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Specificity studies of an antibody developed against a mucin-type glycoprotein

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The specificity of a new anti-epiglycanin antibody (AE-3) which recognizes a mucin-type glycoprotein, the Human Carcinoma Antigen, found in the blood of patients with carcinomas, was studied. Information regarding the chemical nature of the antibody binding site was obtained by altering the structure of epiglycanin by chemical or enzymic means and testing the product in a competitive binding assay for inhibition of the binding of AE-3 to epiglycanin. The need for a high molecular weight antigen containing clustered T disaccharide, Galβ1-3GalNAc, was demonstrated. The specificity was further explored by inhibition studies with glycopeptides having one to three mono- to disaccharides. The results were interpreted using computer graphics molecular modeling which predicted the specific recognition of hydroxyl groups on oligosaccharides on adjacent amino acids. Thus T antigen O-linked glycopeptide tumour markers can be designed to be distinguished by antibodies by the amount of clustering of their oligosaccharides.

Keywords: **diagnostics, epiglycanin, Human Carcinoma Antigen, immunoglobulin M, mucin-type glycoprotein**

Abbreviations: **BSA: bovine serum albumin, HCA: Human Carcinoma Antigen, PBS: phosphate buffered saline**

Introduction

Mucin-type glycoproteins are large and highly glycosylated. They appear to be ubiquitous to all epithelial cell surfaces [1], but the surfaces of malignant cells have been found to contain abnormal mucins [2]. During recent years a large number of different monoclonal antibodies against carcinoma-associated mucin-type glycoproteins have been prepared. The properties of some of these antibodies which recognize the protein cores have been reviewed [3]. Comparisons of some carcinoma specific antibodies have also been performed [4], relatively little is known, however, regarding the detailed structures of their epitopes.

The finding that a mucin-type glycoprotein, epiglycanin, of the allotransplantable TA3-Ha mouse mammary adenocarcinoma ascitic cell strongly influenced the malignant properties of this cell [5,6], led to investigation of its structural [7–10], immunochemical [11], and immunological [12] properties. In the latter study it was found that a rabbit anti-epiglycanin polyclonal antibody recognized a mucin-type glycoprotein (Human Carcinoma Antigen, HCA) present in the sera and ascitic fluids of patients with carcinomas. Subsequently, more than forty anti-epiglycanin monoclonal antibodies were prepared for the purpose of developing a reliable immunoassay for human carcinomas [13]. In order to select the antibody with the greatest specificity and sensitivity, to be used in the test, each of the antibodies was tested against a panel of sera from patients with or without carcinomas. The antibody with the most favorable characteristics was AE-3. An electron microscopic investigation of the pathway taken by epiglycanin from its sites of biosynthesis to its location at the cell surface had previously demonstrated the capacity of AE-3 to identify epiglycanin both within and on the outside of the cell [14]. In addition, a competitive binding assay involving this antibody demonstrated its unique capacity to identify the Human Carcinoma Antigen (HCA) [15], and to distinguish between normal sera and sera of individuals who were without evidence of the disease [16]. This assay has also proven reliable in both prospective and retrospective studies as an aid in monitoring the state of the disease in

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patients with carcinomas (Codington, Kuter, Nikrui, Haavik, Matson, in preparation). The purpose of the present study is to describe the characteristics of the binding site of the AE-3 antibody.

Materials and methods

Biotin labelled peanut lectin, T-disaccharide, soybean trypsin inhibitor, bovine serum albumin type V, biotin hydrazide, iodoacetamide and p-nitrophenyl phosphate were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Alkaline-phosphatase-labelled goat anti-mouse IgM (μ-chain), anti-mouse Ig (Fab), and peroxidase-labelled goat anti-rabbit immunoglobulins were obtained from Boehringer Mannheim (Indianapolis, IN, USA). Alkaline phosphatase labelled streptavidin and anti T-antibody HBT1 were obtained from DAKO (Glostrup, Denmark). Rabbit anti-mouse κ-chain was obtained from Bethesta Research Laboratories (Gaithersburg, MD, USA). Microtitre plates (NUNC Maxisorp) were obtained from Nunc (Copenhagen, Denmark). CNBr-activated Sepharose, molecular weight markers for gel filtration, and SDS-PAGE were obtained from Pharmacia AB, (Uppsala, Sweden).

The synthetic glycopeptides were gifts from Dr. Hans Paulsen, University of Hamburg, Institute for Organic Chemistry, Hamburg, Germany. GF-intestinal mucin from germfree rats [17] was a gift from Dr. Jens K. Wold, Institute of Pharmacy, University of Oslo, Norway.

Epiglycanin

Epiglycanin was isolated from the ascitic fluid of A/WySn mice 7 days after injection of 10^5 TA3-Ha ascitic cells, as described previously [18].

Monoclonal anti-epiglycanin antibody

Antibody AE-3 was prepared as described [14]. It was later subcloned again and purified by HPLC on a DEAE 5 PW column (Waters, Milford, MA, USA).

Preparation of reduced mono- and dimeric IgM fragments

Purified mouse IgM (1 mg/ml) was shaken for 2 h at 37 °C with 10 mM cysteine, 2 mM EDTA in 50 mM tris-HCl, pH 8.0. Iodoacetamide was then added to a final concentration of 15 mM and shaking continued for 30 min. at 37°C. Reduced fragments of IgM were isolated by chromatography on a column (7.5 × 300 mm) of TSK G 4000 SW eluted at 0.4 ml/min with 0.1 M Na-phosphate pH 6.80. The column was calibrated for MW determination the gel filtration standards thyroglobulin (MW 670,000), gamma globulin (MW 158,000), ovalbumin (MW 44,000), myoglobin (MW 17,000) and vitamin B_{12} (Mw 1,350) (Bio Rad, Richmond, CA, USA).

Preparation of $F(ab')_2$ fragments

Mouse IgM (1 mg/ml) was incubated in 0.1 M Na-acetate and 0.1 M NaCl containing 40 μg/ml pepsin, pH 4.60, at 37 °C for 14 h. $F(ab')_2$- fragments were isolated from the incubation mixture by TSK G 4000 SW chromatography as described above.

Preparation of Fab fragments

IgM (1 mg/ml) was incubated in 50 mM tris-HCl, pH 8.0, buffer containing 0.1 M NaCl, 2 mM mercaptoethylamine, 2 mM EDTA and 0.1 ml/ml TPCK-trypsin-Sepharose (10 mg trypsin/g CNBr-Sepharose) for 16 h at 37 °C. 40 μg soybean trypsin inhibitor was added, and incubation continued for 15 min at 37 °C, iodoacetamide was added to a final concentration of 5 mM and incubation continued for 30 min. at 37 °C. The trypsin-Sepharose was then removed by centrifugation and Fab-fragments were purified from the supernatant by TSK G 4000 SW chromatography as described above.

Antibody capture assay (ELISA) of anti-epiglycanin activity

The wells of a Nunc Maxisorp 96 well microtiter plate were coated with 100 μl of epiglycanin (10 μg/ml) in PBS pH 7.50 for 16 h at 4 °C. The coating solution was then removed, and the wells blocked by incubation with BSA (5 mg/ml) for 1 h at 4 °C. After incubation with anti-epiglycanin antibody, rabbit anti-mouse κ-chain diluted 1:2,000 was added, and incubation continued for 1.5 h at 37 °C. This solution was then removed, the wells washed two times with PBS, pH 7.50, and peroxidase-labelled goat anti-rabbit Immunoglobulins diluted 1 : 2,000 was added. Incubation was continued for 1.5 h at 37°C. The wells were then washed three times with PBS, pH 7.50, and substrate solution for peroxidase (0.003% H_2O_2, 0.1% o-phenylene diamine in 0.1 M citrate-phosphate buffer pH 4.0) was added. The reaction was stopped by addition of 0.33 M citric acid, and absorbance read at 490 nm. Control experiments demonstrating that the rabbit anti-mouse κ-chain bound to the tested fragments were performed.

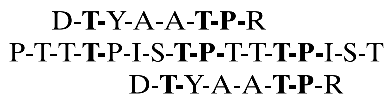
Competitive binding assays

To wells coated with epiglycanin (10 ng/well) and blocked with BSA were added: a) 50 μl of a standard solution of epiglycanin or inhibitor to be tested (oligosaccharide, glycopeptide or glycoprotein); and b) 50 μl solution of AE-3 (200 ng/ml). The mixture was incubated on a shaker for 16 h at 20°C. The wells were washed three times with PBS containing 0.05% Tween 20 and incubated for 2 h at 20°C with alkaline phosphatase labelled goat anti-mouse IgM dissolved in PBS containing 0.5% BSA. The wells were washed three times and then 100 μl of substrate solution containing 1 mg/ml p-nitrophenyl phosphate in 0.1 M ethanolamine pH 10.0 was added. The absorbance was read

at 405 nm. For the inhibition of the binding between peanut lectin and epiglycanin, AE-3 was replaced by biotin labelled peanut lectin (1 μglml). Binding was detected by incubation with alkaline phosphatase labelled streptavidin and subsequent incubation with the substrate for alkaline phosphatase as described above.

Computer modeling of glycopeptide

The peptide was constructed using Insight II (Molecular Simultations Inc., San Diego, CA), by homology with the sialoglycopetide bound to wheat germ agglutinin [19] deposited as 2CWG in the Brookhaven Protein Databank [20]. The sialoglycopeptide sequence was aligned against the double length peptide sequence P-T-T-T-P-I-S-T-P-T-T-T-P-I-S-T and found to give two possible alignments which overlap the sequence of interest:



The latter alignment was chosen for use, and a peptide of sequence S-T-P-T-T-T-P-I was generated with the protein backbone coordinates of the sialoglycopeptide. A double copy of this peptide was generated, so that a peptide sequence P-T-T-T-P-I-S-T could then be excised. The sugar residues were added to the structures from a previously generated library [21], using the geometries for the same residues in the asialoglycopeptide to produce glycopeptide glycosylated as in P112. This glycopeptide was subjected to a molecular dynamics simulated annealing simulation under periodic boundary conditions in a 25A box containing water as solvent by modification to the AMBER forcefield [22] using Discover (Molecular Simultations inc., San Diego, CA). The simulation minimised the complex, then heated the system to 1000 degrees K over a 1ps equilibration step. 50ps molecular dynamics at 1000 degrees K were performed, after which further 20ps intervals were performed at temperature decreasing by 20 degree intervals to 0 degrees K.

Results

Inhibition of binding between AE-3 and epiglycanin by various glycoproteins

The inhibitory activity on a weight basis of asialoglycophorin in the enzyme competitive binding assay was equal to that of epiglycanin (Fig. 1). Asialo GF-rat intestinal mucin had an inhibitory activity that was about 5×10^{-4} times that of epiglycanin. The GF-rat intestinal mucin has been shown to have a high content of T-disaccharide (Galβ1→3)GalNAc) [17].

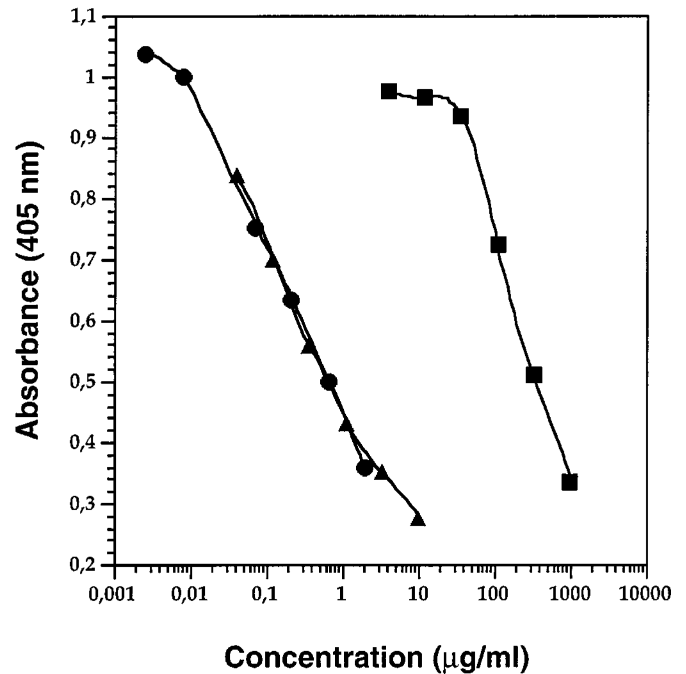


Figure 1. Inhibition of binding between AE-3 and epiglycanin in the enzyme competitive binding assay by epiglycanin (●), asialoglycophorin (▲) and neuraminidase treated GF rat intestinal mucin (■).

Comparison of binding between AE-3 and epiglycanin and between peanut lectin and epiglycanin

About 10^8 times higher concentration of the Galβ(1→3) GalNAc disaccharide than of epiglycanin was needed in order to get comparable inhibition in the enzyme competitive binding assay (Fig. 2a). In an equivalent competitive binding assay using biotinylated peanut lectin instead of AE-3 the relative inhibitory activity of the T-disaccharide was at least 10^4 times higher than in the AE-3 based assay (Fig. 2b).

Inhibition of binding between AE-3 and epiglycanin by synthetic glycopeptides

The eight tested glycopeptides showed different abilities to inhibit binding between AE-3 and epiglycanin in the enzyme competitive binding assay (Fig. 3). The following relative inhibitory activities were observed: P88 > P92 > P112 > P89 > P90 > P91 > P93 > P24. P24 possessed almost no detectable inhibitory activity in the concentrations tested. The relative inhibitory activity on weight basis of P88 was about 10^{-8} times that of epiglycanin (Fig. 3b).

Computer modeling of gycopeptide P112

Figure 5 shows the glycopeptide segment of the 24-mer formed by triplicating the amino acid sequence: Pro-Thr-Thr-Thr-Pro-lle-Ser-Thr in order to minimise end effects. The central peptide was glycosylated as in P112 using the

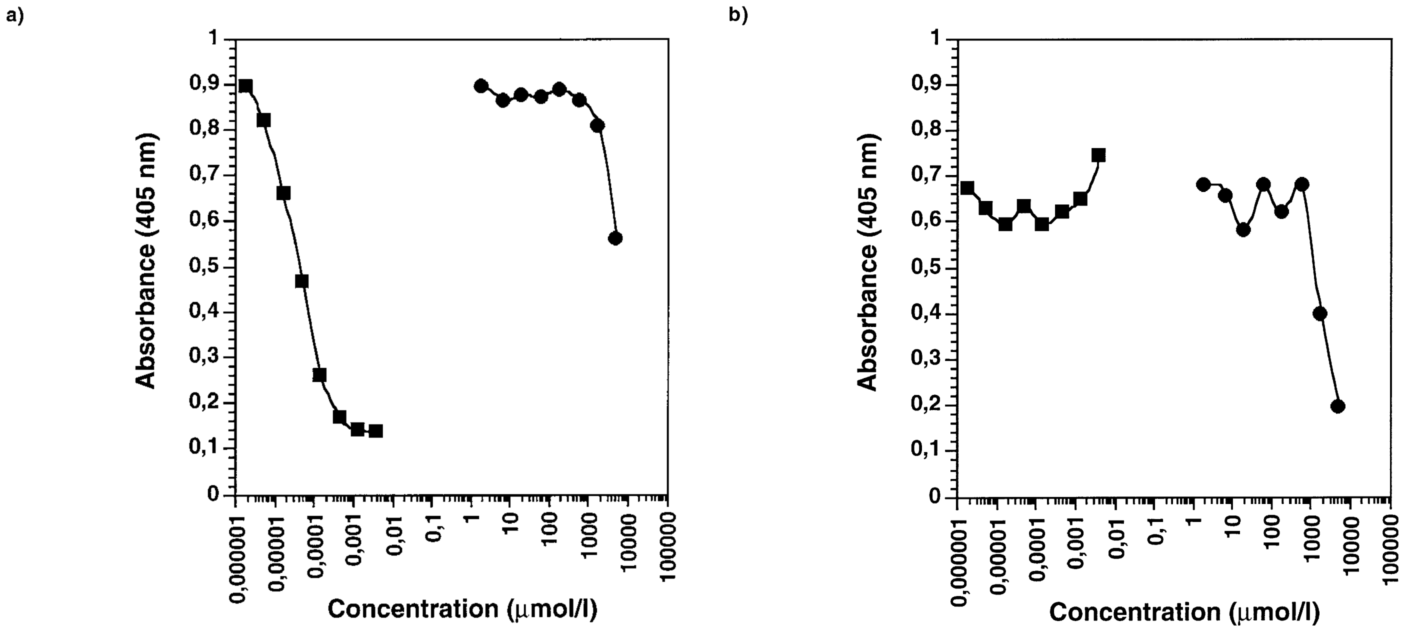


Figure 2. Inhibition of binding between a) AE-3 and epiglycanin b) peanut lectin and epiglycanin by epiglycanin (■) and T-disaccharide (Galβ1-3)Gal-NAc)(●). The experiment was performed as described in the Materials and Methods section.

starting geometry from a similar peptide of glycophorin which had been crystallized [19]. The glycosylated 24-mer was then subjected to simulated annealing in water as described in Materials and Methods. From the left hand side of the model can be seen the Gal and GalNAc on Thr 2, Pro 1, the tetraglycosylated dipeptide Thr 3. Thr 4 (with the glycosylation of Thr 2 in the foreground), and then the Pro-lle-Ser-Thr peptide. Extrapolating to the most inhibitory glycopeptide P88, it can be seen that going from bottom to top of the middle glycopeptide segment the Gal (on Thr 3), the GalNac (on Thr 3) and the Gal-GalNAc (on Thr 4) together form a distinct glycopeptide motif.

Epiglycanin binding activities of mono- and divalent fragments of the anti-epiglycanin antibody AE-3

Mono- and divalent fragments of AE-3 were prepared and tested for binding to immobilized epiglycanin in order to

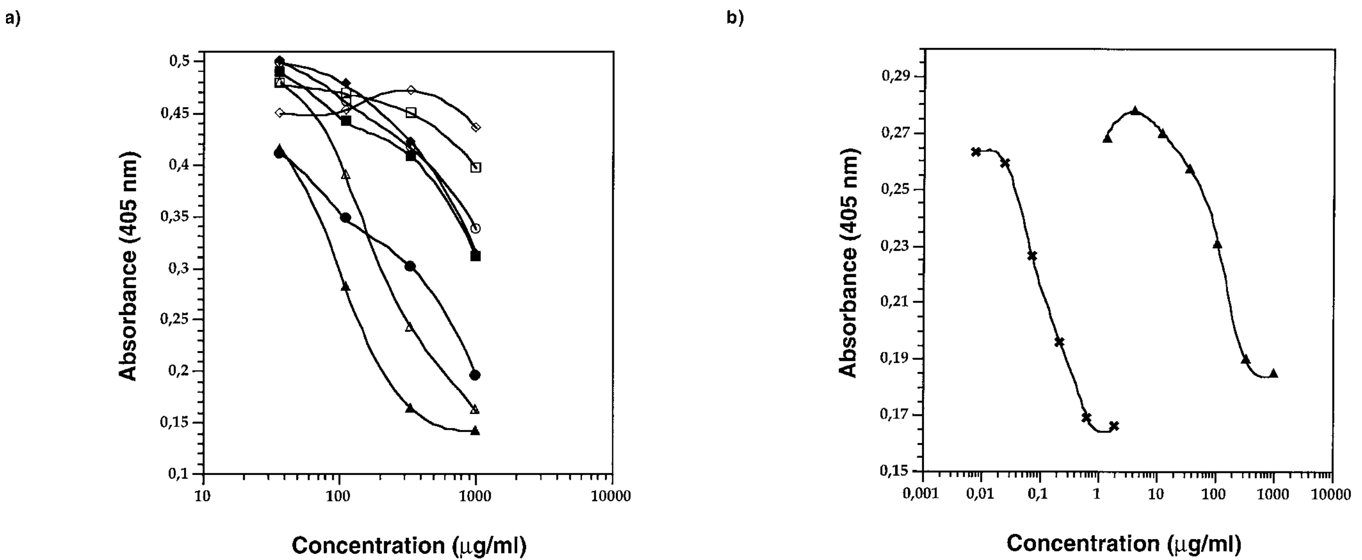


Figure 3. Inhibition of binding between AE-3 and epiglycanin by glycopeptides in the competitive binding assay (structures given in fig. 6). a) P 24 (◇), P 88 (▲) P 89 (■), P 90 (◆), P 91 (○), P 92 (△), P 93 (□), P 112 (●). b) epiglycanin (✕), P 88 (▲).

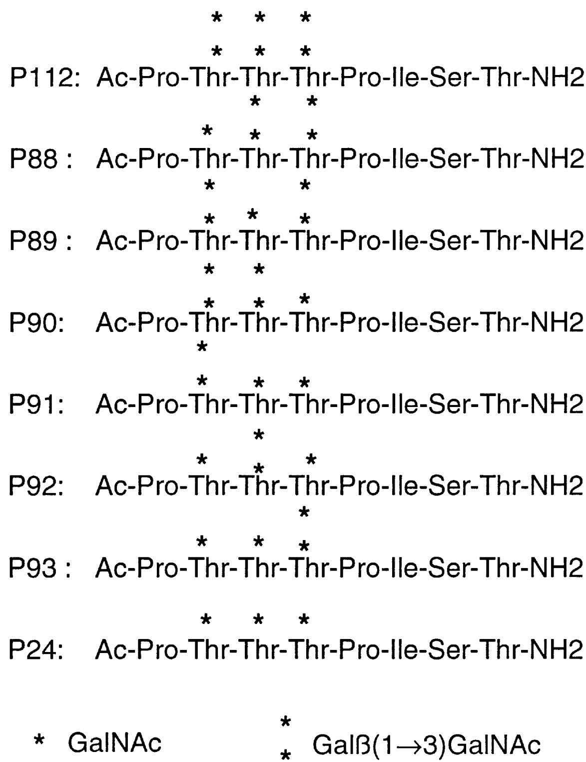


Figure 4. Structures of glycopeptides tested in fig. 3 [33].

study the importance of multivalency of the IgM antibody (AE-3) for its binding to epiglycanin (Fig. 6). In the assay, a more than 100 times higher concentration of the reduced 180 kD fragment was required than of the native antibody in order to obtain the same absorbance. About 10 times higher concentration of the $F(ab')_2$-fragment was required than of the 180 kD fragment; whereas the Fab-fragment possessed very low antigen binding activity.

Discussion

Interest in the anti-epiglycanin monoclonal antibody AE-3 stems from its ability to recognize a glycoprotein, the Human Carcinoma Antigen (HCA), which is associated with malignancies of human epithelial tissues. This property is manifested in its role in a competitive binding assay [16]. This assay has been used effectively to monitor the condition of patients with breast carcinoma (Codington, Kuter, Nikrui, Matson, in preparation). Because of the specificity of AE-3 for HCA, it was of interest to determine the molecular characteristics of the epitope recognized by AE-3. Since both HCA and epiglycanin appear to contain the same epitope, most of these studies have been performed with epiglycanin, which is more readily available in purified form for experimentation.

AE-3 binds to a T-disaccharide containing epitope on epiglycanin. Several other anti-T antibodies have previously been prepared by various methods. For example, the antibody 49H.24 was induced by immunization with neuraminidase-treated erythrocytes [23], and HH8 which resulted from immunization with glycolipids [24]. Human sera also contain anti-T antibodies that are thought to be produced in response to immunogens in gastrointestinal bacteria [25]. The relationship between the different anti-T antibodies, however, is still poorly understood. The inability of the T-disaccharide to inhibit binding between AE-3 and epiglycanin seems to distinguish AE-3 from the anti-T antibody 49H.24. In contrast to our results with AE-3, the simple monovalent glycoside Galβ1→3)GalNAcα-O-$(CH_2)_8CONHNH_2$ was found to be a potent inhibitor of binding between 49H.24 and antigen [23].

Peanut lectin is known to bind the T-disaccharide and is considered an anti-T reagent [26]. Since the difference in relative inhibitory activity for epiglycanin and the T-disaccharide is more than 10^4 times greater for the AE-3-epiglycanin interaction than for the peanut lectin-epiglycanin interaction (Fig. 2), the AE-3-epiglycanin interaction is clearly different and much more specific than the binding between peanut lectin and epiglycanin. One possibility is that the antibody binds to more than one disaccharide unit on epiglycanin which would explain the low avidity of the mono- and divalent fragments (Fig. 6).

The importance of multivalency for strong (and specific) binding has also been described for other biological systems, such as that for the binding of E-selectin to sialyl Lewis^x -containing ligands [27]. Three consecutively glycosylated serine- or threonine residues were essential for Tn antigenicity for the IgG3 anti-Tn antibody MLS 128 [28]. In order to study the possible involvement of more than one T-disaccharide in the epitope of of AE-3 eight synthetic glycopeptides containing zero to three T-disaccharides were compared in the competitive binding assay (Fig. 3 and 4). The most active glycopeptide, P88 contained two T-disaccharides on neighbouring threonine residues. The activity of P92 which only contained one T-disaccharide was higher than that of P112 which contained three T-disaccharides and that of P89 and P90 both carrying two disaccharides (Fig. 3a). This is explained by the modeling which shows that the Pro-Thr-Thr-Thr-Pro sequence forms a particular glycopeptide motif where the glycosylation on the second and third Thr residues form a compact epitope for antibody recognition. It can therefore be envisaged that each antibody or lectin can have a distinct glycopeptide recognition domain which is specific for both carbohydrate and peptide sequence. In the case of these O-linked glycopeptide motifs, the conformation is determined by hydrophobic interactions between the protein and the oligosaccharides and the hydroxyls accessible for interaction with water or antibody. Favourable entropic factors derived from the displacement of bound water in the antibody binding site would thus contribute to the binding energy. This would be in contrast to results from several

a)

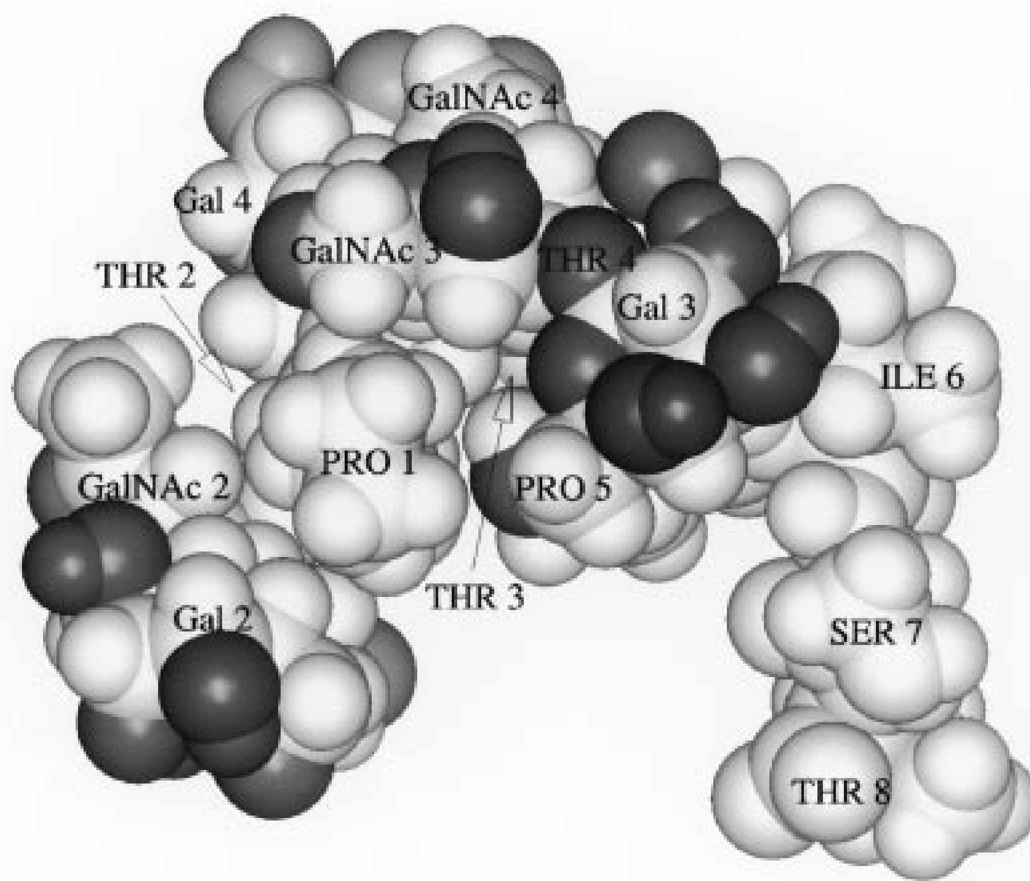


Figure 5. Three dimensional models of glycopeptide P112 showing in darker shade a) the O-H groups and ring oxygens of the oligosaccharides for hydrophilic interactions, and b) the hydrophobic regions of the oligosaccharides and amino acids.

studies of oligosaccharide binding to IgG antibodies where there is a significant hydrophobic interaction in the binding site.

IgM antibodies have been considered less useful than IgG antibodies due to their multivalency and tendency for non specific binding [29]. It is plausible that these disadvantages might be circumvented by the preparation of $F(ab')_2$ and Fab-fragments of the IgMs. The use of such fragments for *in vivo* imaging and therapy of tumors has been reported [30]. As the previously reported procedures for fragmentation of mouse IgM [31,32] were not effective for this antibody, it was necessary to develop new procedures for the preparation and isolation of sufficiently pure AE-3 fragments. Higher yields were obtained by digestion with trypsin in the presence of 2 mM mercaptoethylamine, rather than cysteine. When highly purified IgM was used as starting material pure fragments could be obtained from the digests by high performance gel filtration on a TSK G 4000 column.

Our studies have shown that multivalency is important for the properties of the anti-epiglycanin IgM antibody. The dependency of multivalency may be a common phenomenon in biological interactions where high molecular weight multivalent glycoconjugates are involved. This phenomenon might be one of the reasons why it has proved to be difficult to design effective inhibitors for such biological recognition events even if the structures of the monomeric units that participate are known.

Acknowledgments

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b)

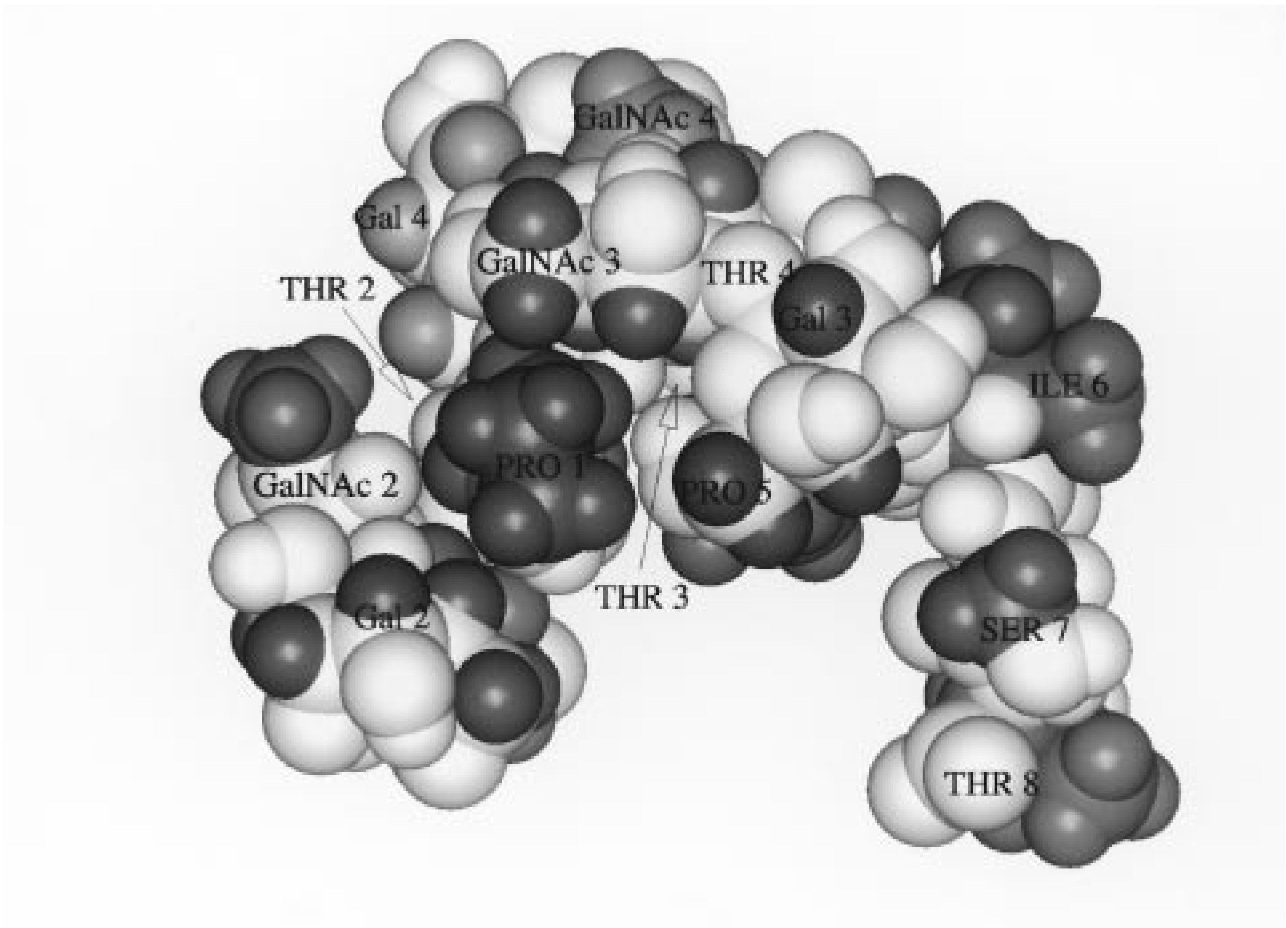

Figure 5. (Continued)

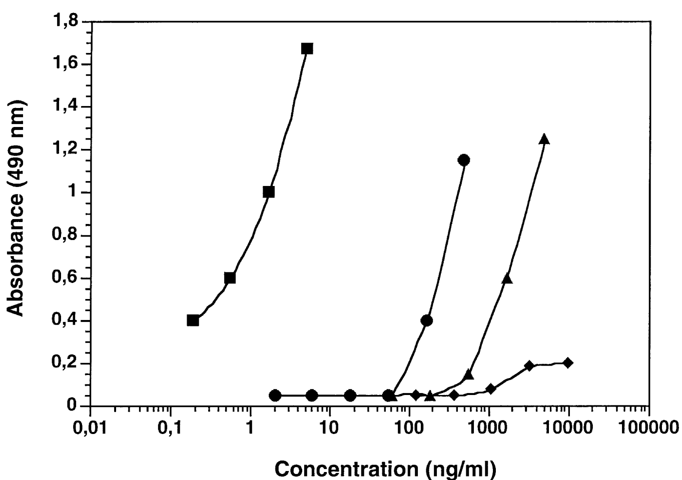

Figure 6. Binding of AE-3 and fragments of AE-3 to epiglycanin. Binding was detected by sequential incubation by rabbit anti-mouse κ-chain and peroxidase labeled goat anti-rabbit immunoglobulins. Native AE-3 (■), 180 kDa fragment (●), $F(ab')_2$-fragment (▲), Fab-fragment (◆).

ledged for their respective gifts of glycopeptides and GF-intestinal mucin.

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