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Antibody recognition of epitopes on wild-type and mutant β-(1→4)-galactosyltransferase-1

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Abstract

The epitopes present on β-(1→4)-galactosyltransferase-1 (β4Gal-T1) have been explored using a panel of monoclonal antibodies (mAbs) raised against the soluble form of the human enzyme. Reactivity of the antibodies with site-specific and truncated mutants of human β4Gal-T1 suggests the presence of a major immunogenic epitope cluster consisting of four epitopes within the stem region and mapping between amino acids 42 and 115. The catalytic activity of the enzyme is increased in the presence of stem region-specific antibody. Two of the epitopes were further localized to a region between amino acids 42 and 77, sequences which are not shared with the recently cloned β4Gal-T2 and β4Gal-T3 enzymes. An epitope located close to or within the catalytic domain is also identified, and the mAb to this region binds synergistically with antibodies to the stem region.

Keywords: Galactosyltransferase; Monoclonal antibodies; Golgi; Surface plasmon resonance; Glycosyltransferase; Synergistic antibody binding

1. Introduction

UDP-galactose:β-*N*-acetylglucosamine β-(1→4)-galactosyltransferase (β4Gal-T, EC 2.4.1.38/90), one of a large number of glycosyltransferases involved in the biosynthesis of glycoconjugates, catalyses the transfer of galactose from UDP-galactose to non-reducing terminal *N*-acetylglucosamine (GlcNAc). The C-terminal globular catalytic domain of the enzyme is exposed to the lumen of the Golgi apparatus, this is followed by an extended stem region, a hydrophobic transmembrane domain and a short cytoplasmic tail. Human β4Gal-T1 cDNA has been expressed in a variety of prokaryotic and eukaryotic systems including large-scale production in *Saccharomyces cerevisiae*, with the recombinant enzyme showing similar characteristics

Abbreviations: β4Gal-T; UDP-galactose:β-*N*-acetylglucosamine β-(1→4)-galactosyltransferase (β4Gal-T, EC 2.4.1.38/90); rhβ4Gal-T1, recombinant human β4Gal-T1; mAbs, monoclonal antibodies; GlcNAc, *N*-acetylglucosamine; PNPP, *p*-nitrophenyl phosphate; SPR, surface plasmon resonance

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to the human milk β4Gal-T [1–3]. Studies on β4Gal-T1 gene knock-outs in mice suggested the existence of other β4Gal-T genes [4,5], and additional human β4Gal-T enzymes (designated β4Gal-T2, β4Gal-T3 and β4Gal-T4) have very recently been cloned [6,7], although the fine specificity of each enzyme remains to be fully established. These developments suggest that much of the previously published data using or analyzing non-recombinant enzyme requires cautious interpretation, and to reflect this in the present publication we use the term β4Gal-T1 only when referring to recombinant enzyme or information derived from nucleic acid-based analyses, otherwise the more general term β4Gal-T is used.

There is a single N-linked glycosylation consensus sequence in human β4Gal-T1. O-linked sugars have also been described in human milk β4Gal-T, with blood group determinants expressed in accord with the blood types of the donors [8]. The function of the carbohydrates on β4Gal-T is unknown. They may serve in the correct folding of β4Gal-T1, as a lower expression was achieved with the non-glycosylated β4Gal-T1 compared to its glycosylated form [3]. However, the carbohydrate moiety on β4Gal-T has been shown not to be involved in its catalytic activity [9–11] or in the binding of the α-lactalbumin regulator protein [12] which modifies the enzyme to produce lactose synthase activity (EC 2.4.1.22).

The β4Gal-T enzyme has been localized predominantly to the *trans* Golgi [13]. However, in some cells the β4Gal-T distribution has been found to overlap into the *trans* Golgi network [14]. The soluble form of β4Gal-T is derived from the membrane-bound enzyme by proteolytic cleavage [15,16]. Cleavage sites in human milk β4Gal-T1 occur at amino acid 42 in the transmembrane/stem region junction [17] and at amino acid 77 in the stem region [18]. A soluble form of another galactosyltransferase, α3Gal-T, has recently been shown to be functionally active inside the Golgi [19].

β4Gal-T has also been detected on the plasma membrane of certain cell types using biochemical and immunodetection techniques [20,21]. It is unlikely that either cell surface β4Gal-T or extracellular soluble β4Gal-T galactosylates structures in vivo as sufficient concentrations of UDP-galactose are unlikely to exist in the extracellular fluid. However, the β4Gal-T has the potential to bind glycoconjugates terminating in GlcNAc and could therefore act as a lectin with other cell surface or cell matrix molecules bearing the appropriate ligand [22]. Suggested functional roles for cell surface β4Gal-T have included involvement in cell migration [20,23], developmental growth [24], neurite outgrowth [25] and signal transduction [26].

The substrate binding sites of β4Gal-T1 are postulated to center around a hydrophobic pocket and include residues Tyr^{284}, Tyr^{309} and Trp^{310} involved in GlcNAc binding with Tyr^{309} additionally involved in the binding of UDP-galactose [10]. Further studies around this hydrophobic area identified Phe^{305}, Pro^{306}, Asn^{307} and Asn^{308} as contributing to the UDP-Gal binding site, though they did not affect the binding of Mn^{2+} [27].

In this study we have used four newly produced and characterized monoclonal antibodies (mAbs) recognizing human β4Gal-T1, together with a previously characterized mAb, to define immunogenic epitopes on the enzyme.

2. Results

Production and characterization of the mAbs.—All four of the cloned hybridomas produced specifically for this study secreted approximately 10–15 μg/mL specific antibody of the IgG1κ isotype and recognized both human and bovine β4Gal-T (Fig. 1). However, except for UCLgt1H11, the detection of reactivity with bovine β4Gal-T was only evident if the ELISA reaction was allowed to proceed overnight. This was still considered a valid reaction because non-specific binding observed with a isotype-matched control mAb remained at background levels. Each of the mAbs recognized bovine β4Gal-T to different extents but showed no reactivity with either IgG or *a*-lactalbumin [Fig. 1(b)], proteins which are often present in low amounts in commercially available β4Gal-T [28]. All the mAbs were also able to detect denatured human (Fig. 2) and bovine (not shown) β4Gal-T following SDS–PAGE and transfer to nitrocellulose membranes, recognizing a broad band between 47 and 54 kDa.

Influence of carbohydrate structures on antibody binding.—Each of the anti-β4Gal-T1 mAbs was able to detect non-glycosylated *E. coli*-expressed soluble rhβ4Gal-T1. Two major bands of 36 and 34 kDa were detected in Western blots in agreement with the size of the recombinant protein (not shown).

Endo H-mediated cleavage of the *N*-glycans from purified rhβ4Gal-T1 from *S. cerevisiae* [3]

resulted in a gel shift from a high molecular diffuse band (of approximately 100–200 kDa) to a major band at 51 kDa and two bands at 43 and 42 kDa [Fig. 2(a)]. Except for GT2/36/118, the anti-β4Gal-T1 mAbs reacted equally well with the rhβ4Gal-T1 before and after treatment with Endo H [Fig. 2(b), lanes 2 and 3], again suggesting that the carbohydrate structures were not involved in the epitopes recognized by these mAbs. Periodate oxidation of β4Gal-T also did not result in decreased binding of the mAb, confirming that the mAbs recognize protein epitopes (not shown). Binding of mAb GT2/36/118 to rhβ4Gal-T1 from *S. cerevisiae* is inhibited in the presence of the large *N*-glycans present on this material, but is able to bind following endo-H treatment of the enzyme. A number of low molecular weight bands (approximately 30 and 14.3 kDa) in the β4Gal-T preparations were detected only with the UCLgt1H11 mAb [Fig. 2(b)]. These were most likely degradation products of β4Gal-T still containing the epitope uniquely recognized by UCLgt1H11.

Cellular localization of the epitopes recognized.— Each of the mAbs gave a pattern of staining consistent with the Golgi localization of β4Gal-T using immunofluorescence on fixed and permeabilized B cells [Fig. 3(a)]. The staining intensity achieved was similar for UCLgt1B6, UCLgt1E7 and UCLgt5G4 mAbs but weaker with the UCLgt1H11 mAb, possibly indicating a less accessible epitope and/or poor binding affinity to β4Gal-T in situ. The over-

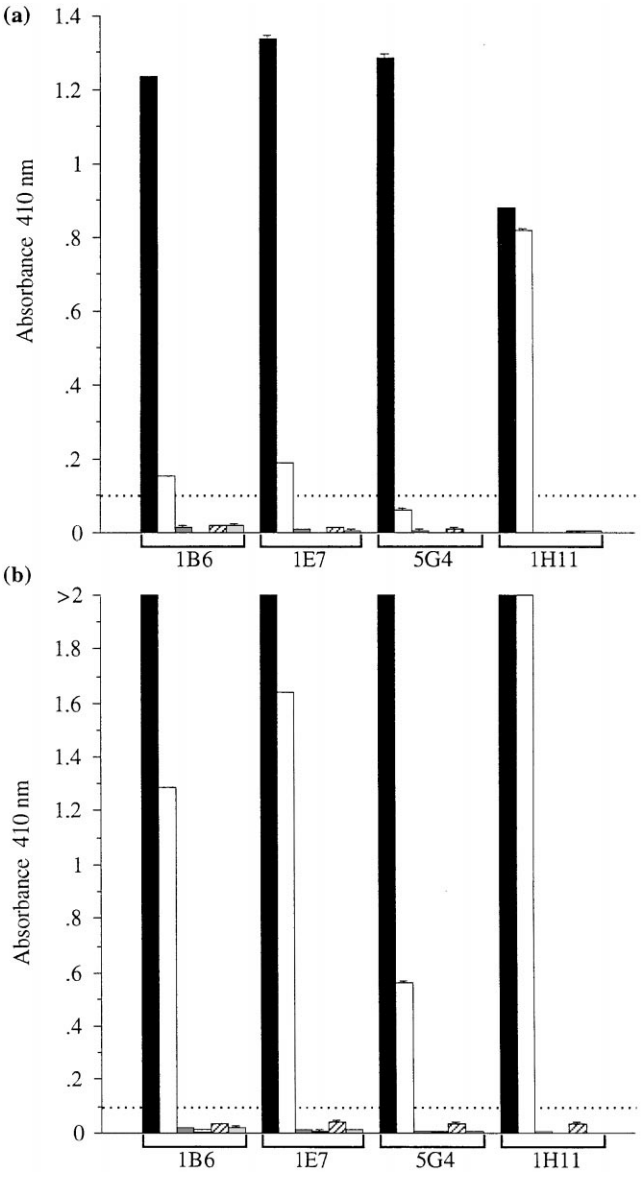

Fig. 1. Specificity of the anti-β4Gal-T1 mAbs. Human milk β4Gal-T (■); bovine milk β4Gal-T (□); human α-lactalbumin (■); bovine α-lactalbumin (▤); human IgG (▨) or BSA (▩) were coated onto ELISA plates at 5 μg/mL. The ELISA substrate reaction was allowed to proceed for (a) 1 h or (b) 16 h. One standard error of the mean is indicated.

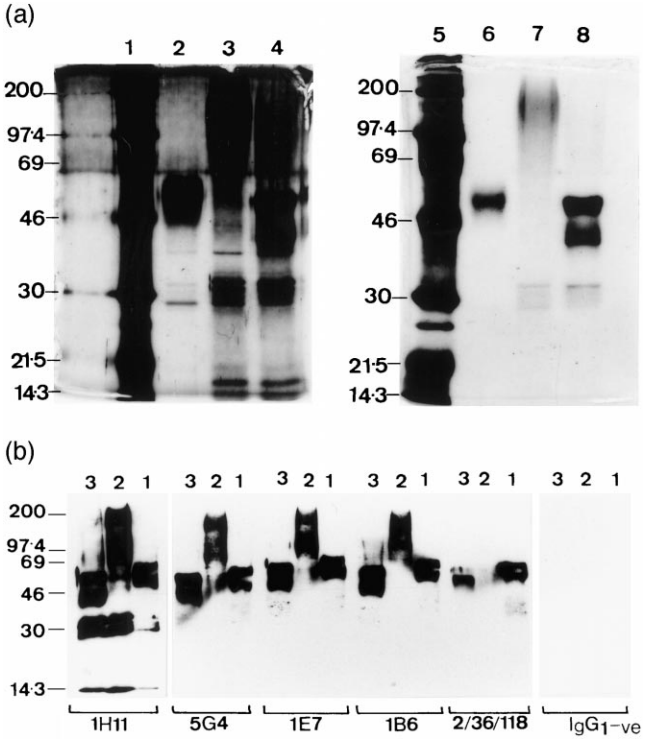

Fig. 2. Western blot of endo-H treated β4Gal-T. 10% SDS–PAGE gel under reducing conditions. (a) Gel stained with silver (lanes 1–4) or Coomassie blue (lanes 5–8). Molecular weight markers in kDa (lanes 1 and 5); human milk β4Gal-T (lanes 2 and 6); rhβ4Gal-T1 from *S. cerevisiae* with (lanes 4 and 8) and without Endo H treatment (lanes 3 and 7). (b) ECL detection of Western blot probed with anti-β4Gal-T mAbs. Each lane contains 1 μg of either purified human milk β4Gal-T (lane 1); rhβ4Gal-T1 from *S. cerevisiae* (lane 2) or endo-H treated rhβ4Gal-T1 from *S. cerevisiae* (lane 3).

expression of rhβ4Gal-T1 in COS-7 cells resulted in some β4Gal-T localized at the cell surface [Fig. 3(b)]. In all of these experiments the isotype-matched control mAb failed to stain the cells.

Binding kinetics.—Surface plasmon resonance (SPR) was used to monitor the association (k_{ass}) and dissociation (k_{diss}) rate constants of immobilized mAb with soluble rhβ4Gal-T over real time (Fig. 4). The k_{diss} data for human milk β4Gal-T and Endo H-treated rhGalT1 (Table 1) fitted best when using a two-site binding model where the majority ($>85\%$) of the sites had slow dissociation rate constants (in the $10^{-4}\,s^{-1}$ range). The mAbs were all of moderate affinity (apparent binding constant, K_B, approximately $10^8\ M^{-1}$) when interacting with the immunogen, human milk β4Gal-T (Table 1). The rhβ4Gal-T1 preparations and bovine milk β4Gal-T all exhibited lower affinities (K_B approximately $10^7\,M^{-1}$) with the mAbs when compared to human milk β4Gal-T. These lower apparent binding constants were most frequently attributable to larger changes in the k_{diss} rates rather than to changes in the k_{ass}. UCLgt1E7 and UCLgt5G4 mAbs had apparent k_{ass} and k_{diss} rate constants which were very similar to each other when tested against all the different types of β4Gal-T. The K_B values for the mAbs were always in a similar range for each of the β4Gal-T proteins assayed.

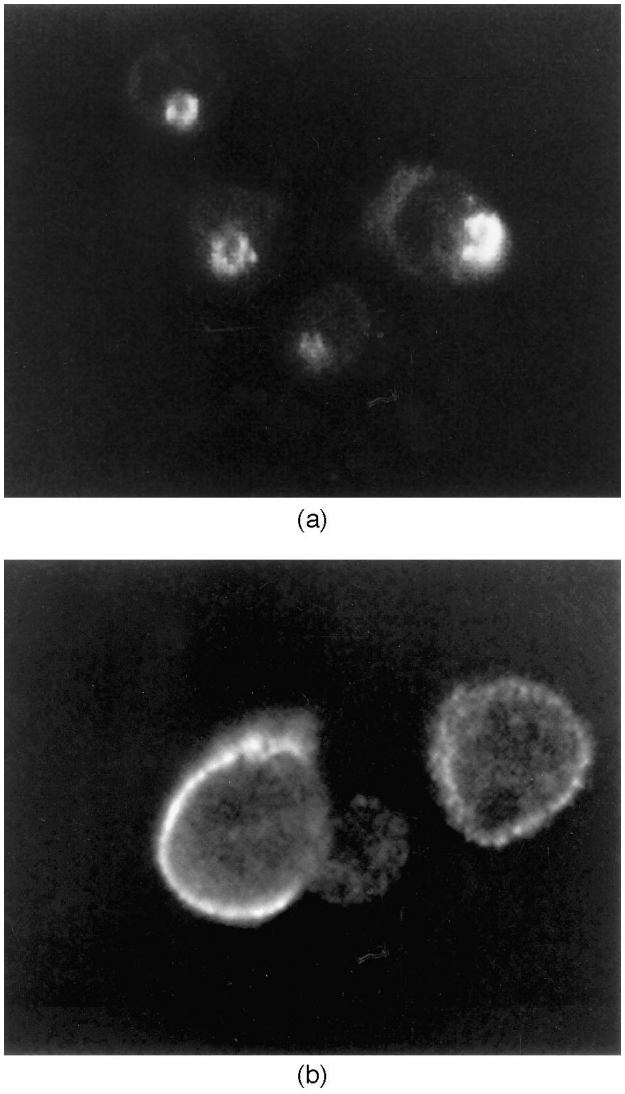

Fig. 3. Cellular localization of β4Gal-T. Immunofluorescence staining with UCLgt1E7 mAb and rabbit $F(ab')_2$ anti-mouse IgG-FITC. Cells were mounted onto glass slides and photographed under UV light. (a) Fixed and permeabilized EBV-transformed B cell line 2B6. (b) Plasma membrane staining of non-permeabilized COS-7 cells transfected with β4Gal-T1 cDNA in pcDNA3.

Ligation of β*4Gal-T by UCLgt1E7 enhances enzyme activity* .—Any attempt to assess the influence on enzyme activity of antibody binding to β4Gal-T could be complicated if the antibody possesses exposed GlcNAc residues which might theoretically act as a competitive substrate for β4Gal-T in the activity assay. Thus, before carrying out this experiment the mAbs were checked for exposed GlcNAc residues using the biotinylated lectins *Bandeiraea simplicifolia* II (to detect terminal GlcNAc) and *Ricinus communis* agglutinin (to detect galactose). The UCLgt1E7 mAb was the only one which lacked exposed GlcNAc, and was therefore the only anti-β4Gal-T1 mAb considered appropriate for this experiment. Only extremely low levels of exposed GlcNAc were detected on the MOPC-21 antibody used as an isotype-matched negative control. The UCLgt1E7 mAb produced a 45% enhancement in enzyme activity when 1 nmol/100 μL/h of β4Gal-T was used (Fig. 5). In contrast, and despite a lack of exposed GlcNAc, the isotype control antibody unexpectedly showed up to 30% inhibition of β4Gal-T activity with this concentration of enzyme (Fig. 5).

Both the stem region and catalytic domain bear immunogenic epitopes.—UCLgt1B6, UCLgt1E7 and UCLgt5G4 strongly ($>95\%$) inhibited binding of each other to human milk β4Gal-T (Fig. 6), suggesting that the epitope(s) they recognize are closely associated. In contrast, UCLgt1H11 interacted with a distinct epitope, as $\leq 10\%$ inhibition of binding was observed in the presence of the other mAbs using either human or bovine β4Gal-T (Fig. 6). The binding of biotinylated UCLgt1B6, UCLgt1E7 and UCLgt5G4 mAbs to β4Gal-T was inhibited by about 20–25% in the presence of

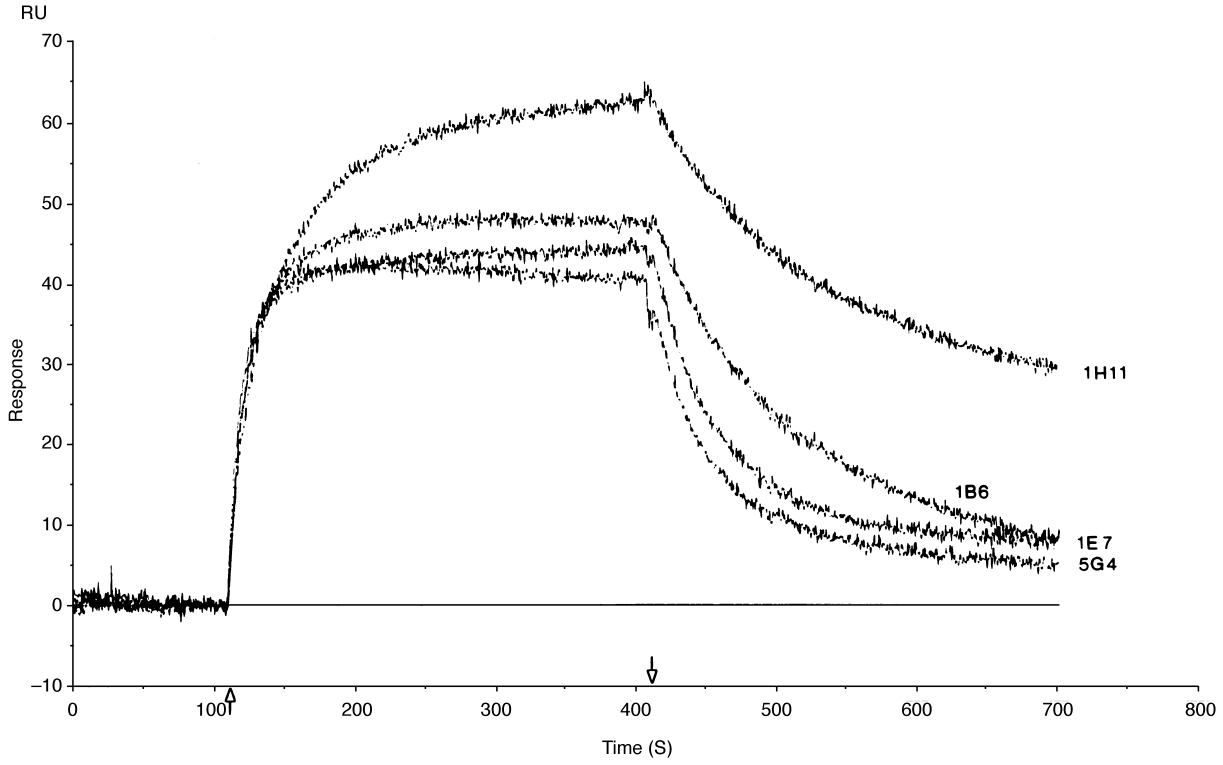

Fig. 4. BIAcore analysis of antibody binding. An overlay of four separate sensorgrams showing immobilized anti-β4Gal-T1 mAbs interacting with soluble rhβ4Gal-T1. The rhβ4Gal-T1 was injected (between the two arrows) and associated with the mAb, then the rhβ4Gal-T1 injection was replaced with buffer (downward arrow) and the dissociation rates of mAb-rhβ4Gal-T1 complexes measured.

Table 1
k_{ass} and k_{diss} of the mAbs for their respective epitopes

	UCLgt1B6	UCLgt1E7	UCLgt5G4	UCLgt1H11
hβ4Gal-T				
k_{ass}	7.12×10^{4}	6.09×10^{4}	7.05×10^{4}	3.21×10^{4}
k_{diss}	5.7×10^{-4}	2.9×10^{-4}	4.3×10^{-4}	4.7×10^{-4}
K_B	1.25×10^{8}	2.1×10^{8}	1.64×10^{8}	6.83×10^{7}
rhβ4Gal-T1				
k_{ass}	1.01×10^{5}	1.45×10^{5}	2.35×10^{5}	4.27×10^{4}
k_{diss}	1.18×10^{-2}	2.22×10^{-2}	3.64×10^{-2}	7.24×10^{-3}
K_B	8.56×10^{6}	6.53×10^{6}	6.46×10^{6}	5.9×10^{6}
rhβ4Gal-T1[a]				
k_{ass}	nf	nf	nf	nf
k_{diss}	7.77×10^{-4}	3.23×10^{-4}	7.25×10^{-5}	4.95×10^{-4}
rhβ4Gal-T1[b]				
k_{ass}	4.14×10^{4}	3.64×10^{4}	4.62×10^{4}	1.07×10^{4}
k_{diss}	6.08×10^{-3}	1×10^{-2}	1.13×10^{-2}	1.31×10^{-3}
K_B	6.81×10^{6}	3.64×10^{6}	4.09×10^{6}	8.17×10^{6}
bβ4Gal-T				
k_{ass}	3.84×10^{4}	3.67×10^{4}	4.03×10^{4}	1.53×10^{4}
k_{diss}	1.59×10^{-3}	2.75×10^{-3}	3.05×10^{-3}	8.62×10^{-4}
K_B	2.42×10^{7}	1.33×10^{7}	1.32×10^{7}	1.77×10^{7}

Summary of anti-β4Gal-T1 mAbs apparent association (k_{ass}, $M^{-1}s^{-1}$) and dissociation (k_{diss}, s^{-1}) rates with different β4Gal-T preparations. The apparent binding constant (K_B, M^{-1}) indicates the relative affinity of the mAbs. Human milk β4Gal-T (hβ4Gal-T), recombinant human β4Gal-T1 expressed in *S. cerevisiae* (rhβ4Gal-T1), rhβ4Gal-T1 treated with Endo H ([a] rhβ4Gal-T1), rhβ4Gal-T1 that has been mutated to remove the *N*-glycosylation site ([b] rhβ4Gal-T1) and bovine milk β4Gal-T (bβ4Gal-T).
nf, data could not be fitted.

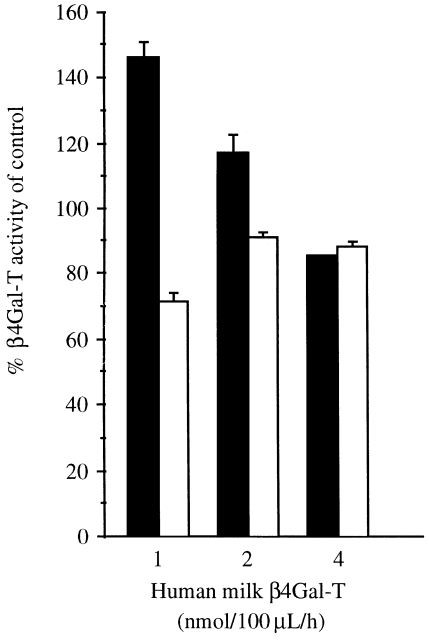

Fig. 5. Effect of anti-β4Gal-T1 antibody on enzyme activity. β4Gal-T activity in the presence of UCLgt1E7 mAb (50 μg/mL, black bars) or an irrelevant isotype control MOPC-21 mAb (50 μg/mL, white bars). The "control" β4Gal-T activity (assigned 100%) was measured in the absence of any mAbs. Error bars indicate one standard error of the mean for duplicate samples. The figure is a representative example of three separate experiments.

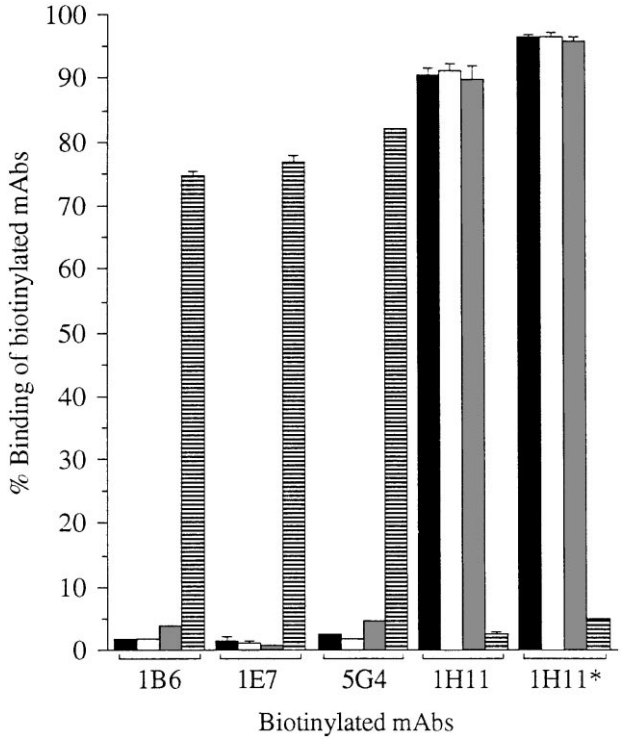

Fig. 6. Epitope mapping. Competition ELISA of biotinylated anti-β4Gal-T1 mAbs binding to human milk β4Gal-T in the presence of unlabelled anti-β4Gal-T1 mAbs UCLgt1B6 (■); UCLgt1E7 (□), UCLgt5G4 (▩) and; UCLgt1H11 (▤). UCLgt1H11 was additionally assayed against bovine milk β4Gal-T*. 100% binding for each biotinylated mAb represents binding in the absence of other mAbs. Error bars indicate one standard error of the mean for duplicate samples. The figure is a representative example of three separate experiments.

unlabelled UCLgt1H11 mAb. Synergistic binding to β4Gal-T was observed when biotinylated anti-β4Gal-T1 mAbs UCLgt1B6 and UCLgt1H11 were jointly bound to the streptavidin-coated ELISA plates (Fig. 7).

Cleveland digests of purified human milk β4Gal-T were performed, transferred onto PVDF membranes and probed with the mAbs. Consistent band patterns of β4Gal-T were generated following several independent digests with V8 protease with characteristic binding patterns for each of the anti-β4Gal-T1 mAbs tested (UCLgt1E7, UCLgt1H11 and GT2/36/118), again suggesting non-identity of the epitopes recognized (data not shown).

β4Gal-T1 mutant proteins expressed in *E. coli* were used in a direct binding ELISA to further define the epitope regions recognized (Fig. 8). The anti-β4Gal-T1 mAbs were raised against the soluble form of human milk β4Gal-T (amino acids 42–400). UCLgt1H11 mAb binding was unaffected by the different β4Gal-T1 deletion mutants. Thus the first 115 amino acids in β4Gal-T1 are not involved in the epitope recognized which therefore most likely lies within the catalytic domain of the enzyme. UCLgt1H11 also bound to the TSSS mutant which involved a loss of negative charge a few amino acids downstream from a hydrophobic pocket, the proposed UDP-galactose binding site [10]. The mAb UCLgt5G4 showed very little binding to any of the mutant proteins, suggesting the epitope recognized lies in the stem region between amino acids 42 and 77. The UCLgt1E7 mab did not show substantial binding to either of the deletion mutants bearing wild-type sequence, but did show a low level of reactivity with the TSSS mutant. UCLgt1B6 showed weak binding to the deletion mutants, and an intermediate level of binding to the TSSS mutant, suggesting some amino acids absent in the mutants partially contribute to the epitope recognized by UCLgt1B6. No cross-reaction to bovine β4Gal-T was seen with GT2/36/118 mAb. This mAb therefore recognizes an epitope which is entirely different to that recognized by UCLgt5G4 but also maps to amino acids 42–77 within the stem region (Fig. 9).

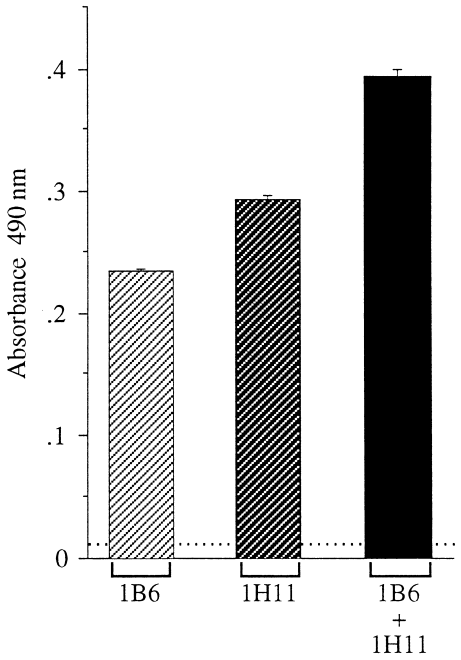

Fig. 7. Synergistic binding of monoclonal antibodies. Biotinylated anti-β4Gal-T1 mAbs UCLgt1B6 (▨, 2.5 μg/mL), UCLgt1H11 (▨, 2.5 μg/mL) or a combination of UCLgt1B6 and UCLgt1H11 (■, each at 1.25 μg/mL) were bound onto streptavidin-coated ELISA plates. Captured human milk β4Gal-T (15 μg/mL) was detected with an affinity-purified rabbit anti-β4Gal-T protein antibody. Non-specific binding with an irrelevant biotinylated isotype control mAb (2.5 μg/mL, dotted line). Error bars indicate one standard error of the mean for duplicate samples. The figure is a representative example of three separate experiments.

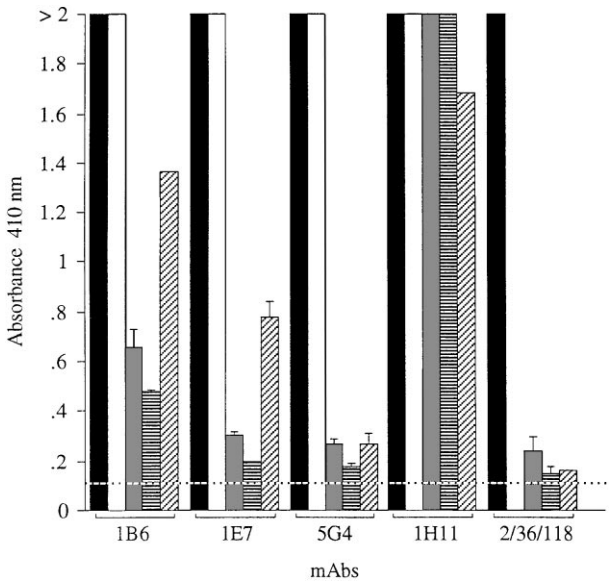

Fig. 8. Localization of epitopes using β4Gal-T1 mutants. Reactivity of the mAbs with the truncated and mutant proteins in an ELISA using human milk β4Gal-T (■); bovine milk β4Gal-T (□); rhβ4Gal-T1 produced in *E. coli* with *N*-terminal deletions rhβ4Gal-T1^{78} (■); rhβ4Gal-T1^{116} (▤) and a quartet mutant of rhβ4Gal-T1^{78} TSSS (▨). The readings obtained were after 16 h development, with the background value indicated by the dotted line. Error bars indicate one standard error of the mean for duplicate samples. The figure is a representative example of three separate experiments.

3. Discussion

Monoclonal antibodies against normal human β4Gal-T [29] and against an ovarian tumor-associated human β4Gal-T [30] have been previously described, as have mAbs against rat [31] and bovine [32] β4Gal-T. Such antibodies can be used for detecting the enzyme on membrane surfaces or in solution, for isolation of the enzyme, and potentially used to differentiate between different members of the β4Gal-T family. The four anti-human β4Gal-T1 mAbs generated for the present study were protein-specific. Furthermore, the large *N*-glycans on rhβ4Gal-T1 expressed in *S. cerevisiae* were shown to have no effect on the binding of these mabs although they did interfere with the binding of a previously generated mAb, GT2/36/118. The single N-linked glycosylation site in β4Gal-T1 is located in the stem region, the most heterogeneous region in β4Gal-T1 between species. The fact that GT2/36/118 did not cross-react with bovine β4Gal-T, which has two N-linked glycosylation sites, and was precluded from binding to heavily glycosylated rhβ4Gal-T1, suggests the oligosaccharides were masking the epitope recognized by this antibody.

All four of the newly generated anti-human β4Gal-T1 mAbs also recognized bovine milk β4Gal-T, which shares an overall 89% sequence homology with the human enzyme. However, only UCLgt1H11 did so to a similar extent as seen with the human immunogen, suggesting the epitopes recognized by the other three mAbs are similar but non-identical between the two species. Cross-inhibition studies clearly indicated that the epitope recognized by UCLgt1H11 was distinct from those recognized by the other mAbs. The 20–25% inhibition exhibited by UCLgt1H11 on the other mAbs may have been due to a slight steric hindrance or a conformational change following UCLgt1H11 interaction. Western blots of β4Gal-T from denaturing gels were still recognized by all the mAbs, suggesting that the relevant epitopes include linear amino acid sequences. It is possible, however, that limited renaturation of the protein had occurred following transfer onto nitrocellulose. The binding of UCLgt1H11 was unaffected by the deletion of the first 115 amino acids in β4Gal-T1, whereas

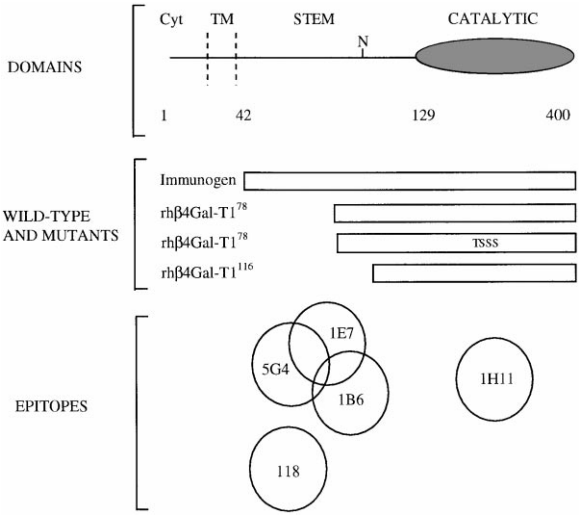

Fig. 9. Diagrammatic representation of the wild-type and mutant β4Gal-T1 constructs. The top panel indicates the domain structure of β4Gal-T1. Soluble human milk β4Gal-T used for the immunization comprises amino acids 42–400 and therefore lacks the transmembrane (TM) and cytoplasmic (Cyt) domains. Residue Cys^{129} marks the boundary between the stem region and the catalytic domain. The middle panel represents the deletion mutants which comprise amino acids 78–400 and 116–400. The bottom panel indicates the approximate location of the β4Gal-T1 epitopes defined in the present study (not to scale). GT2/36/118 recognizes an epitope which maps to the same region as UCLgt5G4, 1E7 and 1B6, but unlike these mAbs it fails to bind glycosylated bovine Gal-T. The position of the N-linked oligosaccharide (N) at Asn^{112} is indicated.

GT2/36/118 and UCLgt5G4 failed to recognize any of the mutants. UCLgt1E7 exhibited a similar binding as UCLgt5G4 to the β4Gal-T1 mutants except with $rhGalT^{78}$ TSSS which UCLgt5G4 did not recognize. These results suggest that human β4Gal-T1 bears immunogenic epitopes between amino acids 42–115 which are recognized by GT2/36/118, UCLgt5G4 and UCLgt1E7, with the epitope recognized by UCLgt1E7 possibly biased towards the carboxyl end of this domain. UCLgt1B6 showed a degree of reactivity with truncated enzyme. Taken together with the cross-inhibition results, UCLgt1B6 appears to detect an epitope partially overlapping with the region recognized by the UCLgt5G4 and UCLgt1E7 but extending C-terminal to amino acid 116. Thus, human β4Gal-T1 bears an epitope cluster composed of a number of non-identical, though overlapping immunogenic epitopes recognized by UCLgt5G4, UCLgt1E7, UCLgt1B6 and GT2/36/118. That four of the five mAbs tested recognized epitopes within the stem region accords with previous observations in which a fusion protein of β-galactosidase with truncated β4Gal-T1 (lacking amino acids 1–201) was much less immunogenic than a similar fusion protein containing most of the stem region (lacking only amino acids 1–59) [33]. It is known that the degree of sequence homology between the human and bovine enzymes is higher in the catalytic domain than in the stem region. The spatially distinct epitope recognized by UCLgt1H11 (Fig. 9) is likely to be identical or near identical in human and bovine milk β4Gal-T as the reactivity of this antibody with these two antigens was indistinguishable.

UCLgt1E7 was found to enhance β4Gal-T activity at low concentrations of β4Gal-T, perhaps by stabilizing the orientation of β4Gal-T1 and thereby enhancing its catalytic activity. The reason for the observed inhibition with the isotype control was unclear as this mAb did not recognize β4Gal-T, neither were there significant amounts of exposed GlcNAc residues on this mAb which could act as a competitive substrate. It is possible that the mAb was interacting with another component of the β4Gal-T activity assay, such as the acceptor substrate. Using crude hybridoma supernatants, a slight enhancement of β4Gal-T activity by anti-human β4Gal-T mAbs (including GT2/36/118) has previously been observed [29]. Other mAbs, raised against bovine milk β4Gal-T, did not affect β4Gal-T activity [32]. It could be of interest to investigate further the differences in the glycosylation status of the anti-β4Gal-T1 mAbs if they are shown to recognize murine β4Gal-T. All but one of the mAbs possessed readily detectable levels of exposed GlcNAc and it is possible that interaction with β4Gal-T1 or other β4Gal-Ts as the mAbs transit the hybridoma Golgi prevents efficient galactosylation of the antibodies. It has previously been shown using microinjection of mRNA encoding the heavy and light chain of an anti-Golgi mAb that the presence of specific antibody in the Golgi can interfere with protein transport [34].

The highest affinity binding constants, measured by SPR, for all the anti-β4Gal-T1 mAbs were observed with human milk β4Gal-T ($10^8\,M^{-1}$) followed by bovine milk β4Gal-T ($10^7\,M^{-1}$) and then rhβ4Gal-T1 (with or without *N*-glycosylation, $10^6\,M^{-1}$–$10^7\,M^{-1}$). In some instances, similar K_B were observed between the anti-β4Gal-T1 mAbs, such as the binding of UCLgt5G4 and UCLgt1H11 to rhβ4Gal-T1, yet there were five-fold differences in their k_{ass} and k_{diss} values. The change in K_B among the mAbs were due primarily to increased k_{diss} rates. Although broadly similar

characteristics have been reported for rhβ4Gal-T1 protein and human milk β4Gal-T [3], there is a 2.5–3-fold increase in K_m for GlcNAc and a 50% decrease in the K_m for glucose (in the presence of 1 mg/mL α-lactalbumin) with rhβ4Gal-T1, suggesting that subtle changes in the folding of β4Gal-T1 were slightly affecting the acceptor binding domains. Such differences along with a modest shift in circular dichroic spectra in *N*-deglycosylated β4Gal-T1 compared to the human milk β4Gal-T [3] may have influenced the high k_{diss} observed with the anti-β4Gal-T1 mAbs.

The recent cloning of additional β4Gal-T enzymes [6,7] suggests that many previous studies which refer to "β1,4 galactosyltransferase" will need to be interpreted carefully. We have referred to the mAbs used in the present study as anti-β4Gal-T1 because, although they were raised to soluble human milk β1,4 galactosyltransferase, we have demonstrated that they react with recombinant β4Gal-T1. We do not yet know if they are specific only for β4Gal-T1, or if the epitopes recognized are present on any other members of the β4Gal-T family. With respect to the catalytic domain specific UCLgt1H11 it is possible that this antibody may react with the other recently cloned β4Gal-T enzymes which, between amino acids 116–400, show 52% (β4Gal-T2), 41% (β4Gal-T3) and 45% (β4Gal-T4) homology to β4Gal-T1. However, it would seem extremely unlikely that the epitopes defined in the stem region of β4Gal-T1 would also be present on either β4Gal-T2 or β4Gal-T3 as these enzyme share no sequence homology with amino acids 42–77 of the β4Gal-T1 sequence (the area to which UCLgt5G4 and GT2/36/118 have been mapped). Thus, these mAbs are likely to distinguish the "classical" β4Gal-T (β4Gal-T1) from the newly described β4Gal-T2 and β4Gal-T3 members of the β4Gal-T family. With respect to β4Gal-T4, although this enzyme shows an overall sequence homology of 37% with β4Gal-T1, the stem region homology is only 16% and therefore it is quite possible that the stem region epitopes defined in the present study are also absent in β4Gal-T4.

4. Experimental

β4Gal-T proteins.—Bovine milk β4Gal-T was obtained from Sigma (Poole, Dorset, UK) and human milk β4Gal-T was purified as described by Gerber and colleagues [17].

Wild-type (wt) recombinant soluble human β4Gal-T1 (rhβ4Gal-T1, from amino acid 45 through to the C-terminus), and rhβ4Gal-T1 in which the Asn^{112} in the single *N*-glycosylation site was mutated to Asp (*N*-aglycosyl rhβ4Gal-T1) were expressed in *Saccharomyces cerevisiae* and purified as previously described [3]. Endoglycosidase H treatment of wt rhβ4Gal-T1 was carried out by incubating 1 mU recombinant Endo H (Boehringer Mannheim) per 2 μg of rhβ4Gal-T1 in 100 mM sodium citrate buffer, pH 5.4, for 2 h at 37 °C.

Bacterially-expressed rhβ4Gal-T1 proteins, including two truncated enzymes, rhβ4Gal-$T1^{78}$ and rhβ4Gal-$T1^{116}$, whose *N*-terminal amino acids were Thr^{78} and Val^{116}, respectively; and a quartet mutant generated by site-directed mutagenesis a few amino acids downstream from the proposed UDP-galactose binding site, Glu^{315}Thr/Asp^{316}Ser/Asp^{317}Ser/Asp^{318}Ser (TSSS), were produced in *E. coli* as previously described [27].

Antibodies.—Affinity purified β4Gal-T-specific rabbit polyclonal antibody raised to a fusion protein consisting of soluble human rhβ4Gal-T1 linked to β-galactosidase, and mAb GT2/36/118 raised to soluble human milk β4Gal-T, were prepared as previously described [29,33]. For the production of the other mAbs, 6-week old Balb/c mice were also immunized with soluble human milk β4Gal-T, a form of the enzyme which lacks the cytoplasmic and transmembrane regions (see Fig. 9). The mice were injected subcutaneously with 5 μg of antigen in Ribi adjuvant, followed 2 weeks later by a second identical injection. A final injection of 15 μg of antigen in saline was given intravenously after another 2 weeks and spleens removed 3 days later. Fusion between X63-Ag8.653 myeloma cells and the spleen cells was carried out using a simplified polyethylene glycol matrix fusion adapted from the classical method [35]. Following the fusion, cells were cultured at approximately 7×10^5 cells/mL in complete medium [comprising RPMI-1640 with sodium bicarbonate (Sigma R-7580), 15% heat-inactivated Myoclone super plus fetal bovine serum (Life Technologies, Paisley, Renfrewshire, UK), 5% heat-inactivated horse serum (TSH-free, Sigma H-1263), 2 mM L-glutamine (Life Technologies), 1 mM sodium pyruvate (Sigma S-8636), 1 μg/mL fungizone (Life Technologies), 50 IU penicillin and 50 μg/mL streptomycin (Life Technologies)] with an equal volume of double strength HAT medium [0.5 mM

hypoxanthine with 80 μM thymidine HT (Sigma H-0137) supplemented with 4 μM aminopterin (Sigma A-5159)]. One hundred μL of cells were plated out into wells containing 100 μL of 10% hybridoma enhancing supplement (HES) of macrophage origin (Sigma H-8142) in HAT medium. The culture medium was changed at 4–7 day intervals. Supernatants were screened for β4Gal-T binding using either ELISA or RIA and the positive hybridomas cloned twice by limiting dilution. For the second limiting dilution and expansion of clones, the serum in the complete medium was replaced with a serum-free supplement of 1:100 (v/v) Nutridoma (Boehringer Mannheim, Lewes, East Sussex, UK).

Quantification of IgG secretion.—ELISA plates were coated with 100 μL of sheep $F(ab')_2$ anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) at 5 μg/mL in 0.15 M phosphate-buffered saline pH 7.4 (PBS) overnight at 4 °C. Plates were washed four times with PBS containing 0.05% Tween 20 (PBS-T), blocked with 200 µL PBS-1% bovine serum albumin (BSA) for 1 h at 37 °C then washed. One hundred μL of mouse standard serum of pre-determined IgG concentration (The Binding Site, Birmingham, UK) in three-fold dilutions from 3 μg/mL of IgG or samples diluted in PBS-T was loaded onto the plates and incubated for 1 h at 37 °C. After washing, IgG was quantified using 100 μL of 1:30 000 sheep $F(ab')_2$ anti-mouse IgG-alkaline phosphatase (Sigma A-3563) in PBS-T for 1 h at 37 °C. The plates were then washed and developed with 100 μL of *p*-nitrophenyl phosphate (PNPP, Sigma 104–105) at 1 mg/mL in 50 mM bicarbonate buffer pH 9.6, with 2 mM $MgCl_2.6H_2O$ and absorbances read at 410 nm.

Isotyping of the antibodies.—One hundred µL of a 50% saturated ammonium sulfate cut from the hybridoma supernatant, at 5 μg/mL in PBS-T, was added to human milk β4Gal-T-coated ELISA plates for 2 h at 37 °C and the isotype of the bound antibodies determined using horseradish peroxidase-labeled goat anti-mouse Ig subclass specific antibodies (isotyping kit, Sigma ISO-2) and goat anti-mouse κ or λ light chains (Southern Biotechnologies). After washing, the plates were developed using 100 μL of 1 mg/mL o-phenylenediamine (OPD) in 0.1 M citrate phosphate buffer, pH 5.0 with 0.03% H_2O_2. The reaction was stopped with 50 μL 3N H_2SO_4 and absorbances read at 490 nm using a Dynatech MR5000 ELISA plate reader.

Specificity of anti-β4Gal-T1 mAbs.—One hundred µL of Protein G-purified mAbs at 1 μg/mL in PBS were incubated for 1 h at 37 °C on ELISA plates coated with 500 ng/well of human milk β4Gal-T; bovine β4Gal-T; human α-lactalbumin; bovine α-lactalbumin; human IgG; or BSA. For analysis of the regions of the enzyme which are recognized, 100 μL of total periplasmic fractions from *E. coli* expressing truncated rhβ4Gal-$T1^{78}$, rhβ4Gal-$T1^{116}$ or the TSSS mutant were directly coated onto Nunc maxisorp ELISA plates. These crude supernatants contained 15.39, 37.33 and 6.2 ng/mL of rhβ4Gal-$T1^{78}$, rhβ4Gal-$T1^{116}$ or the TSSS mutant, respectively. Binding of the mAbs was detected using sheep anti-mouse IgG-alkaline phosphatase as above.

Epitope mapping of anti-β4Gal-T1 mAbs.—The mAbs were biotinylated using *N*-hydroxysuccinimide ester biotin [36]. Biotinylated mouse IgG1κ MOPC-21 (Sigma M-9269) was used as a non-specific isotype-matched control. One hundred μL of biotinylated anti-β4Gal-T1 mAbs at a concentration which gave 50% maximum binding (40–210 ng/mL depending on the mAb used) were incubated for 2 h at 37 °C on human milk β4Gal-T coated ELISA plates in the presence of unlabelled anti-β4Gal-T1 mAbs at 10 μg/mL in PBS-T. Binding of biotinylated mAb was detected using horseradish peroxidase-streptavidin. Results are expressed as a percentage of binding, taking 100% binding as the absorbance obtained with biotinylated anti-β4Gal-T1 mAbs in the absence of unlabelled competitor mAbs.

Synergistic binding of β4Gal-T using pairs of mAbs coated onto ELISA plates was also employed as a means of assessing binding to spatially distinct epitopes. Fifty μL of streptavidin (Sigma S-4762) at 5 μg/mL was coated overnight at 4 °C, washed four times with PBS-T, then blocked with 100 μL of PBS-1% BSA for 2 h at 37 °C. After washing, one half of the plate was incubated with 50 μL of biotinylated isotype-matched control MOPC-21 at 5 μg/mL in PBS, with the other half of the plate incubated with 50 μL of a mixture of biotinylated anti-β4Gal-T1 mAbs, UCLgt1B6 and UCLgt1H11, each at 2.5 μg/mL in PBS. Plates were incubated for 2 h at 37 °C and then overnight at 4 °C. Following washing, the plates were incubated with 50 μL of 15 ng/mL human milk β4Gal-T. Plates were incubated for 2 h at 37 °C, then washed and incubated with 50 μL affinity-purified rabbit anti-human β1,4-GalT at 5 μg/mL in PBS-

T-1% BSA for 1 h at 37 °C. After washing, the plates were incubated with 50 μL of goat F(ab′)$_2$ anti-rabbit IgG-horseradish peroxidase (Jackson ImmunoResearch Laboratories) at 1:2000 in PBS-T-1% BSA for 1 h at 37 °C. Plates were washed and developed as above.

Surface plasmon resonance.—"On rates", "off rates" and binding affinities were determined by surface plasmon resonance (SPR) using BIAcore (Pharmacia Biosensor, Uppsala, Sweden). Briefly, a sensor chip was equilibrated in running buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20) at a constant flow rate of 5 μL/min. The matrix was activated by injecting 80 μL of a 1:1 mixture of 11.5 mg/mL *N*-hydroxysuccinimide and 75 mg/mL N-ethyl-N′-(3-dimethylaminopropyl)-carbodiimide to enable it to covalently bind free amino groups of proteins. This was followed by a 80 μL injection of a 20 μg/mL solution of polyclonal rabbit anti-mouse IgG1 (Pharmacia) in acetate buffer, pH 4.5. Any remaining unreacted sites were blocked with an injection of 40 μL of 1 M ethanolamine. Mouse antibodies were then captured on the immobilized anti-IgG1 by injection of a 100 μg/mL solution in running buffer. Saturation of all available sites was confirmed by injection of additional antibody until no further increase in the signal was observed. Antigen was then injected over the captured antibodies at a concentration of 0.3 μM. After the end of the protein injection (25 μL), running buffer resulted in dissociation of bound material, enabling the determination of the dissociation rate constant. The association rate constant could be determined from the sensorgram and the relative affinities of the various antibodies calculated (binding constant $K_B = k_{ass}/k_{diss}$). To correct for non-specific binding and drift in the signal, a control run was performed with a non-specific antibody and the response obtained with this control antibody was subtracted from that obtained with specific antibody. Fits of the on and off rate were applied using the corrected data.

β4Gal-T enzyme activity in the presence of anti-β4Gal-T1 mAb.—The enzyme activity assay was performed as previously described [37] using the UCLgt1E7 anti-β4Gal-T1, or an irrelevant isotype control mAb, at 50 μg/mL in the assay buffer which contained known amounts of β4Gal-T.

Western blotting.—Samples were reduced in Laemmli buffer containing a final concentration of 5% 2-mercaptoethanol, separated on a 10% SDS–PAGE gel, and proteins electrophoretically transferred to a 0.45 μm pore nitrocellulose membrane (NC, Schleicher and Schuell). Free-sites on the blot were blocked with 5% dried skimmed-milk powder in PBS-T 0.2%. Following incubation with primary antibody and with horseradish peroxidase-conjugated secondary antibody, the blots were developed using enhanced chemiluminescence (ECL, Amersham International, UK) followed by exposure to Kodak X-OMAT-AR film.

Cleveland digests.—Twenty five micrograms of purified human milk β4Gal-T was separated using SDS–PAGE under reducing conditions, the enzyme band excised, and then equilibrated in 125 mM Tris-HCl, pH 6.8, 0.1% SDS, 1 mM EDTA for 30 min. The excised gel bands were then loaded onto a second SDS–PAGE with 10 μL of V8 protease at 0.5 mg/mL. Following separation of the digested fragments, the gel was blotted onto NC membrane as described above.

Transient transfection with human β4Gal-T1.—COS-7 cells were transiently transfected with β4Gal-T1 cDNA (subcloned into the BamH I/Xho I sites of pcDNA3) using a modification of the method of Aruffo et al. [38]. Briefly, cells were transfected for 2 h at 37 °C in transfection medium (10% NuSerum, 250 μg/mL dextran, 200 μM chloroquine and 5 μg/mL DNA), shocked with 10% DMSO for 2 min, then re-cultured in fresh medium and analyzed 48 h post-transfection.

Immunofluorescence.—2.5×10^5 cells (either the 2B6 EBV-transformed B cell line [39] or the transfected COS-7 cells) were resuspended with 100 μL of test mAb or isotype-matched control mAb at 20 μg/mL in wash buffer (PBS–0.1% BSA with 15 mM NaN_3) and incubated for 45 min on ice. Following two 1 mL washes, 50 μL of FITC-labelled rabbit F(ab′)$_2$ anti-mouse IgG, diluted 1:10 in wash buffer, was used to resuspend the cell pellet and incubated for 45 min on ice. Cells were washed twice in wash buffer and resuspended in 250 μL PBS. For intracellular staining, cells were fixed and permeabilized using PermeaFix (Ortho Diagnostic Systems, Buckinghamshire, UK) before being stained. The staining procedure was as described above except that an additional 30 min wash step was included following antibody staining. Stained cells were mounted onto slides using a drop of fluorescence mounting medium (Dako). Bio-Rad MRC600 Confocal microscope images were captured using Biosis software (Bio-Rad) and exported into NIH-Image 1.59 (http://rsb.info.nih.gov/nih-image/) for processing.

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