\mu g\,l^{-1}$ streptomycin in a 5% CO_2 atmosphere. The cells were grown to confluence, the medium removed, the adherent cells washed with phosphate buffered saline (PBS: 8 g NaCl, 0.34 gl^{-1} KH_2PO_4, 1.21 gl^{-1} K_2HPO_4; pH 7.3) and detached from the plastic container by treatment for 5 min at 37 °C with trypsin (0.05%):EDTA (0.02%). The detached cells, suspended in a small volume of DMEM, were spun at 2000 × g, washed with PBS, resuspended in PBS and counted in a haemocytometer. For enzyme assays the requisite number of cells were suspended in 0.15M saline containing 0.25% Triton X-100 and left at 4 °C for 1 h before addition to the reaction mixtures.

Spent culture medium from large scale growths of A431 cells in DMEM was kindly provided by Dr M. Waterfield (ICRF Laboratories, London). The cell-free medium was concentrated approximately 20-fold to a final volume of 4200 ml on an Amicon LP-1A hollow fibre ultrafiltration apparatus fitted with an H1P5-20 filter. The concentrate was stored at −70 °C until used.

Protein estimations

Protein was estimated by the method of Read and Northcote [25] with bovine serum albumin as standard.

α-2-Fucosyltransferase assays

α-2-Fucosyltransferase activity was assayed by a modification of the method of Chester *et al.* [26]. The incubation mixtures contained in a total volume of 60 µl; 5 µmol phenyl β-D-galactoside; 0.2 nmol (70000 counts min^{-1}) GDP-[^{14}C]fucose; 1 µmol $MnCl_2$; 0.5 µmol neutral ATP; 50 µg NaN_3; 5 µmol sodium cacodylate:HCl buffer, pH 7.3; 5 µl 1% Triton X-100 and 20 µl containing 0.8 × 10^6 A431 cells solubilized in Triton X-100 or 20 µl spent culture medium. The reaction mixtures containing the A431 cells were incubated for 30 min, and those containing the culture medium for 16 h, at 37 °C and the products were then separated by descending paper chromatography for 4 h on Whatman No 40 paper in solvent **1**. After development the papers were scanned for radioactivity in a Packard radiochromatogram scanner and the radioactive areas were cut out and counted in a Nuclear Chicago scintillation counter.

α-3- and α-4-Fucosyltransferase assays

Low-molecular-weight acceptors. Standard reaction mixtures for α-3- and α-4-fucosyltransferase activity contained in a total volume of 70 µl: 0.5 µmol acceptor sugar; 0.2 nmol GDP-[^{14}C]fucose (70000 counts min^{-1}); 1 µmol $MnCl_2$; 5 µmol sodium cacodylate:HCl buffer, pH 7.3; 5 µg NaN_3; 20 µl of enzyme source (culture medium, purified enzyme or 0.8 × 10^{-6} solubilized A431 cells) and either 0.5 µmol neutral ATP (crude enzyme preparation) or 250 µg bovine serum albumin (purified enzyme preparation). An additional 5 µl Triton X-100 was added to the mixtures containing the solubilized A431 cells. *N*-Acetyllactosamine and 2′-fucosyllactose were the substrates used for routine assays of α-3-fucosyltransferase activity, and lacto-*N*-biose 1 the substrate for routine assays of α-4-fucosyltransferase activity. The reaction mixtures were incubated at 37 °C for between 30 min and 16 h, depending on the activity of the transferases, and then the products were separated by descending paper chromatography in solvent **4** for 4 h. The mobilities of the products were measured relative to lactose (R_{lac}).

The same procedure was used for the other neutral oligosaccharide acceptors but, after the first chromatographic separation, the products were eluted and re-run in a different solvent. In order to separate more than one compound formed with the larger oligosaccharides the radioactive product area was eluted and run in solvent **3** for from three

to 10 days. Mobilities of the products were measured relative to lacto-*N*-tetraose (R_{LNT}) or lacto-*N*-fucopentaose II ($R_{LNFP\,II}$). The products formed with the Type 1 and 2 disaccharides with hydrophobic spacer arms were re-run in solvent **2** for 6 h; both had a mobility of 1.3 relative to fucose.

The products formed with the sialylated oligosaccharides were first subjected to paper electrophoresis on Whatman 3MM paper in 40 mM pyridine acetate buffer, pH 5.4, on a Southern-Shandon electrophoresis apparatus at 4 kV and 80 mA for 2 h. The mobilities of the labelled products were measured relative to a picrate marker ($R_{picrate}$). The radioactive fucosylated products synthesized with 3′-sialyllactose ($R_{picrate}$ 0.55), 3′-sialyl-*N*-acetyllactosamine ($R_{picrate}$ 0.57) or sialyllacto-*N*-tetraose ($R_{picrate}$ 0.40) were well separated from [^{14}C]fucose, which remained at the origin, and GDP-[^{14}C]fucose ($R_{picrate}$ 1.2). After scanning the papers, the radioactive product areas were cut out, eluted and re-run in solvent **2** with standard markers.

Large scale biosynthesis of fucosylated products. The products synthesized with lacto-*N*-biose 1 and 2′-fucosyllactose were made on a larger scale for identification by methylation or ^{1}H-NMR analysis. GDP-Fucose (6 mg), containing 1 200 000 counts min^{-1} GDP-[^{14}C]fucose, was incubated at 37 °C for 48 h with 5 mg acceptor sugar, 50 μmol sodium cacodylate: HCl buffer, pH 7.3, 5 μmol $MnCl_2$, 1 mg bovine serum albumin and purified A431 α-3/4-fucosyltransferase preparation in a total volume of 250 μl. The incubation mixtures were spotted on to Whatman No 40 paper and run in solvent **1** for 40 min. Radioactive fucosylated product was detected with a radiochromatogram scanner, eluted from the paper, dried under reduced pressure at 30 °C, made up to 1 ml with water and loaded on to a Fractogel TSK 40(S) (190 cm × 1 cm) column. The column was eluted with water (15 ml h^{-1}), the radioactive fractions pooled, concentrated, and the purity of the fucosylated derivative was assessed by TLC in solvent **7**. Yield of fucosylated product with lacto-*N*-biose 1, 1.5 mg; yield of fucosylated product with 2′-fucosyllactose, 3 mg.

Glycoprotein acceptors. The reaction mixtures were the same as for the low-molecular-weight acceptors except that 100 μg glycoprotein was used as substrate. The reaction products were separated by chromatography in solvent **4** on Whatman No 40 paper for 20 h. The papers were scanned and counted as above. Radiolabelled proteins remained at the origin of the chromatogram and were separated from the unused GDP-fucose, fucose and other breakdown products. Assay mixtures without acceptor substrate were included to correct for endogenous incorporation of [^{14}C]fucose, but no endogenous incorporation was observed with the purified A431 α-3/4-fucosyltransferase.

Methylation analysis

Methylation with methyl iodide in dimethylformamide with $BaO/Ba(OH)_2$ as catalyst was carried out as described [27]. After hydrolysis with triethylacetic acid for 2 h at 100 °C the monosaccharide methyl esters were separated into neutral and basic fractions by passage through ion exchange columns, and the amino sugar methyl ether fraction was examined on an amino acid analyser [27]. The neutral sugar methyl ethers were reduced with $NaBH_4$ and examined by GLC.

^{1}H-NMR analysis

Samples for ^{1}H-NMR spectroscopy were repeatedly evaporated from solution in ^2H_2O to replace all exchangeable hydrogens. The 500 MHz ^{1}H spectroscopy was carried out by Dr J. Feeney (National Institute for Medical Research, London) on a Bruker AM500 spectrometer with a probe temperature of 295 K as described previously [16]. Chemical shifts were measured from an acetone internal standard and then expressed as ppm from sodium 4,4-dimethyl-4-silapentane-1-sulphonate (taken as 2.225 ppm from the acetone signal).

Fucosidase treatment

α-2- or α-3/4-fucosidases from *Trichomonas foetus* were separated as described [28] and tested for specificity with lacto-*N*-fucopentaose I and lacto-*N*-fucopentaose II. The enzyme preparations (5 μl) were incubated at 37 °C for 1 h with 5 μl of a 1% solution of the oligosaccharides or 5 μl of an aqueous solution of the products of [^{14}C]fucosyl transfer (approximately 10 000 counts min^{-1}). The products were run on Whatman No 40 paper in solvent **4**, and the released fucose was either visualized with alkaline silver nitrate [22] or, for the radioactive products, detected on a radioachromatogram scanner and counted as described above.

Binding of α-3/4-fucosyltransferase to concanavalin A

The purified A431 fucosyltransferase preparation (0.8 ml) in 25 mM sodium cacodylate:HCl buffer, pH 7.3, containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5% glycerol and 0.1% bovine serum albumin was loaded on to a column of concanavalin A-Sepharose 4B (substitution 4 mg ml^{-1}). The column was washed with 0.8 ml of the same buffer and then the bound glycoprotein was released by inclusion in the eluting buffer of 0.25 M α-benzyl *N*-acetylglucosaminide. The capacity of the 0.4 ml column for transferrin was 3 mg ml^{-1}, and no binding was observed with bovine serum albumin.

Results

Fucosyltransferase activity in A431 cells and in the culture medium in which they were grown

A431 cells solubilized in Triton X-100, and the unconcentrated medium in which the cells had been grown, were

Table 1. Fucosyltransferase activities in A431 cells and in the growth medium from the cells.

Acceptor	[^{14}C]*Fucose transferred (disintegrations min*$^{-1}$)	
	A431 cells[a]	*Culture medium*[b]
Galβ1-3GlcNAc	6027	4662
Galβ1-4GlcNAc	2195	545
Phenyl β-Gal	1318	179
Ratio α-3:α-4 activity	1:3	1:9
Ratio α-2:α-4 activity	1:5	1:26

[a] Triton-solubilized cell suspension.
[b] Unconcentrated culture medium after removal of cells. Fucosyltransferases were assayed with standard reaction mixtures and 20 μl solubilized A431 cells or 20 μl culture medium as the enzyme source. The mixtures containing A431 cells were incubated for 30 min, and those containing the culture medium 16 h, at 37 °C.

tested for α-2-, α-3- and α-4-fucosyltransferase activities with, respectively, phenyl β-D-galactoside, *N*-acetyllactosamine and lacto-*N*-biose 1 as substrates (Table 1). No fucosyltransferase activity was detectable with any of these substrates when they were incubated under the same conditions with fresh DMEM culture fluid. Both the cells and the spent culture medium had some activity with all three substrates but showed strongest activity with the Type 1 acceptor, lacto-*N*-biose 1. However, the ratios of the three activities were different in the culture medium from those in the solubilized cell extract. The medium contained relatively more α-4-fucosyltransferase activity than the cell extract and the ratio of 9:1 found for α-4-activity:α-3-activity closely resembled the ratio of these two activities found for the extensively purified α-3/4-fucosyltransferase isolated from human milk [9, 10]. Steps were therefore taken to obtain sufficient quantities of spent A431 growth medium to permit the isolation of the α-3/4-fucosyltransferase by a series of procedures similar to those used for the isolation of the milk α-3/4-fucosyltransferase.

Purification of the α-3/4-fucosyltransferase from the culture medium of A431 cells

All purification steps were carried out at 4 °C.

Step 1. Ammonium sulfate fractionation and hydrophobic chromatography on Phenyl Sepharose 4B. Preliminary experiments showed that α-4-fucosyltransferase activity could be precipitated from the concentrated spent growth medium at 25–55% ammonium sulfate saturation. The bulk concentrate (4200 ml) was therefore precipitated with ammonium sulfate between these limits and the precipitate was removed by centrifugation at 9000 × g for 30 min in a Sorvall RC-2B centrifuge. The precipitate was redissolved in a minimum volume of 25 mM sodium cacodylate:HCl buffer, pH 7.3, containing 0.1 M NaCl and 5% (w/v) glycerol (buffer **A**) and loaded on to a 10 cm × 55 cm column containing 500 ml of Phenyl Sepharose CL-4B that had been equilibrated in buffer **A**. In the purification of the milk α-3/4-fucosyltransferase the enzyme had bound to this hydrophobic adsorbent, and its use had proved to be an efficient means of concentrating the enzyme from a large volume of starting material. In addition, some α-3-fucosyltransferase activity, catalysing the addition of fucose only to *N*-acetylglucosamine residues in Type 2 chains, was removed by elution of the Phenyl Sepharose with buffer at pH 9.1; the α-3/4-fucosyltransferase activity remained adsorbed during this treatment but was subsequently removed by elution with 50 mM sodium cacodylate:HCl buffer, pH 6.5, containing 5% glycerol (buffer **E**) to which Triton X-100 has been added [9]. However, for reasons that are not clear only a very small proportion (5%) of the total α-3/4 activity in the soluble A431 preparation was retained by the hydrophobic gel, and the unbound fraction was therefore the one used for further purification. Although very little overall purification was achieved by the Phenyl Sepharose step, an increase in the total recovered activity suggests the possible removal of an inhibitor of the α-3/4-fucosyltransferase (Table 2).

The unbound fraction was concentrated to 2050 ml on an Amicon LP-1A ultrafiltration apparatus and the pH was adjusted to 6.5 with buffer **E**.

Step 2. Cation exchange chromatography on CM-Sephadex C-50. CM-Sephadex C-50 gel was equilibrated at pH 6.5 with buffer **E** in a 25 cm × 5 cm column and the concentrated enzyme solution that failed to bind to the Phenyl Sepharose CL-4B adsorbent was loaded on to the gel. The gel was washed with 1250 ml of buffer **E**, transferred to a 2.5 cm × 50 cm glass column, washed with a further 750 ml of buffer **E** and then eluted with 600 ml of a linear gradient (0–0.25 M) of NaCl. Fucosyltransferase activity was eluted when the NaCl concentration reached approximately 0.16 M (Fig. 1). The active fractions were pooled to give CM-Sephadex C-50 eluate. The ratios of α-3- to α-4-fucosyltransferase activity when assayed with the Type 1 and Type 2 disaccharide acceptors were virtually unchanged from the starting material; the α-3-fucosyltransferase activity measured with 2′-fucosyllactose was similar to the level of α-4-activity measured with the Type 1 disaccharide (Fig. 1).

Step 3. Affinity chromatography of CM-Sephadex-C 50 eluate on GDP-hexanolamine Sepharose 4B. The CM-Sephadex C-50 eluate was pumped at a rate of 15 ml h^{-1} on to a 0.75 cm × 20 cm column of GDP-hexanolamine Sepharose 4B. The gel was then washed with 50 ml 25 mM sodium cacodylate:HCl buffer, pH 7.3, containing 25% glycerol (buffer **B**) to which 1% Triton X-100 was added, followed by 210 ml of buffer **B**. α-3/4-Fucosyltransferase activity was then eluted slowly under gravity with buffer **B** to which had

Table 2. Summary of purification of α-3/4-fucosyltransferase from culture medium of A431 cells.

Purification step	*Volume (ml)*	*Total protein (mg)*	*Acceptor substrate*	*Total activity (μmol min^{-1})*	*Specific activity (μmol min^{-1} mg^{-1})*	*Purification (fold)*	*Recovery (%)*
1. Culture medium	4200	452000	Galβ1-3GlcNAc	0.27	0.6×10^{-6}	–	100
			Fucα1-2Galβ1-4Glc	0.27	0.6×10^{-6}	–	100
2. Phenyl Sepharose CL 4B: Unbound fraction	2050	219000	Galβ1-3GlcNAc	0.37	1.7×10^{-6}	2.8	136
			Fucα1-2Galβ1-4Glc	0.42	1.9×10^{-6}	3.2	156
3. CM-Sephadex C-50	240	3672	Galβ1-3GlcNAc	0.28	7.6×10^{-5}	126	100
			Fucα1-2Galβ1-4Glc	0.26	7.1×10^{-5}	120	94
4. GDP-Hexanolamine-Sepharose 4B:							
(i) Total active fraction	30	0.86	Galβ1-3GlcNAc	0.19	2.2×10^{-1}	372000	72
			Fucα1-2Galβ1-4Glc	0.20	2.3×10^{-1}	386000	74
(ii) Peak fraction	5	0.13	Galβ1-3GlcNAc	0.06	4.6×10^{-1}	730000	20
			Fucα1-2Galβ1-4Glc	0.06	4.8×10^{-1}	760000	24

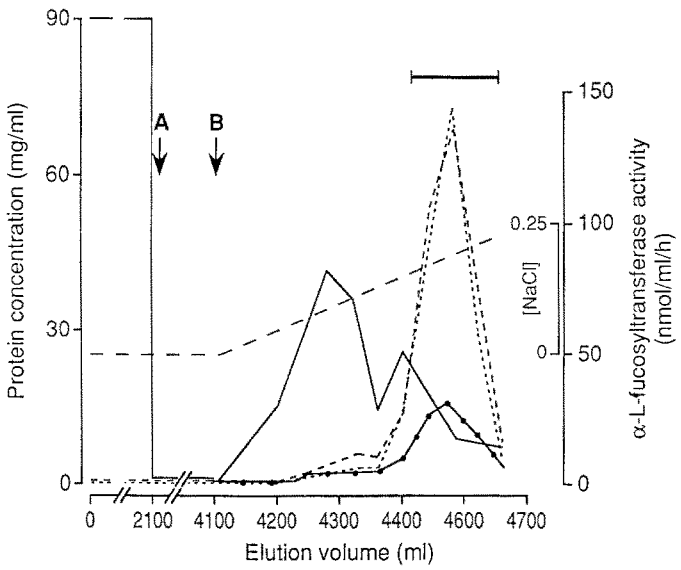

Figure 1. Elution profile of α-3/4-fucosyltransferase from the culture medium of A431 cells after cation-exchange chromatography on CM-Sephadex C-50. Fucosyltransferase assays were carried out under standard conditions. —●—, α-3-fucosyltransferase activity measured with Galβ1-4GlcNAc as substrate; ------, α-3-fucosyltransferase activity measured with Fucα1-2Galβ1-4Glc as substrate; – – – – –, α-4-fucosyltransferase activity measured with Galβ1-3GlcNAc as substrate; ———, protein concentration. The arrows indicate the points of application of: **A**, 50 mM sodium cacodylate: HCl buffer, pH 6.5, containing 5% (w/v) glycerol; **B**, NaCl gradient (0–0.25 M). The bar indicates the fractions that were pooled to give CM-Sephadex C-50 eluate.

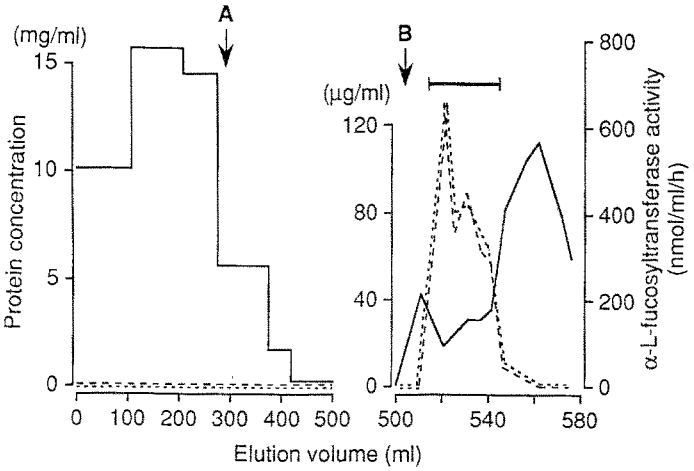

Figure 2. Elution profile of α-3/4-fucosyltransferase from the culture medium of A431 cells after affinity chromatography of CM-Sephadex C-50 eluate on GDP-hexanolamine Sepharose 4B. Fucosyltransferase assays were carried out under standard conditions. ------, α-3-fucosyltransferase activity measured with Fucα1-2Galβ1-4Glc as substrate; – – – – –, α-4-fucosyltransferase activity measured with Galβ1-3GlcNAc as substrate; ———, protein concentration. The arrows indicate point of application of: **A**, 25 mM sodium cacodylate:HCl buffer, pH 7.3, containing 1 mM NaCl and 25% (w/v) glycerol; **B**, 10 μM GDP-fucose containing 1 mM $MnCl_2$. The bar indicates the fractions that were pooled to give GDP-hexanolamine Sepharose eluate.

been added 10 μM GDP-fucose and 1 mM $MnCl_2$ (Fig. 2). Testing the individual fractions for protein concentration and activity with lacto-*N*-biose 1 and 2′-fucosyllactose showed that the most active fraction had an overall purification factor of 700000 with respect to these two substrates (Table 2). The values for the degree of purification, specific activity and recovery of enzyme were closely similar for lacto-*N*-biose 1 and 2′-fucosyllactose at each stage of purification, and the ratio of α-3 activity measured with *N*-acetyllactosamine remained virtually constant. The total active fractions from the GDP-hexanolamine Sepharose 4B column (Fig. 2) were pooled (overall purification 400000) and used for studies on the properties of the purified enzyme.

Table 3. Substrate specificity of α-3/4-fucosyltransferase purified from the culture medium of A431 cells; low-molecular-weight acceptors.

Substrate	*Relative rate*[a]	*Apparent* K_M (*mM*)	*Apparent* V_{max} ($\mu mol\ mg^{-1}\ ml^{-1}$)
1. Galβ1-3GlcNAc	100	3.0	1.4
2. Galβ1-3GlcNAc-*O*-$(CH_2)_8$COOMe	227	0.3	2.5
3. Galβ1-3GlcNAcβ1-3Galβ1-4Glc	163	1.3	1.9
4. NeuAcα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc	46	0.8	0.6
5. Fucα1-2Galβ1-3GlcNAcβ1-3Galβ1-4Glc	180	0.8	2.1
6. Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4Glc	96	11	2.0
7. Galβ1-4GlcNAc	11	23	0.3
8. Galβ1-4GlcNAc-*O*-$(CH_2)_8$COOMe	2	–	–
9. NeuAcα2-3Galβ1-4GlcNAc	15	28	0.6
10. NeuAcα2-6Galβ1-4GlcNAc	0	–	–
11. Fucα1-2Galβ1-4GlcNAc	72	5	1.0
12. GalNAcα1-3Galβ1-4GlcNAc	27	7	0.2
13. Galβ1-4GlcNAcβ1-3Galβ1-4Glc	46	19	1.6
14. Galβ1-4[Fucα1-3]GlcNAcβ1-3Galβ1-4Glc	63	15	1.7
15. Galβ1-4Glc	22	53	1.9
16. NeuAcα2-3Galβ1-4Glc	15	22	0.7
17. NeuAcα2-6Galβ1-4Glc	0	–	–
18. Fucα1-2Galβ1-4Glc	115	6	2.0
19. GalNAcα1-3[Fucα1-2]Galβ1-4Glc	123	n.d.	n.d.
20. Galβ1-3Glc	18	35	1.1
21. GDP-Fucose		0.006	

[a] Activity relative to Galβ1-3GlcNAc at 10 mM concentration except for the two disaccharides, 3 and 4, with attached hydrophobic spacer arms, $(CH_2)_8$COOMe), which were tested at a concentration of 0.8 mM.
n.d., not determined.

Properties of the purified α-3/4-fucosyltransferase secreted by A431 cells

Substrate specificity with low-molecular-weight acceptors. The acceptor specificity was tested with a range of Type 1 and Type 2 oligosaccharide acceptors (Table 3). The enzyme showed a complex specificity pattern similar to that given by the purified α-3/4-fucosyltransferase isolated from human milk after separation of contaminating *N*-acetylglucosaminide:α-3-fucosyltransferase activity by gel filtration chromatography [10]. In general, the enzyme exhibited a preference for transfer of fucose to acceptors based on Type 1 chains but substitution of the terminal β-galactosyl residues of either Type 1 or Type 2 structures influenced the apparent K_M and V_{max} values. The ratio of 9:1 for α-4-:α-3-fucosyltransferase activities when the unsubstituted Type 1 (**1**, Table 3) and Type 2 (**7**, Table 3) disaccharides were used as acceptors was similar to that found for the enzyme in the unpurified A431 culture medium (Table 1) and for the extensively purified milk α-3/4-fucosyltransferase [10]. As was also found for the milk enzyme, the best acceptor of those tested was the Type 1 disaccharide bearing the hydrophobic spacer arm $(CH_2)_8$COOMe (**2**, Table 3); the corresponding Type 2 disaccharide (**8**, Table 3) was a very poor acceptor. Substitution of the Type 1 oligosaccharide lacto-*N*-tetraose with α-2 linked fucose, as in lacto-*N*-fucopentaose 1 (**5**, Table 3) slightly enhanced the activity but substitution with α-2,3 linked sialic acid on the terminal β-galactosyl residue (**4**, Table 3) reduced the apparent V_{max} and relative rate in comparison with the parent compound. Substitution of the terminal disaccharide residue of the Type 2 structure with α-2,3 linked sialic acid (**9**, Table 3) gave less marked change in apparent K_M than that observed for the purified milk enzyme, but substitution with α-2 linked fucose (**11**, Table 3) gave a very definite increase in affinity and apparent V_{max} for the Type 2 structure.

Lactose (**15**, Table 3) itself was a poor acceptor substrate for the A431 enzyme, but substitution of the terminal β-galactosyl unit with α-2 linked fucose (**18**, Table 3) increased both the rate of transfer and the affinity of the transferase for this substrate to such an extent that the activity at 10 mM concentration was as good as that found with the unsubstituted Type 1 disaccharide. Further substitution of the galactose residue with α-3 linked *N*-acetylgalactosamine, as in the blood group active A tetrasaccharide GalNAcα1 - 3[Fucα1 - 2] - Galβ1 - 4Glc

Table 4. ^{1}H-NMR shifts of the substituent monosaccharides of the alditols of the products synthesized by the purified A431 α-3/4-fucosyltransferase with lacto-*N*-biose 1 (LNB 1) and 2′-fucosyllactose (2′-FL).

Residue	*Reporter group*	*Alditols of fucosylated products formed with*		*Alditols of reference compounds*		
		LNB 1	*2′-FL*	*LNB 1*	*4-FLNB1*[a]	*LDFT*
Fuc[1-2]	H-1	–	5.417	–	–	5.417
	H-5	–	4.213	–	–	4.212
	H-6	–	1.223[b]	–	–	1.223
Fuc[1-3]	H-1	–	5.070	–	–	5.069
	H-5	–	4.185	–	–	4.185
	H-6	–	1.223[b]	–	–	1.221
Fuc[1-4]	H-1	5.077	–	–	5.076	–
	H-5	4.225	–	–	4.223	–
	H-6	1.205	–	–	1.203	–
Gal	H-1	4.505	4.566	4.499	4.503	4.566
	H-2	3.557	3.681	3.552	3.575	3.680
	H-3	3.670	–	3.674	3.668	3.864
	H-4	3.926	3.890	3.900	3.924	3.889
GlcNAc	H-2	4.309	–	4.251	3.307	–
	H-3	4.169	–	4.166	4.167	–
	H-4	4.013	–	3.551	4.015	–
	NAc	2.046	–	2.033	2.044	–
Glc	H-2	–	4.140	–	–	4.140
	H-3	–	4.061	–	–	4.061

[a] 4-FLNB1, Galβ1-3[Fucα1-4]GlcNAc, synthesized by milk α-3/4-fucosyltransferase [10].
[b] Fucose methyl doublets were not resolved.
Other reference compounds were authentic samples of lacto-*N*-biose 1 (LNB 1) [10] and lactodifucotetraose (LDFT) [16].

(**19**, Table 3) did not decrease the activity of the enzyme.

As has been found previously for the milk α-3/4-fucosyltransferase [10], substitution of the terminal β-galactosyl residue of Type 2 structures with α-2,6 linked sialic acid (**10** and **17**, Table 3) completely abolished the capacity of the compounds to function as acceptors for the A431 enzyme.

Some of the larger oligosaccharides were good acceptors for the A431 enzyme, but further characterization was necessary to establish which of the potential acceptor sites had been utilized by the transferase (see below).

Characterization of products

*Lacto-*N*-biose 1* and *2′-fucosyllactose products.* Larger scale preparations were made, as described in the Materials and methods section, of the fucosylated products synthesized by the A431 enzyme with a mixture of unlabelled and ^{14}C labelled GDP-fucose as the sugar donor and lacto-*N*-biose 1 (yield 1.5 mg) and 2′-fucosyllactose (yield 3.0 mg) as acceptors. The transferred fucose in both compounds was susceptible to hydrolysis by the α-3/4-fucosidase and not by the α-2-fucosidase. Methylation and hydrolysis of the lacto-*N*-biose 1 product (R_{lac} 0.70 in solvent **1**) gave 2,3,4-*O*-methylfucose, 2,3,4,6-*O*-methylgalactose and 6-*O*-methylglucosamine, demonstrating that the fucose residue had been transferred to the *O*-4 position of *N*-acetylglucosamine to give the Le[a] trisaccharide structure, Galβ1-3[Fucα1-4]GlcNAc. ^{1}H-NMR analysis of the reduced trisaccharide established that the product was identical with that formed with this substrate by the purified milk α-3/4-fucosyltransferase [10]. Comparison of the chemical shifts for the reduced fucosylated derivative with that of the reduced parent compound showed that the values for galactose were little changed, whereas large changes in the value for H-4 of GlcNAc-ol were consistent with the addition of fucose on to the *N*-acetylglucosamine residue (Table 4).

Examination of the ^{1}H NMR spectrum of the reduced product formed with 2′-fucosyllactose (R_{lac} 0.40 in solvent **1**) showed that it was identical with that given by the product formed by the milk α-3/4-fucosyltransferase with this substrate [10] and with that given by an authentic specimen of lactodifucotetraose [16] (Table 4). Structural examination of these two compounds therefore confirmed that the purified A431 enzyme preparation had transferred fucose to the *O*-4 position of *N*-acetylglucosamine in the Type 1

chain acceptor and to the *O*-3 position of glucose in the lactose-based acceptor. The remaining products were synthesized only on a micro-scale with GDP-[^{14}C]fucose as the sugar donor.

*Lacto-*N*-tetraose and lacto-*N*-neotetraose products.* The fucosylated product (R_{lac} 0.2 in solvent **1**) formed with lacto-*N*-tetraose was eluted and re-chromatographed for three days in solvent **2**. Two radioactive spots were separated; the major product accounted for 84% of the radioactivity and co-chromatographed with an authentic specimen of lacto-*N*-fucopentaose II (R_{LNT} 0.52 in solvent **2**). The mobility of the minor product (R_{LNT} 0.56 in solvent **2**) was consistent with an attribution of lacto-*N*-fucopentaose V (Galβ1-3GlcNAcβ1-3Gal1-4[Fucα1-3]Glc) [29] and, although no authentic sample of this compound was available for comparison, the mobility was the same as that of the minor product synthesized by the milk α-3/4-fucosyltransferase with this substrate [10]. By contrast, the radioactive product (R_{lac} 0.2 in solvent **1**) formed with lacto-*N*-neotetraose gave only one radioactive spot on re-chromatography in solvent **2** (R_{LNT} 0.55) for three days. This spot was clearly separable from an authentic sample of lacto-*N*-fucopentaose III (R_{LNT} 0.50 in solvent **2**) and was tentatively identified as lacto-*N*-fucopentaose 'VI' (Galβ1-4GlcNAcβ1-3Galβ1-4[Fucα1-3]Glc).

*Lacto-*N*-fucopentaose I and lacto-*N*-fucopentaose II products.* The radiolabelled fucosylated product formed with lacto-*N*-fucopentaose I (**5**, Table 3) was re-chromatographed in solvent **2** for three days and gave a single spot that co-chromatographed (R_{LNT} 0.26) with authentic lacto-*N*-difucohexaose I (Fucα1-2Gal-β1-3[Fucα1-4]GlcNAcβ1-3Gal*β*1-4Glc), showing that the fucose has been transferred to the *O*-4 position of *N*-acetylglucosamine.

The product synthesized with lacto-*N*-fucopentaose II (**6**, Table 3) similarly gave only one radioactive spot on re-chromatography in solvent **2** for three days (R_{LNT} 0.29). The mobility of this product was identical with that of authentic lacto-*N*-difucohexaose II (Galβ1-3[Fucα1-4]GlcNAcβ1-3Galβ1-4[Fucα1-3]Glc), consistent with the interpretation that the fucose has been transferred to the *O*-3 position of the reducing glucose residue.

*Sialyllactose: sialyl-*N*-lactosamine and sialyllacto-*N*-tetraose products.* The radioactive products synthesized with the sialylated acceptors were separated from other components of the reaction mixtures by paper electrophoresis, eluted and chromatographed in solvent **1**; the mobilities corresponded to the values obtained for the more rigorously characterized products synthesized by the purified milk α-3/4-fucosyltransferase. The product (R_{lac} 0.25 in solvent **1**) formed with 3′-sialyllactose (**16**, Table 3) co-chromatographed with NeuAcα2-3Galβ1-4[Fucα1-3]Glc and the product (R_{lac} 0.27) in solvent **1**) formed with 3′-sialyl-*N*-acetyllactosamine (**4**, Table 3) co-chromatographed with NeuAcα2-3Gal[Fucα1-3]GlcNAc; the structures of these two oligosaccharides had been established by ^{1}H-NMR analysis for the milk enzyme products [10]. The product ($R_{LNFP\,II}$, 0.46 in solvent **2**) formed with sialyllacto-*N*-tetraose (**4**, Table 3) corresponded to that of the major product, NeuAcα2 - 3Galβ1 - 3[Fucα1 - 4]GlcNAcβ1 - 3Glc, formed with the milk enzyme which was characterized by the mobilities of its enzymatic degradation products with neuraminidase and α-3/4-fucosidase [10].

Other products. The radioactive products synthesized with the following compounds were characterized by their chromatographic mobilities relative to lactose in solvent **1**; Fucα1-2Galβ1-4GlcNAc (**11**, Table 3) product (R_{lac}, 0.40); GalNAcα1-3Galβ1-4GlcNAc (**12**, Table 3) product (R_{lac}, 0.35); GalNAcα1-3[Fucα1-2]Galβ1-4Glc (**19**, Table 3) product (R_{lac}, 0.20); Galβ1-3Glc (**20**, Table 3) product (R_{lac} 0.50); Galβ1-4Glc (**15**, Table 3) product (R_{lac} 0.70).

Substrate specificity with glycoprotein acceptors

The purified A431 fucosyltransferase gave virtually no incorporation of [^{14}C]fucose into the glycoproteins fetuin, α_1-acid glycoprotein and transferrin, which carry predominantly Type 2 (Galβ1-4GlcNAc) terminal structures [30–32], and removal of sialic acid from the terminal β-galactosyl units did not improve significantly the incorporation. The Type XIV pneumococcal polysaccharide, which carries multiple Type 2 branches [33], also failed to function as an acceptor for the purified A431 enzyme. Of the glycoproteins tested, only the ovarian cyst blood group precursor glycoprotein which carries both Type 1 and Type 2 chains [34] gave good incorporation of [^{14}C]fucose: this incorporation was increased by removal of sialic acid (Table 5)

Effect of pH and divalent cations on the activity of the A431 α-3/4-fucosyltransferase

The enzyme had a fairly broad pH optimum with maximum activity at pH 8.8 (Fig. 3) in comparison with an optimum of 9.0 for the α-3/4-fucosyltransferase purified from human milk [10]. Tested with a range of divalent cations at 20 mM concentration, the α-3/4-fucosyltransferase activity was enhanced by Mn^{2+}, Ca^{2+}, Mg^{2+}, and Co^{2+} ions and inhibited by Cu^{2+}, Zn^{2+} and Ni^{2+} ions (Table 6). This pattern of activation and inhibition is similar to that obtained for the purified α-3/4-fucosyltransferase from milk [10]. Manganese and calcium chloride concentration curves obtained with the A431 enzyme demonstrated maximum activity with Mn^{2+} ions at 10–40 mM and with Ca^{2+} ions at 20–100 mM (Fig. 4).

Effect of N*-ethylmaleimide and* p*-hydroxymercuribenzoate on the activity of the purified A431 α-3/4-fucosyltransferase*

The purified A431 α-3/4-fucosyltransferase was strongly inhibited by *N*-ethylmaleimide at 10 mM concentration and

Table 5. Substrate specificity of α-3/4-fucosyltransferase purified from A431 cells with glycoprotein acceptors.

Substrate	*Major chain type*	*Relative activity*[a]
Transferrin	2	0.4
Asialo-transferrin		0.4
α_1-Acid glycoprotein	2	0.6
Asialo-α_1-acid glycoprotein		0.5
Fetuin	2	0.8
Asialo-fetuin		2.9
Ovarian cyst glycoprotein No 484	1 and 2	12.2
Asialo-ovarian cyst glycoprotein		19.7
Type XIV pneumococcal polysaccharide	2	0.8
Lacto-*N*-biose 1	1	100

[a] Incorporation of [^{14}C]fucose into 100 μg glycoprotein compared with the incorporation into 0.5 μmol lacto-*N*-biose 1 (100%) under identical conditions. Assays were carried out with standard reaction mixtures except that the concentration of GDP-fucose was increased to 14 μM.

Table 6. Influence of divalent cations on the activity of the α-3/4-fucosyltransferase purified from the growth medium of A431 cells.

Divalent cation	*Relative activity*[a]
No added cation	100
Mn^{2+}	325
Ca^{2+}	185
Mg^{2+}	217
Ni^{2+}	46
Co^{2+}	148
Cu^{2+}	4
Zn^{2+}	18

[a] Activity relative to that found in the absence of added cations.
α-4-Fucosyltransferase activity was assayed with lacto-*N*-biose 1 as acceptor substrate under standard conditions except that 20 mM $MnCl_2$ was replaced by chlorides of other divalent cations at the same concentration.

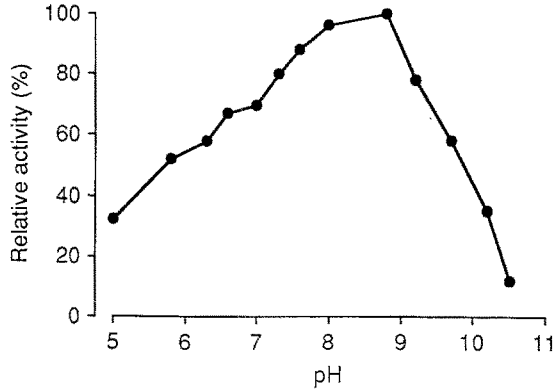

Figure 3. Effect of pH on the activity of the purified α-3/4-fucosyltransferase from the culture medium of A431 cells. Fucosyltransferase assays were carried out with Galβ1-3GlcNAc as substrate under standard conditions except that the pH of the buffer was varied as shown.

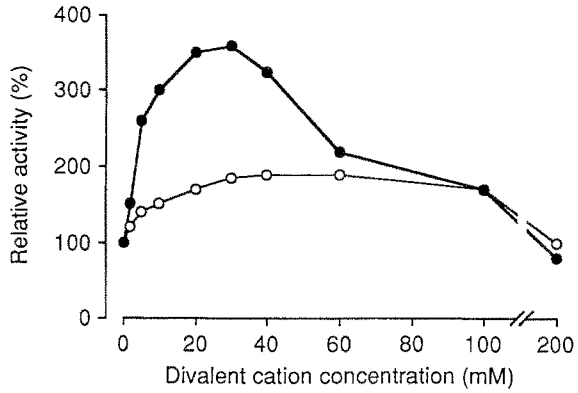

Figure 4. Effect of Mn^{2+} and Ca^{2+} ion concentration on the activity of the α-3/4-fucosyltransferase purified from the culture medium of A431 cells. Fucosyltransferase activity was assayed with Galβ1-3GlcNAc as substrate under standard conditions except that the concentrations of divalent cations were varied as shown. •, Mn^{2+}; o, Ca^{2+}.

by *p*-hydroxymercuribenzoate at 0.1 mM concentration (Fig. 5). This is in contrast to the purified milk α-3/4-fucosyltransferase which is relatively more resistant to inhibition by these sulfhydryl reagents [10].

Gel exclusion chromatography of the purified A431 enzyme on Sephadex G-150

The purified enzyme (50 μl) was diluted to 1 ml with 0.15 M NaCl containing 0.01% bovine serum albumin, loaded on to a column (70 cm × 1.5 cm) of Sephadex G-150 and run in 0.15 M NaCl at a flow rate of 29 ml h^{-1}. The fractions (1 ml) were assayed for α-3 activity with 2′-fucosyllactose and *N*-acetyllactosamine and for α-4 activity with lacto-*N*-biose 1. The activities with lacto-*N*-biose 1 and 2′-fucosyllactose both gave a similar broad elution pattern with a major peak of apparent molecular weight 95 000 and two unresolved smaller peaks of apparent molecular weights of 88 000 and 112 000. The fractions tested with *N*-acetyllactosamine showed only a low level of activity, but the shape of the elution pattern was nevertheless similar to that given by the other two substrates and there was no evidence for the separation of *N*-acetylglucosaminide α-3-fucosyltransferase, as occurred at this stage in the purification of the milk α-3/4-fucosyltransferase [9] (Fig. 6).

Binding of purified A431 α-3/4-fucosyltransferase to concanavalin A-Sepharose 4B

A column of concanavalin A-sepharose 4B (0.4 ml; 4 mg ml^{-1} substitution) was equilibrated in 25 mM sodium cacodylate:

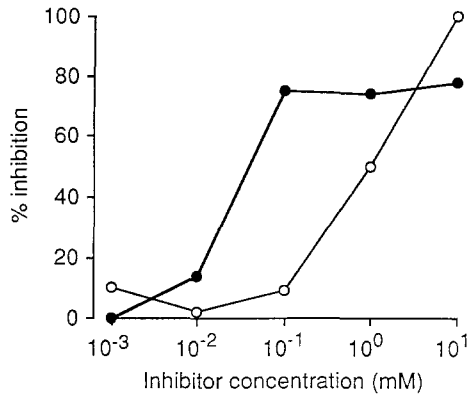

Figure 5. Effect of sulfhydryl-binding reagents on the activity of α-3/4-fucosyltransferase purified from the culture medium of A431 cells. Varying concentrations of ○, *N*-ethylmaleimide and ●, *p*-hydroxymercuribenzoate were added to reaction mixtures containing the enzyme preparation and the standard ingredients except BSA. The tubes were left at 4 °C for 45 min and then BSA was added and the mixtures were incubated for 3 h at 37 °C.

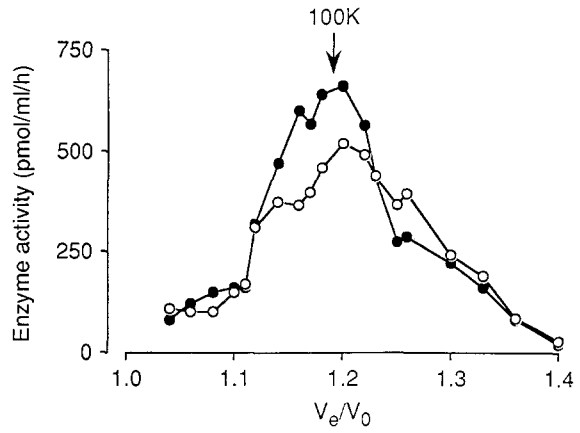

Figure 6. Gel exclusion chromatography of α-3/4-fucosyltransferase purified from the culture fluid of A431 cells on Sephadex G-150. Fucosyltransferase assays were carried out by standard procedures with ○, Fucα1-2Galβ1-4Glc and ●, Galβ1-3GlcNAc as substrates. The arrow indicates the 100 000 molecular size calculated from the elution volumes of molecular weight markers.

HCl buffer, pH 7.3, containing 0.5 M NaCl, 1 mM, $MgCl_2$, 1 mM $CaCl_2$ and 5% glycerol. The purified A431 enzyme (0.8 ml) diluted in the same buffer containing 1 mg ml^{-1} bovine serum albumin was loaded on to the concanavalin A-Sepharose and the column was washed through with 0.8 ml of the same buffer. Benzyl *N*-acetyl-α-glucosaminide was then added to the buffer to give a final concentration of 25 mM and the solution was used to elute bound glycoprotein from the column. Seventy one per cent of the α-4-fucosyltransferase activity measured with lacto-*N*-biose 1 bound to the concanavalin A column and 14% of the bound activity was recovered in the benzyl *N*-acetyl-α-glucosaminide eluate. The A431 enzyme thus resembled the milk α-3/4-fucosyltransferase [10] in that it behaved as expected of a glycoprotein bearing *N*-linked oligosaccharide chains.

Discussion

The striking feature of the α-3/4-fucosyltransferase secreted into the culture medium during the growth of the human A431 cell line is that the ratio of activities with simple Type 1 and Type 2 disaccharide acceptor substrates (Table 1) closely resembles the ratio of activities found for these substrates with the extensively purified α-3/4-fucosyltransferase purified from human milk [10]. At 10 mM acceptor substrate concentration both enzyme preparations exhibit a marked preference for the Type 1 acceptor, Galβ1-3GlcNAc, with an activity nearly tenfold greater towards this substrate than towards the Type 2 disaccharide, Galβ1-4GlcNAc. In human milk the co-presence in the secretion of a considerable amount of a second fucosyltransferase, an *N*-acetylglucosaminide:α-3-fucosyltransferase, necessitated a number of purification steps before the final ratio of activities with these substrates became apparent [9, 10]. However, although the highly purified, secreted, A431 enzyme has not been shown to be homogeneous, the fact that the ratio of activities with the two disaccharide substrates remained constant throughout the purification procedure, suggests that the enzyme secreted into the medium in all probability consists of one major fucosyltransferase species.

Triton-solubilized preparations of the harvested A431 cells have proportionately much more activity directed towards *N*-acetylglucosamine in Type 2 chains (Table 1) than is found in the culture medium. In the cells therefore there is at least one other species of α-3-fucosyltransferase with the capacity to synthesize X (Galβ1-4[Fucα1-3]GlcNAc-R) structures, but this enzyme is apparently not released into the culture medium to any significant extent and at no stage was *N*-acetylglucosaminide:α-3-fucosyltransferase activity separated from the A431 α-3/4-fucosyltranferase by procedures that removed this activity from the milk α-3/4-fucosyltransferase preparation [9, 10].

The acceptor specificity of the purified A431 α-3/4-fucosyltransferase with a range of low-molecular-weight oligosaccharide acceptors gave further evidence of the close resemblance between this enzyme and the purified milk α-3/4-fucosyltransferase (Table 3). Both enzymes utilized substrates with sialic acid linked α-2,3 to the terminal β-galactosyl residue in Type 1 structures, although the substitution with this sugar reduced the efficiency of the compounds as acceptors. In contrast, the presence of a fucose substituent in α-1,2 linkage to the terminal β-galactosyl residue slightly enhanced the efficiency with which the Type 1 compounds were used as substrates. Lactose itself was a poor acceptor for the A431 enzyme, but a fucose substituent on the terminal nonreducing β-galactosyl residue, as in 2′-fucosyllactose, lowered the

apparent K_M and gave a very marked increase in the capacity of the enzyme to transfer fucose to the terminal reducing glucose unit. The high apparent K_M of the enzyme for the Type 2 disaccharide was lowered also when a fucose or *N*-acetylgalactosamine unit was substituted on the β-galactosyl residue but sialic acid in α-2,3 linkage did not enhance the utilization of Type 2 structures. In agreement with results found for other fucosyltransferases [35, 36] substitution of the terminal sugar with sialic acid in α-2,6 linkage abolished the capacity of the oligosaccharides to function as acceptors. In compounds with two potential acceptor sites, such as lacto-*N*-tetraose, which has a subterminal *N*-acetylglucosamine residue with a free O-4 position and a terminal nonreducing glucose with a free O-3 position, the A431 enzyme showed a distinct preference for transfer of fucose to the O-4 site of *N*-acetylglucosamine. Unfortunately, no substrates were available to test for the transfer of fucose to internal *N*-acetylglucosamine structures in oligosaccharide chains containing tandemly repeating units of either Type 1 or Type 2 structures.

Measurement of the incorporation of [^{14}C]fucose into glycoprotein acceptors (Table 6) gave even more striking evidence that the enzyme was transferring fucose primarily to *N*-acetylglucosamine units in Type 1 oligosaccharide chains. Results which were similar to those obtained with the milk α-3/4-fucosyltransferase [10] revealed that none of the glycoproteins tested with *N*-linked oligosaccharide chains terminating in Type 2 sequences functioned as acceptors for the purified fucosyltransferase (Table 5). The Type XIV pneumococcal polysaccharide with multiple repeating Type 2 disaccharide branches [33], which had proved to be an excellent acceptor for the milk *N*-acetylglucosaminide:α-3-fucosyltransferase but not for the milk α-3/4-fucosyltransferase [10], similarly failed to act as a substrate for the purified A431 enzyme. The mucin-type ovarian cyst glycoprotein with O-linked oligosaccharide chains containing both Type 1 and Type 2 terminal nonreducing sequences [34] was, however, a good substrate for the A431 enzyme, as it was also for the milk α-3/4-fucosyltransferase [10]. The inference can therefore be drawn that the fucose had been transferred to the O-4 position of the *N*-acetylglucosamine residues in the Type 1 sequences in the cyst glycoprotein.

The properties of the purified A431 α-3/4-fucosyltransferase with respect to pH optimum (Fig. 3), divalent cation requirements (Fig. 4) and ability to bind to concanavalin A-Sepharose 4B were also similar to those recorded for the enzyme isolated from human milk [10]. The molecular size (~95 000) of the major A431 enzyme species, as measured by gel filtration, was slightly larger than the value recorded for the milk enzyme (90 000) [9] which was assessed by a similar procedure. An α-3/4-fucosyltransferase specimen, isolated from milk by Eppenberger-Castori *et al.* [37] and given a final purification step on an HPLC gel filtration column, was estimated to have a molecular weight of 98 000. The cDNA believed to correspond to the Lewis gene, isolated by Kukowska-Latallo *et al.* [8], encodes a 42 000 protein, and therefore if the milk and the A431 α-3/4-fucosyltransferases are the protein products of this gene they are presumably dimeric forms and the exact size may be dependent on the degree of glycosylation and/or other post-translational modifications. The fact that the enzymes are soluble indicates that they are lacking the hydrophobic transmembrane segment encoded by the gene [8]. The predicted amino acid sequence analysis of the cloned cDNA indicates two potential *N*-glycosylation sites [8], and both the milk and A431 enzymes behave with respect to concanavalin A binding as would be expected of glycoproteins with *N*-linked oligosaccharide chains.

The most evident difference between the A431 enzyme and the purified milk α-3/4-fucosyltransferase is the increased susceptibility of the A431 enzyme to inhibition by *N*-ethylmaleimide and *p*-hydroxymercuribenzoate. However, this property was measured on the milk enzyme preparation obtained immediately prior to the final gel filtration step that separated out further amounts of residual *N*-acetylglucosaminide:α-3-fucosyltransferase activity [10]. It is therefore not entirely clear whether the increased susceptibility represents a genuine difference in behaviour between the two α-3/4-fucosyltransferase preparations or whether the presence of the contaminating enzyme in some way protected the partially purified milk α-3/4-fucosyltransferase from inactivation by the sulfhydryl reagents. In any event, the A431 fucosyltransferase was still more resistant to inactivation by *N*-ethylmaleimide than other fucosyltransferases; for example the plasma *N*-acetylglycosaminide:α-3-fucosyltransferase, purified over a million-fold, was completely inhibited by 0.01 mM *N*-ethylmaleimide [11], whereas 10 times this concentration was required to give equivalent inhibition of the A431 α-3/4-fucosyltransferase. Overall, of the fucosyltransferases so far described, the enzyme released into the culture medium of A431 cells most closely resembles the α-3/4-fucosyltransferase species synthesized by the mammary gland and secreted into milk. At least *in vitro*, with the oligosaccharides and glycoproteins tested as substrates, these enzymes react primarily with Type 1 chains to form structures with fucose linked α-1,4 to *N*-acetylglucosamine, namely Le^a, Le^b and sialyl-Le^a determinants. Both these enzymes also have the property of transferring fucose to the O-3 position of terminal reducing glucose residues in lactose-based chains to synthesize the type of oligosaccharides with fucosylated glucose units that are found as free entities in milk and urine [38, 39]. However, although the enzymes have this dual function and therefore contradict the rule, first proposed by Hagopian and Eylar [40], of 'one glycosyltransferase–one glycosidic linkage', in *in vitro* experiments they appear to have only a limited capacity to transfer fucose to the O-3 position of subterminal *N*-acetylglucosamine residues in Type 2 sequences to form X, Y or sialyl-X structures.

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Note added in proof

Small scale experiments carried out since this paper was submitted have shown that the α-3/4-fucosyltransferase activity in the culture medium from A431 cells does bind to Phenyl Sepharose 4B. It is unclear why the enzyme in the large scale preparation used for the purification procedure described in this paper failed to bind to this adsorbent.

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