

Glycosylation and epitope mapping of the 5T4 glycoprotein oncofoetal antigen

David M. SHAW*, Andrew M. WOODS*, Kevin A. MYERS*¹, Caroline WESTWATER*, Veena RAHI-SAUD*, Michael J. DAVIES†², David V. RENOUF†, Elizabeth F. HOUNSELL† and Peter L. STERN*³

*CRC Immunology Group, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Wilmslow Road, Manchester M20 4BX, U.K., and

†School of Biological and Chemical Sciences, Birkbeck College, University of London, Gordon House, 29 Gordon Street, London WC1H 0PP, U.K.

The human 5T4 oncofoetal antigen is a focus for development of several antibody-directed therapies on the basis of the murine monoclonal antibody against 5T4 (mAb5T4), which recognizes a conformational epitope. 5T4 molecules are highly N-glycosylated transmembrane glycoproteins whose extracellular domain contains two regions of leucine-rich repeats (LRRs) and associated flanking regions, separated by an intervening hydrophilic sequence. Using a series of deletion and mutated cDNA constructs as well as chimaeras with the murine homologue, we have mapped the mAb5T4 epitope to the more membrane-proximal LRR2 or its flanking region. Analysis of the glycosylation of the seven consensus Asp-Xaa-Ser/Thr sites was consistent with all of the sites being glycosylated. A combination of two high-mannose chains (predominantly octasaccharide) and five mostly sialylated bi-, tri- and tetra-antennary complex chains with minor quantities

of core fucose were detected. The two glycosylation sites, which are the most likely to have predominantly high-mannose chains, are in the only two regions that show significant differences between the human and the 81% identical mouse sequence. A site-directed mutation, which abolished glycosylation at one of these sites (position 192), did not alter antigenicity. The other, which is nearest to the N-terminus in the human, has an Asn-Leu-Thr to Asn-Leu-Leu conversion in the mouse, so cannot be glycosylated in the latter species. The large complex glycosylation at the other sites is likely to influence the antigenicity and tertiary structure generating the 5T4 epitope.

Key words: leucine-rich repeat protein, metastasis, oncotrophoblast antigen, oligosaccharide, tumour-associated antigen.

INTRODUCTION

The expression of specific trophoblast cell-surface molecules is likely to contribute to the controlled invasion of maternal tissue and/or avoid immune recognition [1]. Such functional molecules, where expressed in malignant tissues, may allow the tumours to invade surrounding normal tissue whilst avoiding host immunity. Such similarities in the properties of embryonic cells, like trophoblast and tumour cells, have stimulated the search for common functional oncofoetal antigens.

The human 5T4 oncofoetal antigen is a 72 kDa leucine-rich repeat (LRR) glycoprotein that is strongly expressed by trophoblasts throughout gestation [2–4]. This antigen is only weakly expressed in adult tissues, but strongly expressed in a number of human carcinomas [3,5–10], and its expression is prognostic in gastric, colorectal and ovarian tumours [5–7,9]. Expression of 5T4 molecules in fibroblast and epithelial cell lines localizes to microvillous projections, and overexpression of the cDNA encoding the human gene results in increased cell motility and altered morphology [11,12], consistent with a role in tumour and trophoblast invasion.

LRR proteins are a diverse family of approx. 60 members and, although no common function has been ascribed, many are involved in protein–protein interactions [13–15]. The tertiary structures of several LRR proteins have now been solved by X-ray crystallography [15–18]. Despite low levels of sequence

identity between the different proteins, a remarkable degree of structural similarity is observed [15]. The basic LRR structure is of repeating units of β -strand and anti-parallel α -helix, and this structure has been used to model other family members [16,19,20]. However, due to the presence of other functional domains and differences in the lengths of the LRRs, the precise structure varies. The short 20–22-amino-acid repeat length of the YopM protein of *Yersinia pestis* results in a conformation where most of the repeats have no α -helical component and the β -strand is less twisted than in porcine ribonuclease inhibitor, which has the longest repeat length of 29 amino acids. 5T4, having a repeat length of 24 amino acids, might be expected to show an intermediate conformation between these two extremes [4,14,17].

5T4 antigen is a highly N-glycosylated protein [21], with seven putative consensus N-linked glycosylation sites in the extracellular domain [4]. Therefore the diversity of the oligosaccharide structures may influence functional aspects both by protecting the antigen from proteolytic attack or promoting interactions with other molecules important to the invasive process.

The murine monoclonal antibody against 5T4 (mAb5T4), which defines the human protein, recognizes an epitope that is dependent upon both glycosylation of the molecule and intramolecular disulphide interactions [21], consistent with it being a conformational epitope. Comparison of the human and mouse 5T4 sequences [22] shows that the structure is highly conserved between the two species. The cytoplasmic and transmembrane

Abbreviations used: 2-AB, 2-aminobenzamide; ECL[®] (Amersham Biosciences), enhanced chemiluminescence; GU, glucose units; HPAEC–PAD, high-pH anion-exchange chromatography–pulsed amperometric detection; LRR, leucine-rich repeat; mAb5T4, murine monoclonal antibody against 5T4; PNGase F, peptide N-glycosidase F; RT-PCR, reverse transcriptase-PCR; StMPM, syncytiotrophoblast microvillous membrane; SHRP, streptavidin–horseradish peroxidase; WGA, wheatgerm agglutinin.

¹ Present address: Oxford Biomedica, Medeware Centre, The Oxford Science Park, Oxford OX4 4GA, U.K.

² Present address: Lonza Biologics, 228 Bath Road, Slough SL1 4DY, U.K.

³ To whom correspondence should be addressed (e-mail pstern@picr.man.ac.uk).

domains are identical and the molecules as a whole share 81% identity at the amino acid level. Six of the seven predicted N-linked glycosylation sites of the human molecule are conserved in the mouse; the most N-terminal glycosylation site is absent, but there is another in the C-terminal flanking region [22]. The murine protein contains an additional six amino acids adjacent to the glycosylation site in the hydrophilic domain, which is a direct repeat of the preceding six amino acids. Despite this high level of identity between 5T4 molecules of the two species, mAb5T4 does not recognize the murine molecule, suggesting that these small differences significantly influence the antigenicity of the molecule.

In the present study, we report the characterization of the oligosaccharide structures present on the human 5T4 antigen by a combination of chromatographic methods involving sensitive analysis of fluorescent derivatives. Furthermore, we have investigated the location of the antigenic epitope within the extracellular domain of human 5T4 by using a series of variant cDNA constructs.

MATERIALS AND METHODS

5T4 purification

Human 5T4 was purified from syncytiotrophoblast microvillous membranes (StMPM) by wheatgerm agglutinin (WGA) affinity chromatography, eluting with 0.3 M *N*-acetylglucosamine, and was purified further by mAb5T4 immunoaffinity chromatography, eluting in 8 M urea, as described by Hole and Stern [21]. Purity of the 5T4 antigen was assessed by SDS/PAGE [23]. 5T4-containing fractions were pooled and desalted using Centrifix 30 centrifugal concentrators (Millipore, Watford, Herts., U.K.) and proteins were quantified by a modified Bradford assay [24].

Concentrated desalted material was freeze-dried prior to further analysis.

Cloning of variant 5T4 constructs

Variant 5T4 cDNA constructs (Figure 1) were generated and expressed in various eukaryotic cell lines using the vector pCMV α neo. Truncated 5T4 variants L1, L1H and TM⁻ were prepared by PCR using sequence-specific primers encompassing the LRR1 (amino acids 1–184), LRR1 plus the hydrophilic domain (amino acids 1–200) and the entire extracellular domain (amino acids 1–335) respectively. A 5T4 variant with a truncated cytoplasmic domain CYT⁻ was made by digestion with *Nsi*I [12]. Site-directed mutations, with deletion of the consensus N-linked glycosylation site (Asn¹⁹²) and tyrosine phosphorylation site (Tyr⁴⁰¹), were made by PCR using primers introducing substitutions of phenylalanine for Asn¹⁹² and Tyr⁴⁰¹ respectively. A chimaeric 5T4/CD44 molecule was made by fusing the extracellular domain of 5T4 to the transmembrane and cytoplasmic domains of human CD44 (human CD44 cDNA provided by Professor Clare Isacke, Chester Beatty Labs, The Institute of Cancer Research, London, U.K.). The human/mouse chimaeric 5T4 molecule (H/M 5T4) was constructed by PCR, utilizing a nucleotide sequence common to both molecules encoding amino acids 166–172 (bp 499–517) as the splice site. The mouse/human chimaera (M/H 5T4) was made by digestion of both cDNAs with *Bsm*BI at nucleotide 511 (corresponding to amino acid 170) and ligating the 5' fragment of the mouse cDNA with the human 3' region.

Expression of variant 5T4 molecules

Variant 5T4 cDNA constructs were expressed variously in A9 mouse fibroblasts, CL-S1 mouse mammary epithelial cells,

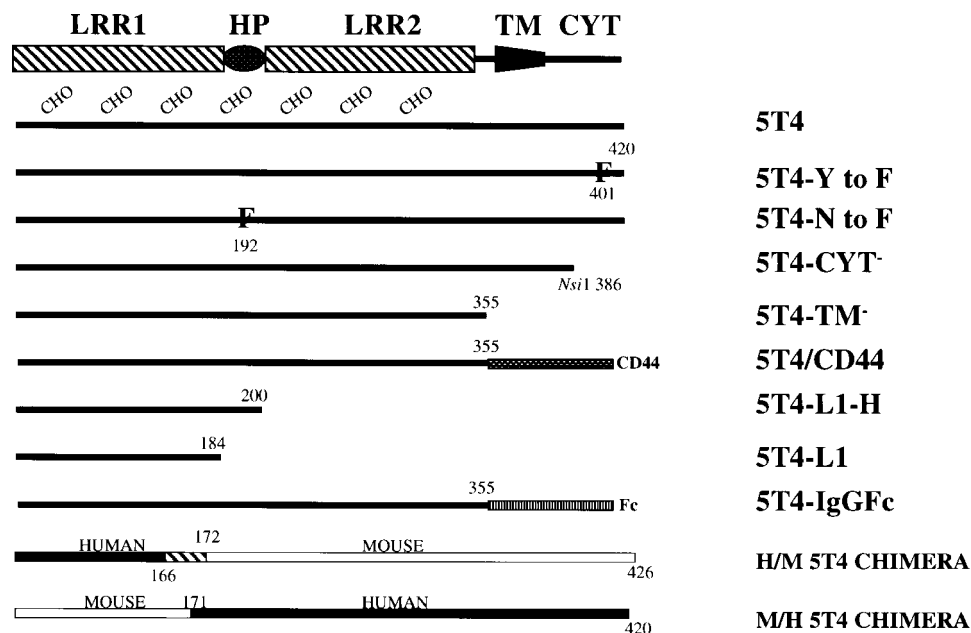


Figure 1 Construction of 5T4 variant cDNA molecules

Truncation, deletion and chimaeric 5T4 molecules were constructed by PCR using specific primers or by restriction digest. The position of carbohydrate (CHO) and hydrophilic (HP), transmembrane (TM) and cytosolic (CYT) domains are indicated. The size of each construct (in amino acids) is given. The location of the substitution of Tyr⁴⁰¹ (5T4-Y to F) and Asn¹⁹² (5T4-N to F) to phenylalanine is indicated (F).

Madin–Derby canine kidney, Chinese-hamster ovary and Cos-7 cells. Cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal-bovine serum and 2 mM L-glutamine in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C. Transfected cells were maintained under antibiotic selection by the addition of 1 mg/ml G418. Cell lysates were prepared at 2 × 10⁶ cells/ml in Tris-buffered saline [50 mM Tris/HCl (pH 8) and 150 mM NaCl] containing 0.5% Nonidet P40 and 0.2 μM PMSF. Cell-culture supernatants for Western- and dot-blot analysis were collected from cultures incubated for 48 h in serum-free Dulbecco's modified Eagle's medium supplemented with L-glutamine and non-essential amino acids. The supernatant was concentrated up to 20 times using centrifugal concentrators. A secreted human 5T4-IgGFc fusion protein was expressed in Cos-7 cells and purified on WGA-agarose and Protein G (Amersham Biosciences, Little Chalfont, Bucks., U.K.) as described previously [25]. The Fc was removed by digestion with Factor Xa protease (Roche Diagnostics, Lewes, East Sussex, U.K.) and Protein G-Sepharose. Disulphide bonds in full-length 5T4, derived from transfected A9 cells, were reduced using 20 mM 2-mercaptoethanol and alkylated with 40 mM iodoacetamide.

Rat polyclonal sera

Rat anti-mouse 5T4 and -human 5T4 polyclonal sera were developed in Lou rats infected with vaccinia encoding the human or mouse 5T4 cDNA (kindly provided by Dr Miles Carroll, Oxford Biomedica, Oxford Science Park, Oxford, U.K.).

FACS analysis

FACS analysis was performed on 2 × 10⁵ cells suspended in FACS buffer (PBS containing 0.2% BSA and 0.1% sodium azide). Antibodies were diluted in this buffer, which was used for subsequent washes; all steps were performed on ice. Cells were post-fixed in 1% paraformaldehyde before analysis. MAb5T4 was used at 1 μg/ml and detected using FITC-conjugated rabbit F(ab')₂ anti-mouse immunoglobulins (1:40; Dako, Denmark House, Ely, Cambs., U.K.). Rat polyclonal anti-mouse 5T4 or -human 5T4 were used at 1:300 dilutions and detected with FITC-conjugated rabbit F(ab')₂ anti-rat immunoglobulins (1:40 dilution; Dako).

Reverse transcriptase (RT)-PCR

Total RNA was extracted from cells using RNeasy B according to the manufacturer's instructions (Biogenesis, Poole, Dorset, U.K.). Removal of genomic DNA was ensured by re-extraction of the RNA with RNeasy B. cDNA was prepared from 5 μg of the RNA using AMV RT (Promega, Chilworth, Southampton, U.K.) in a total reaction volume of 100 μl. For constructs, the presence of 5T4 mRNA in the cells was detected by PCR on 5 μl of the cDNA using primers in the 5T4 LRR1 region, yielding a 338 bp 5T4-specific band.

SDS/PAGE, Western and dot blotting

SDS/PAGE was performed by the method of Laemmli [23] using a mini-gel system. Separated proteins were transferred on to nitrocellulose as described by Towbin and Gordon [26] using a semi-dry transfer system. Membranes were blocked using PBS containing 2% (w/v) non-fat dried milk for at least 1 h before developing, and probed for 1 h with each antibody. MAb5T4 (1 μg/ml) was detected using biotinylated rabbit anti-mouse

immunoglobulins (1:3000 dilution; Dako) followed by strept-avidin-horseradish peroxidase (SHRP; 1:6000 dilution; Dako). Membranes were developed by using enhanced chemiluminescence (ECL[®]; Amersham Biosciences) according to the manufacturer's instructions.

Samples were applied to nitrocellulose by vacuum dot blotting and developed as for Western blots.

Monosaccharide analysis

Neutral and amino monosaccharides were released by hydrolysis with 100 μl of 2 M HCl for 2 h at 100 °C, and sialic acids were released from the protein with 100 μl of 0.1 M HCl for 1 h at 70 °C. The samples were dried and washed three times with 100 μl of water prior to analysis by high-pH anion-exchange chromatography-pulsed amperometric detection (HPAEC-PAD) [27]. HPAEC-PAD of neutral and amino monosaccharides was carried out using a CarboPac PA1 column (4 mm × 250 mm; Dionex, Camberley, Surrey, U.K.) fitted with amino trap [28] and borate trap guard columns [29]. Sialic acids were analysed with a CarboPac PA1 guard column and a borate trap guard column. Neutral and amino monosaccharides were eluted in 2% 50 mM NaOH/1.5 mM sodium acetate and 98% H₂O for 30 min, followed by regeneration in 100 mM NaOH for 10 min; 300 mM NaOH was added as a post-column reagent to increase sensitivity and baseline stability. Detection was with a PED-2 detector (Dionex). Potentials for the gold electrode were as follows: E₁ = 0.1 V; E₂ = 0.7 V; and E₃ = -0.3 V. Sialic acids were eluted in a gradient from 100 mM NaOH to 300 mM sodium acetate/100 mM NaOH in 30 min, followed by regeneration in 100 mM NaOH for 10 min. All neutral and amino monosaccharides samples were analysed with 2-deoxyglucose added as a reference peak after hydrolysis and quantified against hydrolysed monosaccharide mixtures.

Oligosaccharide release

Portions of affinity-purified 5T4 were desalted further and concentrated on Microcon 10 centrifugal concentrators (Millipore). 5T4 glycoprotein was denatured by boiling for 2 min in 200 μl of a solution containing 1% (w/v) SDS, 0.5% 2-mercaptoethanol, 20 mM Na₂HPO₄, 50 mM EDTA and 0.02% sodium azide (pH 7.5). To this, 2 units of peptide N-glycosidase F (PNGase F; Oxford GlycoSciences, Abingdon, Oxon., U.K.) and 10 μl of 10% (v/v) Nonidet P40 were added prior to incubation for 72 h at 37 °C. The digested glycoproteins were centrifuged briefly at 13000 g and transferred to Microcon 10 centrifuge concentrators, pre-spun with 500 μl of water to remove glycerol. The samples were centrifuged at 13000 g for 20 min and the retentate was washed with a further three times with 100 μl of water. The retained material was freeze-dried and assayed for monosaccharides by HPAEC-PAD as described above. The filtrate and washes were pooled, concentrated and purified on a Biogel P2 column (2 ml bed volume; Bio-Rad, Hemel Hempstead, Herts., U.K.). Glycans were eluted in the void volume.

Fluorescence labelling of oligosaccharides

Oligosaccharides released from 500 μg–2 mg of 5T4 were labelled with 2-aminobenzamide (2-AB) (Oxford GlycoSciences) as described by Bigge et al. [30]. Briefly, the oligosaccharides were suspended in 5 μl of a solution containing 0.35 M 2-AB, 0.1 M sodium cyanoborohydride and 30% (v/v) acetic acid in DMSO, and incubated for 2 h at 60 °C. Labelled oligosaccharides were purified on a cellulose membrane (supplied with labelling kit) by eluting with decreasing proportions of acetonitrile, and the

samples were freeze-dried. Samples for GlycoSep C HPLC were then analysed directly. Following labelling and Biogel P4 (Bio-Rad) chromatography, neutral oligosaccharides, either naturally occurring or produced by mild acid hydrolysis to remove sialic acid, were then desalted further on a 1 ml mixed bed anion-exchange column of AG1 (triethylamine form) and AG50 in water.

GlycoSep C HPLC

Fluorescently labelled oligosaccharides were fractionated on the basis of charge by chromatography on a GlycoSep C HPLC column (4.6 mm × 100 mm; Oxford GlycoSciences) [31]. Oligosaccharides were eluted with a gradient of 20% acetonitrile/80% water to 20% acetonitrile/30% water/50% 0.3 M ammonium acetate (pH 4.5) over 40 min at a flow rate of 0.4 ml/min, and detected by fluorescence at 330 nm and 420 nm for excitation and emission respectively.

Biogel P4 chromatography

Analytical Biogel P4 chromatography was performed using a RAAM 2000 GlycoSequencer (Oxford GlycoSciences) at a temperature of 55 °C in water at a flow rate of 30 µl/min for 11 ml, ramped to 160 µl/min over 27 ml and held for 7 ml. Detection was by fluorescence at 330 nm and 420 nm for excitation and emission respectively. Data were analysed by the Glyco-link software supplied. Preparative Biogel P4 chromatography was carried out on the same instrument using a constant flow program (45 µl/min). Assignment of glucose units (GU) was made by comparison with a dextran hydrolysate run either concurrently (analytical runs) or separately (preparative runs) and detected by refractive-index detection. Peak assignments were achieved using comparison with the New-Bio2 database of the Eve software supplied with the RAAM 2000 instrument and relating these to the results of the monosaccharide analysis and GlycoSep C.

Glycopeptide mapping

5T4 was desalted as described above and then reduced and carboxymethylated. Trypsin digestion was performed for 5 h at 37 °C in 50 mM ammonium bicarbonate buffer (pH 8) using a 1:100 (w/w) 5T4/enzyme ratio. After freeze-drying, the digest was dissolved in 20 µl of buffer A [0.1% aq. trifluoroacetic acid]. Peptides were separated by HPLC on a graphitized carbon column (HyperCarb, 4.6 mm × 100 mm; Thermo Hypersil, Run-corn, Cheshire, U.K.) using a gradient of 2% buffer B (0.1% trifluoroacetic acid in acetonitrile) in buffer A for 2 min, followed by a linear gradient to 82% buffer B over 80 min at 1 ml/min. The order of elution of the peptides was predicted from their hydrophobicity index [32]. Fractions (1 ml) were collected over 80 min and a sample was added to the wells of a microtitre plate. This was followed by 25 µl of 4% aq. phenol and, after 5 min, 200 µl of concentrated sulphuric acid was added. The absorbance was read at 490 nm and compared with a hexose standard curve. Oligosaccharides from selected fractions were released with 0.01 unit of PNGase F in 40 mM potassium phosphate buffer (pH 7.4) containing 10 mM EDTA for 48 h at 37 °C. The oligosaccharides were desalted, desialylated and fluorescently labelled as described above, prior to analysis by Biogel P4 chromatography.

RESULTS

Epitope mapping

To investigate the contribution of different regions of the 5T4 molecule in the presentation of the epitope for mAb5T4, a number of deletion and mutation variant molecules were constructed (see Figure 1). These were cloned into the vector pCMVα neo and expression was assessed in various stable and transiently transfected mammalian cell lines by RT-PCR, SDS/PAGE and Western blotting, dot blotting and FACS analysis, as summarized in Table 1. All constructs were tested for expression of the mAb5T4 epitope in at least two cell lines.

Deglycosylation of 5T4 leaves a 42 kDa core protein [21], with removal of the carbohydrate by either chemical or enzymic means leading to a loss of mAb5T4 reactivity, but the core protein is still recognized by polyclonal sera. Additionally, disruption of the intramolecular disulphide bonds leads to a loss of mAb5T4 reactivity (Figure 2); these data are consistent with the 5T4 epitope being conformational in nature.

Table 1 Expression of the monoclonal 5T4 epitope in variant 5T4 molecules

The presence (+) of the mAb5T4 epitope was determined at the cell surface by FACS, and in cell lysates or culture supernatants by Western and dot blotting. The presence (+) of mRNA was confirmed by RT-PCR. nt, not tested; —, absence.

5T4 construct	RT-PCR	FACS (cell surface)	Western blot (kDa)		
			Cell lysates	Supernatant	Dot blot
Full-length	+	+	72	72	+
5T4-Cyt ⁻	+	+	67	67	+
5T4-Y to F	+	+	72	nt	+
5T4-N to F	+	+	69	69	+
5T4/CD44	+	+	80	nt	nt
5T4-TM ⁻	+	—	nt	64	—
5T4-IgGfc	+	—	nt	92	+
5T4-L1	+	—	—	—	—
5T4-L1-H	+	—	—	—	—
H/M 5T4 chimaera	+	—*	—*	nt	—
M/H 5T4 chimaera	+	+	75	nt	nt

* Human/mouse chimaera positive with polyclonal sera against both species.

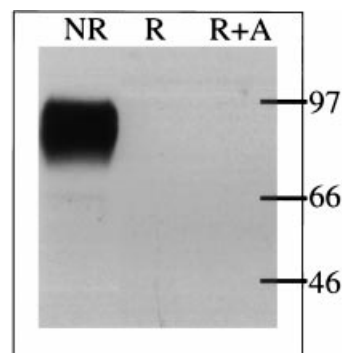


Figure 2 Reduction and alkylation of full-length 5T4

Lysates from A9 cells expressing 5T4 were separated by SDS/PAGE [10% (w/v) gel] under non-reducing (NR), reducing (R) or reducing and alkylating conditions (R + A). Western blots were probed with mAb5T4 (1 µg/ml), biotinylated rabbit anti-mouse immunoglobulins (1:3000 dilution) and SHRP (1:6000 dilution). Blots were developed using ECL[®]. The positions of molecular-mass markers (in kDa) are indicated on the right.

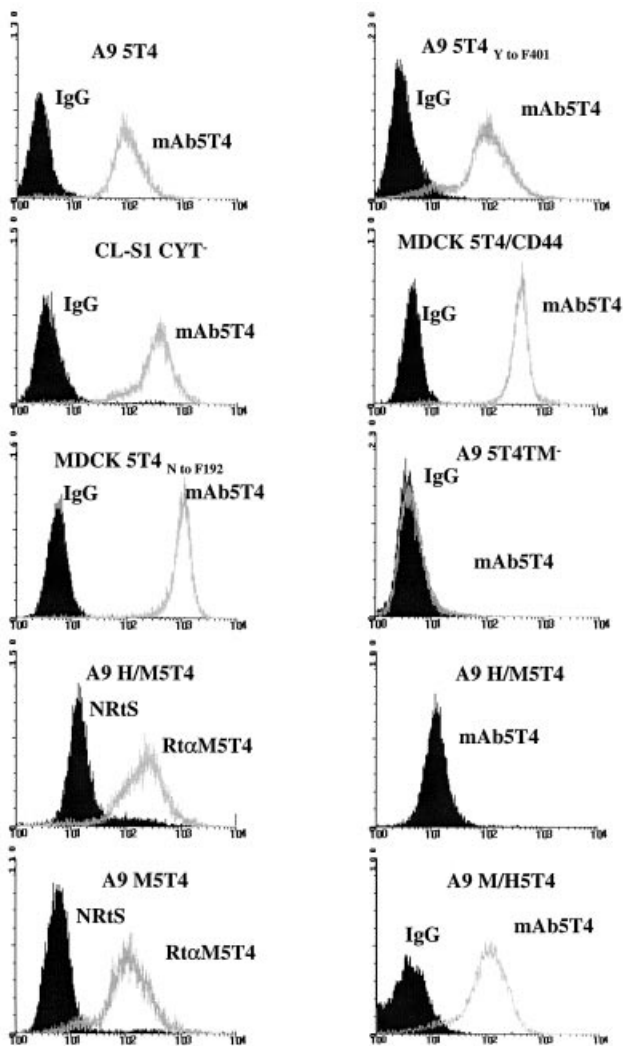


Figure 3 FACS profiles of variant human 5T4 transfectant cell lines

Cells [A9, CL-S1 and Madin–Darby canine kidney (MDCK); 2×10^5] were labelled using mAb5T4 or mouse IgG₁ (IgG) at 1 μ g/ml, followed by FITC-conjugated rabbit F(ab)₂ anti-mouse immunoglobulins (1:40 dilution). Wild-type 5T4, 5T4-N to F (5T4_{NtoF192}) and 5T4-Y to F (5T4_{YtoF401}) site-directed mutants, 5T4-CYT⁻, 5T4/CD44 and human/mouse chimeric 5T4s (H/M5T4 and M/H5T4) were labelled with mAb5T4. Mouse 5T4 (M5T4) and human/mouse chimeric 5T4 (H/M5T4) were labelled using rat anti-(mouse 5T4) (RtaM5T4) or normal rat serum (NRtS) at a 1:300 dilution, followed by FITC-conjugated rabbit F(ab)₂ anti-rat immunoglobulins (1:40 dilution).

Human 5T4 variants predicted to be expressed at the cell surface were assessed by FACS using mAb5T4 or an isotype-matched control. The full-length wild-type 5T4 stained with mAb5T4, but not the control antibody (Figure 3); a neo control transfectant was negative with both antibodies (results not shown). Mutation of the putative tyrosine phosphorylation site in the cytoplasmic tail of 5T4 to phenylalanine (5T4-Y to F) did not alter the expression of the epitope at the cell surface, nor did removal of the entire cytoplasmic domain (5T4-CYT⁻). A 5T4/CD44 chimaera, comprising the extracellular domain of 5T4 and transmembrane and cytoplasmic domains of CD44, also expressed the mAb5T4 epitope. Together, these data show that there is no ‘inside-out’ influence on 5T4 conformation mediated specifically by the 5T4 transmembrane or cytoplasmic domains.

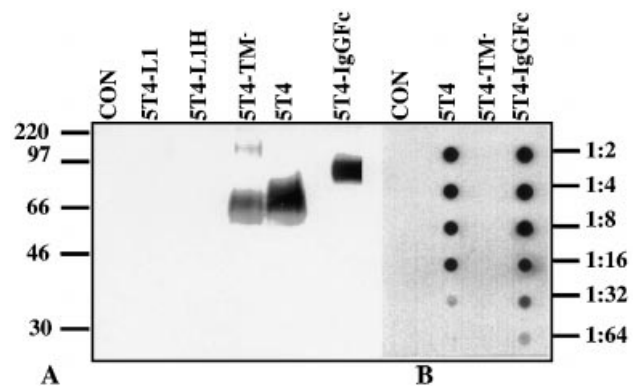


Figure 4 Western and dot blots of key 5T4 variant constructs

(A) Full-length 5T4 and H12 (CON), 5T4-TM⁻, 5T4-IgGFc, 5T4-L1 and 5T4-L1H, expressed in A9 cells, were analysed by Western blotting. The position of molecular-mass markers (in kDa) is indicated on the left. (B) Full-length 5T4 and H12 (CON), 5T4-TM⁻ and 5T4-IgGFc were serially doubly diluted (1:2–1:64) and analysed by dot blotting. Both Western and dot blots were probed with mAb5T4 (1 μ g/ml), biotinylated rabbit anti-mouse immunoglobulins (1:3000 dilution) and SHRP (1:6000 dilution). Blots were developed using ECL[®].

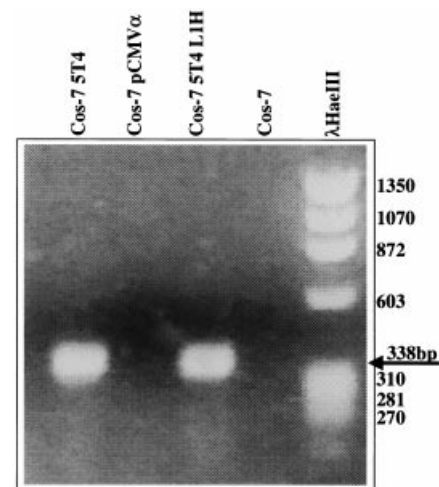


Figure 5 RT-PCR of variant 5T4 transient expression in Cos-7 cells

RT-PCR products were run on a 1% (w/v) agarose gel. Full-length 5T4 and 5T4-L1H contained the 5T4-specific band of 338 bp, whereas Cos-7 and a neo transfectant control (Cos-7 pCMV α) did not. The position of the markers (λ HaeIII) is indicated on the right.

Expression of the monoclonal 5T4 epitope was not detected in the supernatant or lysate from stable transfectants of the two extracellular deletion constructs 5T4-L1 and 5T4-L1H by either Western (Figure 4A) or dot blot (results not shown). Transient expression, over 48 h, of full-length 5T4 and 5T4-L1 and 5T4-L1H cDNAs in Cos-7 cells confirmed expression of the full-length molecule at the cell surface (results not shown), but neither deletion construct could be detected using mAb5T4. The presence of 5T4 mRNA in all of the 5T4 transfectants was shown by RT-PCR using primers specific for the L1 region (LRR1) of the gene, yielding a 338 bp 5T4 band (Figure 5).

The transmembrane deletion construct (5T4 TM⁻) was not detected at the cell surface, but was detected using mAb5T4 in

the supernatants of transfected cells as a 64–67 kDa band by Western blot (Figure 4A). However, it could not be detected in the same samples, or 20 times concentrates thereof, by dot blot (Figure 4B). A fusion protein comprising the extracellular domain of human 5T4 and human IgGFc (5T4-IgGFc) was detected by both Western and dot blotting techniques. Removal of the Fc domain by protease Xa cleavage and purification on Protein G resulted in a product essentially identical with the 5T4-TM⁻ construct, which retained no human Fc immunoreactivity. However, unlike 5T4-TM⁻, this molecule was recognized by mAb5T4 by dot-blot analysis (Table 1).

Murine 5T4 is not recognized by the monoclonal antibody to the human molecule, but can be labelled at the cell surface by polyclonal anti-mouse 5T4 sera in transfected (Figure 3) and naturally expressing cells. A difference in sequence within the hydrophilic domain, where an additional six amino acids are inserted adjacent to the N-glycosylation site in mouse 5T4, suggested that this site might be important in the recognition of human 5T4 by mAb5T4. However, mutation of Asn¹⁹² within this region to phenylalanine (5T4-N to F), deleting the potential glycosylation site, did not affect mAb5T4 binding by FACS analysis, Western or dot blotting. The size of product recognized by mAb5T4 in Western blot was approx. 3–5 kDa smaller than full-length 5T4, confirming that this site is normally glycosylated (Table 1).

A human/mouse 5T4 chimaera (H/M 5T4) was made by replacing the first LRR, LRR1, of mouse 5T4 with that of the human molecule. This construct was not recognized by mAb5T4 by either FACS or Western blotting, but the molecule was detected at the cell surface with polyclonal sera specific for both human and mouse 5T4. The reciprocal mouse/human 5T4 chimaera (M/H 5T4) was shown to express the mAb5T4 epitope, as it was detected at the cell surface of transfected cells by FACS (Figure 3) and in lysates by Western blot.

Thus these data show that the mAb5T4-recognized epitope is dependent solely on the extracellular domain of the molecule, and it is possible to positively assign the epitope to the membrane-proximal LRR2. The 5T4 molecule has been shown to be highly glycosylated, probably exclusively through N-linked structures. The extent and complexity of the glycosylation of the seven potential sites in the extracellular domain was investigated in trophoblast-derived 5T4 molecules.

Carbohydrate studies

The initial extraction of nine full-term placentae yielded 12.95 g of StMPM. Purification of 5T4 from StMPM by lectin affinity chromatography on a WGA column and mAb5T4 column yielded pure 5T4 in 81 1 ml fractions. Two representative fractions were assayed by SDS/PAGE under non-reducing conditions to confirm the isolation of 5T4. The major band eluted at 72 kDa for both fractions, confirming the isolation of the 5T4 antigen. Higher-molecular-mass bands seen in the non-reducing gels include multimeric forms of 5T4, as they were not observed in reducing gels and drop out at 72 kDa in non-reduced/reduced two-dimensional gels (results not shown). Bradford assay revealed that 400 µg of 5T4 antigen had been purified, although this value must be regarded as approximate, due to the lack of quantified 5T4 standards with which to build a calibration curve.

Monosaccharide analysis by HPAEC-PAD revealed the presence of fucose, galactose, glucosamine, mannose and *N*-acetylneuraminic acid (Table 2). The absence of galactosamine in 5T4 confirms the absence of oligosaccharides linked via GalNAc 1-O-Ser/Thr [21]. Oligosaccharides were therefore released from 5T4

Table 2 Monosaccharide compositional analysis of 5T4 before and after PNGase F treatment

Quantification was performed by comparison with a mixture of hydrolysed monosaccharides prepared simultaneously with the glycoprotein hydrolysates. 2-Deoxyglucose was added to each sample prior to injection as a reference peak. nt, not tested.

Sample	Monosaccharide	[Monosaccharide] (nmol)	Monosaccharide/5T4 ratio
5T4 before PNGase F	Fucose	0.04	negligible
	Galactose	16.69	4.62
	Glucosamine	25.67	7.11
	Mannose	10.83	3.00
	<i>N</i> -Acetylneuraminic acid	5.64	1.56
5T4 after PNGase F	Fucose	0	0
	Galactose	2.25	4.00
	Glucosamine	2.98	5.40
	Mannose	1.67	3.0
	<i>N</i> -Acetylneuraminic acid	nt	–

by PNGase F treatment under reducing conditions and purified by centrifugation through 10 kDa molecular-mass-cut-off filters and Biogel P2 chromatography. In addition to releasing the oligosaccharides from untreated 5T4, the oligosaccharides were released from a sample of 5T4 that had been mildly acid hydrolysed to generate a library of neutral oligosaccharides. The efficacy of enzymic release was shown to be 87% by performing HPAEC-PAD monosaccharide compositional analyses on the PNGase F-treated 5T4 protein (Table 2).

Fluorescently labelled oligosaccharides released from 5T4 and purified into charged groups by GlycoSep C HPLC were shown to have one to four (a1–a4) sialic acid residues. Neutral oligosaccharides were collected in the void volume (Figure 6A). The latter were purified further by preparative Biogel P4 chromatography (Figure 6B). Calculation of GU by comparison with an external dextran standard showed an oligosaccharide of approximately a dodecasaccharide, probably a triantennary complex-type chain with core fucosylation (peak 4) and Man₆-type high-mannose chains (peak 2). In addition, the presence of small amounts of Man₅ and Man₇ high-mannose chains was shown (peaks 1 and 3 respectively).

The neutral glycan library obtained by PNGase F digestion of 5T4 after mild acid hydrolysis was analysed by Biogel P4 chromatography (Figure 6C) after fluorescent labelling. Calibration of the resulting peaks with respect to a dextran hydrolysate, detected by refractive index run simultaneously, allows the description of the N-glycans in terms of GU. Comparison of these values with a database of N-linked structures and their GU, coupled with the monosaccharide compositional data (Table 2) and peak area ratios, allowed assignment of the glycan structures as approximately the follows: two high-mannose oligosaccharides of the Man₆-type (with minor amounts of other high-mannose varieties); one each of triantennary oligosaccharides with (compared with Figure 6B) and without core fucose; one each of tetra-antennary oligosaccharides with and without core fucose; and an additional biantennary chain with 50% core fucosylation. The monosaccharide ratios for mannose: galactose were consistent with this ratio of chains.

Other interpretations of the GU data are possible due to the great potential in variation of different fucosylation, branching patterns, bisecting *N*-acetylglucosamine and hybrid chains etc. [33]. The amount of sialylation found (Table 2 and Figure 6) was

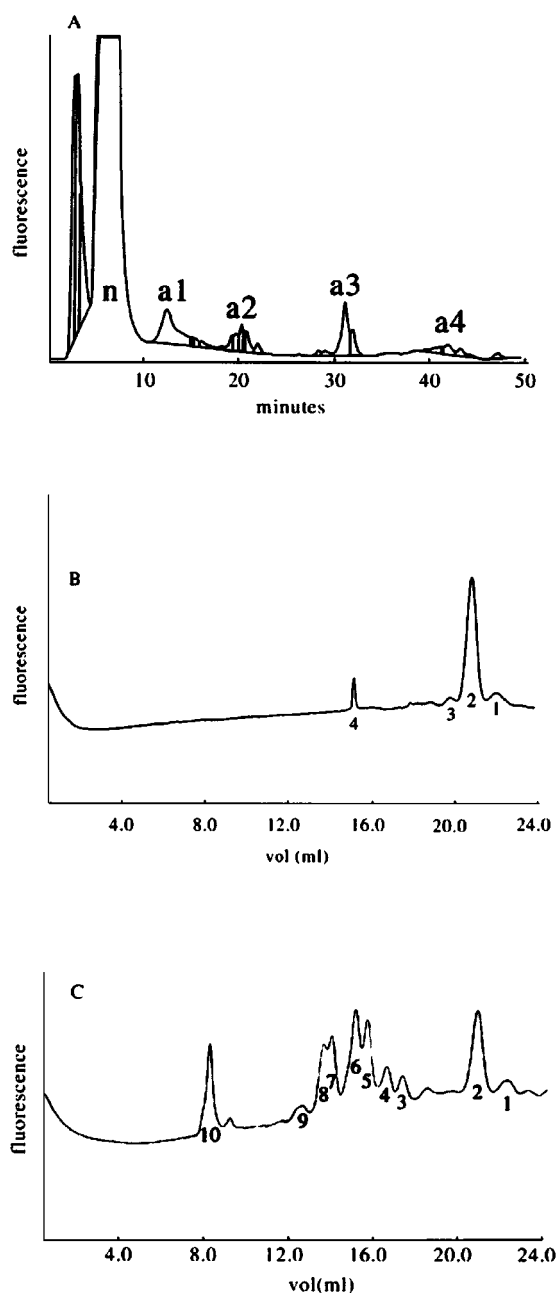


Figure 6 Chromatographic analysis of 5T4 carbohydrate structures

(A) GlycoSep C HPLC of neutral 2-AB-labelled oligosaccharides released from 5T4 by PNGase F calibrated against a dextran hydrolysate. Fractions n and a1–a4 represent neutral and mono- to tetra-sialylated structures. (B) Biogel P4 chromatography of the neutral fraction (n) from (A). Peaks were as follows: 1, Man₅ (6.6 GU); 2, Man₆ (7.6 GU); 3, Man₇ (8.6 GU); and 4, triantennary complex chain (14.4 GU), where GU was calibrated against a dextran hydrolysate. (C) Biogel P4 chromatography of the oligosaccharide pool represented in (A) after the release of sialic acid. Peak assignments were made by comparison with a structural database (Eve New-Bio2; Oxford GlycoSciences), with calibration against a dextran hydrolysate, and from the monosaccharide compositional data as follows: 1, Man₅ (6.4 GU); 2, Man₆ (7.5 GU); 3, biantennary (11.2 GU); 4, biantennary + core fucose (12.2 GU); 5, triantennary (13.5 GU); 6, triantennary + core fucose (14.5 GU); 7, tetra-antennary (16.5 GU); 8, tetra-antennary + core fucose (17.3 GU); 9, penta-antennary + core fucose (19.7 GU); and 10, unidentified.

consistent with mainly complex rather than hybrid chains. As Biogel P4 is primarily a sizing column, we can be sure of the extent of the hydrodynamic mass of the oligosaccharides if

Table 3 Position of carbohydrate chains within the 5T4 sequence

Compositions of the carbohydrate structures at each of the seven N-linked glycosylation sites (N) in the extracellular domain of 5T4 were determined.

HPLC fraction	Peptide	Carbohydrate structure
11–12	81- N LTEVPTDL	Penta-antennary + N-acetylglucosamine
14–16	256- N LTHLESLHLED	Penta-antennary + fucose
25–31	116-PLAELAAL N LSGSRL	High-mannose or penta-antennary ± fucose
25–31	238-HLDLS N NLSVSL	
25–31	267-HLEDNALKVLH N GT	
67–70	166- M ASVSAPS ...	Bi-, tri- or tetra-antennary + fucose
67–70	183-IVPPEDERQ N RS	

information is also known about the occupancy of the N-glycosylation sites. Further information on the latter can be gained by analysis of glycopeptides and peptides from the tryptic digests fractionated by relative hydrophobicity on reverse-phase HPLC. Their predicted cleavage sites and hydrophobicity [32] can be used to assign them to the carbohydrate-containing fractions, as determined by hexose content (Table 3). The results for 5T4 showed that the most hydrophobic peptides having N-glycosylation sites were QNR(S), followed by NLTEVPTDL-PAYVR (Asn¹⁹² and Asn⁸¹ respectively; where single-letter amino-acid notation has been used). These were assigned to the early eluting hexose-positive peaks, which were shown after PNGase F treatment, mild acid hydrolysis, fluorescence labelling and Biogel P4 analysis to have only high-mannose chains.

The largest hexose-containing peak eluted next on the reverse-phase HPLC and was shown to contain predominantly biantennary chains with and without fucose, together with high-mannose chains. From the amount of hexose and the hydrophobicity read-out, it was likely that this would contain the glycopeptides NLTHLESLHLEDNALK, HLDLSNNSLVSL-TYVSFR, VLHNGTLAELQGLPHIR, RPPLAELAALNLS-GSR (where single-letter amino-acid notation has been used), corresponding to amino acid residues 256–271, 238–255, 272–288 and 114–129, all of which are very close in hydrophobicity. This identified complex N-glycosylation at all, or one or more, of the Asn¹²⁴, Asn²⁴³, Asn²⁵⁶ and Asn²⁷⁵ sites.

Hexose was spread thinly over the later fractions of the reverse-phase HPLC and was available in two small amounts for adequate analysis by Biogel P4, but from the hydrophobicity read-out and the above results on mono/oligosaccharide composition, we would predict that they will contain other glycopeptides containing Asn¹²⁴, Asn²⁴³, Asn²⁵⁶ and Asn²⁷⁵ with different peptide cleavage sites [34,35] and the tri-, tetra- and penta-antennary or equivalent size chains discussed above.

There were few trypsin cleavage sites around the seventh N-glycosylation site at Asn¹¹⁶, and therefore it was predicted that glycopeptides containing this site were very large and with much greater hydrophobicity. A late-running cluster of hexose-positive peaks in the reverse-phase HPLC was consistent with these large glycopeptides, and Biogel P4 analysis gave some indication of oligosaccharides of tri- and tetra-antennary size.

DISCUSSION

MAb5T4 is a high-affinity monoclonal antibody that recognizes the oncofoetal antigen 5T4 [2,25]. The restricted expression of this molecule in normal adult tissue and its more widespread expression in malignancy means that it is a suitable vector for the development of antibody-targeted tumour therapy. Strategies using 5T4 specificity on the basis of Fab fragments and single-

chain antibodies to deliver *Staphylococcus aureus* superantigen ('SEA') and other immunological response modifiers to the site of a tumour are under development [25,36]. A better understanding of the nature of the 5T4 antigen, which is dependent on the appropriate folding and its carbohydrate structure, is important in optimizing these approaches.

The epitope for mAb5T4 is conformational in nature, being dependent on both glycosylation and the presence of intact intramolecular disulphide bonds. Despite human and mouse 5T4 sharing 81% identity and a conserved domain structure [22], the monoclonal against the human molecule does not cross-react with the mouse. Comparison of the sequences highlights only two significant differences between the two species, as discussed further below.

We have shown that the 5T4 epitope lies entirely within the extracellular domain, as both the 5T4-TM⁻ construct and 5T4-IgGFc fusion protein, which include only the extracellular domain of 5T4, were recognized by mAb5T4 by Western blot. Further analysis of the soluble molecule, 5T4-TM⁻, has given some insight into the conformational nature of the 5T4 epitope. Although recognized by Western blot, the construct was not detectable by dot blot. In contrast, the fusion protein, 5T4-IgGFc, was recognized in both Western and dot blots. Moreover, proteolytic removal of the Fc yields a protein essentially identical with the 5T4-TM⁻ construct, but that retains the 5T4 epitope by dot blot. Taken together, these results suggest that the most thermodynamically stable conformation of the truncated molecule, when newly synthesized, is one in which the epitope is obscured. Furthermore, the denaturation and subsequent refolding of the mature fully glycosylated molecule during SDS/PAGE and Western blotting leads to the molecule assuming its correct conformation, thus exposing the epitope. In addition, the influence of the transmembrane domain on the correct folding of the protein can be substituted by other sequences, here immunoglobulin Fc, and once this folding has been imprinted on the molecule during translation, it remains stable when the additional sequence is removed.

The extracellular domain deletion constructs, 5T4-L1 and 5T4-L1H, were not expressed at the cell surface and could not be detected in culture supernatants by either Western or dot blotting. Although these constructs were transcribed, the proteins were either not translated, unstable, or did not present the 5T4 epitope through either misfolding or its being located within another region of the extracellular domain.

Analysis of the two human/mouse chimaeric 5T4 constructs indicates that the epitope lies within the more membrane-proximal region of human 5T4. The lack of effect upon expression of the epitope by the removal of the glycosylation site within the hydrophilic domain indicates that the epitope must reside in either LRR2 or its C-terminal flanking domain.

The remaining challenge is to determine which of the carbohydrates are likely to contribute to the final structure and what role, if any, they play in the function of 5T4. Co-translational addition *en bloc* of the core glycan to nascent polypeptides confers stability and regulates folding both directly and through interaction with lectin chaperones [37]. These in turn are involved in the sorting through the Golgi apparatus of the correctly processed mature protein. Incorrect glycan processing may in turn lead to an inability to interact correctly with protein disulphide-isomerase and a failure of disulphide-bond formation [38].

From the studies presented in this report, high-mannose chains predicted at Asn¹⁹² in the hydrophilic domain do not appear to be necessary for presentation of the epitope. This is one of the two sites which differ significantly in mouse and human; the

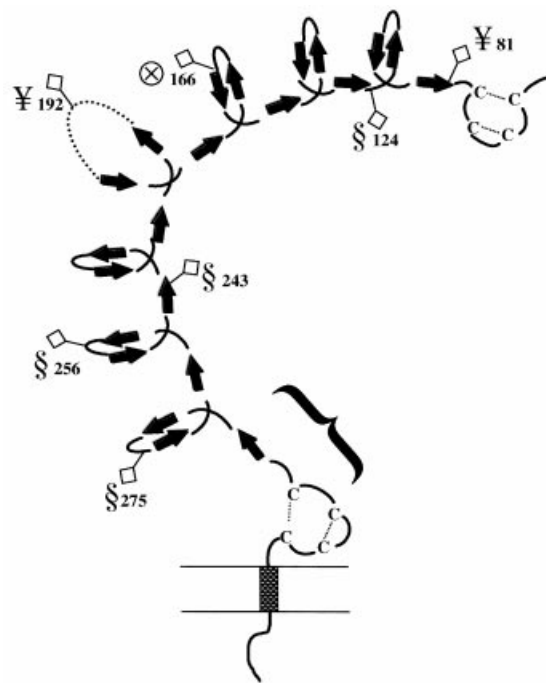


Figure 7 Cartoon diagram of 5T4 structure

Deduced position of the carbohydrate chains and proposed location of the mAb5T4 epitope are indicated. □, asparagine residues; Y, high-mannose; §, biantennary (with and without fucose) and larger complex oligosaccharides; }, mAb5T4 epitope; hatched box, transmembrane domain; dotted line, hydrophilic sequence; ⊗, large sialated complex oligosaccharides. Disulphide bonds (C·····C) are also indicated.

other is the putative high-mannose occupying site at Asn⁸¹, which is absent in the mouse. The mouse has an extra N-glycosylation consensus sequence in the C-terminal membrane-proximal region. Other differences in protein sequence are slight, the only other non-conservative amino acid differences being an increase in acidity in the mouse, with aspartic acid replacing human Phe⁴⁷ and human Gly³¹⁷ and glutamic acid replacing human Val¹⁷⁶. There are some changes in the position of the basic amino acid arginine in residues 50–250 (human numbering), but no differences in number. Thus the major differences between the mouse and the human are likely to be found in the structure of the oligosaccharides. No O-glycosylation was found in the present study in placental 5T4, but two regions of the amino acid sequence in both mouse and human show several close serine and proline residues, which are indicators of potential O-glycosylation sites. One of these is near the N-terminus of the human before the cysteine-rich region and the second leads up to the hydrophilic loop. The complex oligosaccharides discussed in the present study (Figure 7) are candidates for carrying oligosaccharide-recognition motifs, which may vary with different sources of 5T4. Oligosaccharide sequences are well known as tumour-associated antigens and with functions in cell–cell interactions [39]. 5T4 appears to influence cellular motility and organization of the cytoskeleton. This function is dependent on the cytoplasmic domain and may be mediated through a recently identified cytoplasmic-interacting protein [40]. The conformation of the extracellular domain may influence its interaction with other extracellular molecules, either at the plasma membrane or in the extracellular matrix, which may in turn modulate the effects of 5T4 on the cells. From our present results, the site at Asn¹⁶⁶ is

likely to be glycosylated with relatively large sialylated oligosaccharides. This and glycosylation at Asn²⁵⁶ and Asn²⁷⁵, the sites of which are within the LRRs (Figure 7), are most likely to be important in overall structure and antigenicity

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