Antibody recognition of a unique tumor-specific glycopeptide antigen

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Aberrant glycosylation and the overexpression of certain carbohydrate moieties is a consistent feature of cancers, and tumor-associated oligosaccharides are actively investigated as targets for immunotherapy. One of the most common aberrations in glycosylation patterns is the presentation of a single O-linked N-acetylgalactosamine on a threonine or serine residue known as the "Tn antigen." Whereas the ubiquitous nature of Tn antigens on cancers has made them a natural focus of vaccine research, such carbohydrate moieties are not always tumor-specific and have been observed on embryonic and nonmalignant adult tissue. Here we report the structural basis of binding of a complex of a monoclonal antibody (237mAb) with a truly tumor-specific glycopeptide containing the Tn antigen. In contrast to glycopeptide-specific antibodies in complex with simple peptides, 237mAb does not recognize a conformational epitope induced in the peptide by sugar substitution. Instead, 237mAb uses a pocket coded by germ-line genes to completely envelope the carbohydrate moiety itself while interacting with the peptide moiety in a shallow groove. Thus, 237mAb achieves its striking tumor specificity, with no observed physiological cross-reactivity to the unglycosylated peptide or the free glycan, by a combination of multiple weak but specific interactions to both the peptide and to the glycan portions of the antigen.

X-ray crystallography | affinity maturation | crystal structure

Neoplastic transformation often affects the Golgi apparatus, and unusual glycosylation patterns specific to the type and stage of different cancers provide numerous diagnostic and therapeutic venues (for review, see ref. 1). Tumor-associated oligosaccharides have been actively investigated targets for immunotherapy because they are on the cell surface and exposed to immune surveillance; however, carbohydrates are generally T-cell independent antigens and can be poor immunogens (2, 3). As a consequence, monoclonal antibodies, especially IgG, raised against purely carbohydrate antigens, frequently exhibit relatively low binding affinities. In contrast, glycopeptides are capable of eliciting both cellular and humoral immune responses, which allows for affinity maturation and the production of higher-affinity antibodies (4). Glycopeptide antigens thus represent attractive targets for diagnosis, vaccination, and immunotherapy for cancers, and several glycopeptidic epitopes are under active investigation as therapeutics (5). One of the most common aberrations in glycosylation patterns is the presentation of a single O-linked N-acetylgalactosamine (GalNAc) on a threonine or serine residue (GalNAc- α -O-Thr/Ser) known as the "Tn antigen," which has been associated with many cancers, including breast (6), esophagus, stomach, colon, biliary tract, pancreas (7), and metastatic melanoma (8).

The ubiquitous nature of the Tn antigen has made it a natural focus of vaccine research, and there are several reports of immunizations against various cancers using Tn-conjugated

glycopeptides, with significant progress reported in the generation of a protective immune response (9, 10). Characterization of the structural aspects of antibody binding of the Tn antigen has broad implications. There are several reports of monoclonal antibodies specific for the Tn antigen that are used as histological and diagnostic reagents (11–17), but structural information on the recognition of Tn by proteins is limited to binding by lectins (18, 19). Despite their high profile generally as specific immunotherapeutic targets, there are no reported structures of a monoclonal antibody in complex with a glycopeptide. The only available structure is a glycopeptide-specific antibody in complex with its corresponding unglycosylated peptide (20), and a full characterization of the antibody recognition of clinically relevant glycopeptides is overdue.

Whereas antibodies against Tn and Tn-containing glycopeptides have been reported, the epitopes they target are not tumor-specific but are also expressed on embryonic and nonmalignant tissues. The consequent immune tolerance has required the utilization of allo- or xenogeneic immunization and/or conjugation of glycopeptides to carrier molecules; however, even these methods often failed to produce effective antitumor immune responses (21). In contrast, glycopeptidic epitopes created by tumor-specific mutations are exclusively expressed by the malignant cell, are not tolerogenic, and can elicit humoral immune responses during tumor development, making tumor-specific glycopeptidic neoepitopes ideal targets for antibody-based cancer therapies.

The high affinity syngeneic monoclonal antibody (mAb) 237mAb (IgG2a) is specific for Ag104A, an aggressive fibrosarcoma that arose spontaneously in an aging mouse (22). 237mAb was found to be unreactive with another spontaneous tumor, Ag104B isolated from the same mouse, and was not reactive with any other tumor line tested (22–23). 237mAb was shown to recognize a glycopeptide within the extracellular domain of the tumor-associated glycoprotein podoplanin (also known as aggrus, T1alpha, and OTS8; for a review, see refs. 1 and 24) formed as a result of a tumor-specific mutation in the chaperone gene *Cosmc* that had abolished the functionality of the enzyme core 1 beta3-galactosyltransferase. This disruption of O-glycan core 1 synthesis yielded a tumor-specific glycopeptide antigen

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Data deposition: The atomic coordinates and structure factors (codes) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (<http://www.rcsb.org/>).

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protein podoplanin. The antibody does not combine with the peptide alone or with the peptide carrying the Thomsen–Friedenreich (TF) disaccharide antigen [Gal- β -(1-3)-GalNAc- α -Thr/Ser] at the same position (23). Surface plasmon resonance confirmed that the Fab fragment of 237mAb did not bind at detectable levels to the unglycosylated peptide or to free or conjugated GalNAc (Table S3). The ability of the antibody to discriminate between the disaccharide and monosaccharide antigens is clearly based upon steric reasons because the small stereo-specific GalNAc recognition pocket would be unable to accommodate a disaccharide residue (Fig. 2B and C). The lack of observable binding of the unglycosylated peptide appears to be because of the relative lack of specific contacts compared to the whole glycopeptide epitope (Fig. 2A). This binding mode is in contrast to many structurally characterized antiprotein antibodies, which make extensive specific hydrogen bonds to both the main chain and side chains of the peptide antigens (28).

Interestingly, there was no observable binding of antibody to the free GalNAc glycan, yet it could be cocrystallized with the Fab at concentrations orders of magnitude higher than could be accommodated in the surface plasmon resonance experiment (Table S3). The free GalNAc and the GalNAc moiety of the glycopeptide formed identical interactions with the antibody-combining site, which indicates that, whereas the combining site can accommodate the free glycan, the interaction cannot generate sufficient binding energy to be detected at physiological concentrations. Furthermore, the inability of the unglycosylated peptide to bind suggests that the glycan moiety provides primary specificity and acts as an anchor whereas the peptide portion of the epitope provides additional specificity and binding energy by complementary interactions, thus ensuring that the antibody cannot generate a physiologically relevant interaction for either the unglycosylated peptide or for the carbohydrate alone. This binding mode is consistent with lower affinity of 237mAb for the glycopeptide (Table S3) than is traditionally observed for antibodies specific for peptide antigens, because a higher affinity resulting from a larger number of contacts to the peptide would lead to cross-reactivity with the unglycosylated peptide.

The Tn Antigen Dominates Recognition. Aberrant glycosylation of peptide epitopes has long been shown to strongly affect antigenicity. Truncated glycosylation patterns not only expose normally cryptic carbohydrate antigens such as TF, Tn, and sialyl-Tn antigens (29) but can also expose purely peptide sequences that are normally masked (30). Prototypic examples are among those antibodies specific for blood group M or N glycoporphins and blood group A cross-reacting antibodies against the Tn antigen found in human adenocarcinomas (31, 32). The most highly characterized tumor-specific antiglycopeptide antibodies are those directed against human mucin-1 (MUC1), a heavily glycosylated transmembrane protein that is overexpressed and aberrantly glycosylated in many adenocarcinomas (33). However, none of these antibodies has been structurally characterized.

Monoclonal antibody SM3 binds to an amino acid repeat region (sequence PDTR) of the extracellular portion of MUC1, which contains a glycosylated Thr residue. Although SM3 is specific for a glycopeptide, X-ray crystallography and NMR studies revealed that glycosylation was not required for binding, but that the GalNAc O-glycosylation induced conformational changes in the peptide that enhanced its interactions with the antibody (20, 34). Similarly, mAb C595 raised against another peptide epitope in MUC1 (sequence RPAP) was found to have enhanced affinity because of conformational changes induced by Tn glycosylation, which was attributed to the stabilizing of a left-handed-polyproline II helix by di- or triglycosylation of the peptide (35).

237mAb is the first structurally characterized glycopeptide-specific antibody (as well as the first to be absolutely specific for a glycopeptide tumor antigen) that will not bind the

corresponding unglycosylated or normally glycosylated peptide, and this specificity is immediately understood upon examination of the leading role in recognition played by the carbohydrate moiety. NMR spectroscopy showed only minor differences in the spectra of the glycosylated and unglycosylated peptide, showing that there are no significant structural effects of glycosylation in this antibody–antigen interaction (Fig. S1 and Table S4). The podoplanin-derived glycopeptide antigen bound to 237mAb has little regular secondary structure apart from a single tight turn about Pro 6. Furthermore, the carbohydrate moiety makes no significant contacts with the peptide moiety either in the crystal structure or within the unbound glycopeptide as evidenced by the lack of appropriate rotating-frame Overhauser effect contacts. Thus it is unlikely that the high level of discrimination of the glycopeptide over the peptide antigen is because of any influence of the carbohydrate on the peptide structure. Furthermore, it is clear that the carbohydrate moiety dominates binding and that the specific contacts between the antibody and the peptide moiety can form only after the carbohydrate enters the combining site pocket.

That the unliganded Fab was not observed to crystallize and that crystals of the GalNAc-liganded Fab cracked upon removal of GalNAc raises the possibility that there is a conformational rearrangement of the Fab upon complexation of the sugar that forms a recognition pocket for the peptide. However, there was no observed binding when the antibody was presented with a mixture of free GalNAc and free unglycosylated peptide.

237mAb Contains Features of Both a Peptide- and a Carbohydrate-Specific Antibody. Comparison of 237mAb to other peptide- and carbohydrate-specific antibodies revealed that 237mAb contains features of both types of antibodies. The affinity of carbohydrate-specific antibodies is usually found to be in the low micromolar range whereas peptide specific antibodies have been observed to bind in the nanomolar range. This observation can be partially attributed to the fact that peptide-specific antibodies can more readily undergo affinity maturation. 237mAb has an affinity at the high end of carbohydrate-specific antibodies after having undergone extensive affinity maturation (Table S3 and Fig. S2).

237mAb Paratope Is Composed of Germ-Line Residues. Examination of the germ-line gene segments from which 237mAb is likely derived reveals that the antibody has undergone extensive affinity maturation (Fig. S2); however, the residues that mediate contact between antibody and antigen correspond to germ-line usage with residues that have been mutated during affinity maturation lying almost entirely within the framework region.

Although affinity maturation would appear to provide a process whereby binding may be increased by mutation of residues in direct contact with antigen, several studies show that it often results from changes to identity of residues not in direct contact (36–38) and that affinity maturation can enhance binding by stabilizing the conformation of the antibody-combining site (39–41). This trend appears to be true for 237mAb in complex with antigen, which shows that the recognition of the Tn antigen takes place through residues encoded by primordial and conserved germ-line genes, with subsequent affinity maturation leading to increased affinity.

Recognition of a Tn Antigen Containing Glycopeptide. The Tn antigen is one of the most frequently observed carbohydrate antigens associated with carcinomas (42). However, despite the Tn antigens' potential as a diagnostic and therapeutic target, there have been numerous difficulties in using Tn antigen monosaccharides as diagnostic or therapeutic venues. In particular, the use of murine mAbs directed against the Tn antigen frequently do not succeed in recognizing human tumors (43, 44). Additionally, antibodies and lectins that bind to Tn antigen epitopes are often cross-

reactive with other GalNAc containing structures, severely limiting their potential exploitation (45). The use of Tn antigen monosaccharides for vaccination has been ineffective, and it has been suggested that the minimum immunogenic Tn antigen structure in humans may be a cluster of three or four monosaccharides (9, 46). The observed specificity of 237mAb to a glycopeptide caused by a tumor-specific mutation represents a unique approach to Tn antigen recognition, because the requirement for both a peptide and sugar for effective binding may obviate the need for cluster recognition. Such an antibody directed against a glycopeptidic neopeptide will have a higher specificity than either lectins or anti-Tn mAbs and thus will represent a more valuable tool for tumor diagnostics and therapies (45).

Glycopeptide Antigens in Cancer Immunotherapy. The advantages of exploiting a glycopeptide antigen for cancer immunotherapy are readily apparent. Purely carbohydrate tumor antigens such as Tn, TF, Lewis^{x/y/a}, and sialyl-Lewis^{x/y/a} are found in embryonic and normal adult tissues in addition to tumor cells and are unlikely to elicit T-cell help (3, 47). Many tumor-specific antigens resulting from somatic mutations of amino acid sequences are found largely on intracellular proteins and are not sensible targets for antibody-mediated immunotherapy (48). In contrast, aberrant glycopeptides exported to the cell surface by a faulty ER-Golgi complex to be exposed to both T cells and B cells are a common occurrence in tumors (49). The monoclonal antibody 237mAb specific for the Tn carrying glycopeptide antigen has high affinity and is truly tumor-specific (23). Cosmc mutations creating such Tn-containing tumor-specific antigens have now been described not only for various murine tumors (23) but have been found in human cancers (50) as well as in patients with the autoimmune disease Tn syndrome (51). Cosmc mutations alter glycosylation globally on numerous cell-surface proteins, creating many potential Tn-containing specific glycopeptidic neopeptides that can be targeted by monoclonal antibodies. Therefore, the structural characterization and elucidation of the features and nature of monoclonal antibodies such as the 237mAb will help to improve our understanding in immune recognition of cancer and autoimmune diseases.

Although this epitope was derived from a mouse tumor, several human cancers have also been shown to exhibit abnormal glycosylation because of somatic tumor-specific mutations in the Cosmc chaperone gene for core 1 O-glycan glycosyltransferase, raising the possibility that antibodies similar to 237mAb have potential use in human cancer immunotherapy.

Conclusions. The structure of 237mAb represents the structural characterization of a tumor-specific glycopeptide epitope bound by a monoclonal antibody and a glycopeptide antigen in which the sugar moiety is directly recognized by the antibody. Unlike other reported antibodies that recognize a glycopeptide, 237mAb does not rely on a conformational epitope on the peptide generated by glycosylation but on multiple and specific weak interactions between the antibody and both the sugar and peptide moieties to ensure that only the intact glycopeptide will be recognized. Binding is accomplished entirely by using germ-line residues on the antibody, which shows that antibody recognition of terminal GalNAc residues is a strongly conserved inherited feature. 237mAb achieves its high affinity and specificity through extensive affinity maturation of framework residues mostly adjacent to the combining site.

Materials and Methods

Antigen Synthesis, Antibody Production, and Purification. The syngeneic monoclonal antibody 237mAb (IgG2a) was produced by the hybridoma derived from a C3H/HeN mouse as described previously (22). The antibody was purified from mouse ascites by using a protein A column, according to the manufacturer's instructions (Bio-Rad). Further purification of the IgG was carried out by HPLC anion-exchange chromatography (Tsk-DEAE, LKB

Bromma). The synthesis of the dodecapeptide ERGKPPLEELS and the glyco-dodecapeptide ERGT(GalNAc)KPPLEELS antigens is described in detail in [SI Materials and Methods](#).

Purification and Crystallization of Antibody Fragments. 237mAb IgG was dialyzed into 20 mM Tris pH 8.0 and digested with papain (Sigma) in 5 mM DTT and 2 mM EDTA by using a 1:200 (wt/wt) ratio. After 2 h digestion at room temperature, the reaction was quenched with 10 mM iodoacetamide (final concentration). The reaction mixture was dialyzed overnight against 4 L of 20 mM Hepes pH 7.5. To purify the Fab fragments from the Fc, HPLC cation exchange chromatography was used (Shodex CM-825). The Fab eluted as a single peak from the column by using a linear gradient of 20 mM Hepes pH 7.5 to 20 mM Hepes pH 7.5, 0.5 M NaCl. The protein was concentrated to 12 mg/mL and exchanged into 20 mM Hepes pH 7.5 for crystallization trials.

Crystals were grown over a period of 1–2 months from the 12 mg/mL stock solution. For the glycopeptide complex, antibody was mixed with glycopeptide in a 1:5 molar ratio. Crystals appeared in 22% PEG monomethyl ether 5000, 160 mM ammonium sulphate, 40 mM ZnCl₂, and 0.1 M MES pH 6.5. The crystal appeared within 1 week and grew to a size of 0.3 × 0.4 × 0.2 mm after 3 weeks. For the monosaccharide, crystals were grown in >100 mM GalNAc (Sigma), which represents an approximate 400-fold excess. Crystals appeared after 2 weeks and grew to a final size of 0.3 × 0.5 × 0.1 mm after 7 weeks.

Interestingly, the same approximate conditions that gave the crystals of the Fab in complex with the glycopeptide and the monosaccharide did not yield crystals of the unliganded form. All attempts to grow crystals of the unliganded form from other conditions failed to yield crystals that diffracted beyond 4.0 Å resolution.

Data Collection, Structure Solution, and Refinement. Crystals were transferred to a cryoprotectant solution containing mother liquor, with 20% glycerol for the glycopeptide complex and 30% 2-Methyl-2,4-pentanediol for the monosaccharide complex. Crystals were flash frozen to –160 °C by using an Oxford Cryostream 700 crystal cooler (Oxford Cryosystems). Data were collected with a Rigaku R-Axis 4++ area detector coupled to a MM-002 X-ray generator with Osmic “blue” optics and processed by using the Crystal Clear/d*trek (Rigaku/MSO). The structure of the liganded 237mAb Fab was solved by molecular replacement by using PHASER (52) as implemented in CCP4 (53) by using the variable and constant domains of the monoclonal antibody SYAJ6 (Protein Data Bank accession code 1M71) as a model. SetoRibbon (available from S.V.E. at svevans@uvic.ca) was used for manual fitting of σ_A -weighted 2F_o–F_c and F_o–F_c electron density maps and to produce Figs. 1 A, C, and D and 2. Restrained refinement was carried out with REFMAC5 as implemented in CCP4 and with *phenix.refine* as implemented in Phenix (53, 54).

Surface Plasmon Resonance. Interactions of GalNAc and unglycosylated and glycosylated peptide with immobilized 237mAb Fab were determined by surface plasmon resonance by using a BIACORE3000 (GE Healthcare). For peptide samples, 8,200 resonance units (RUs) of 237mAb Fab and 3,200 RUs of unreligated Fab as a reference were immobilized on research grade CM5-sensorchip (GE Healthcare), respectively. For GalNAc, 4,100 RUs of 237Fab and 6,000 RUs of unreligated mouse IgG as a reference protein were immobilized. Immobilizations were carried out at protein concentrations of 50 µg/mL in 10 mM acetate pH 4.5 by using an amine coupling kit supplied by the manufacturer. In all instances, analyses were carried out at 25 °C in 10 mM Hepes, pH 7.4 containing 150 mM NaCl and 0.005% surfactant P20 at a flow rate of 40 µL/min. The surface was thoroughly washed with the running buffer without regeneration solution. Data were analyzed with BIAevaluation 4.1 software (GE Healthcare).

NMR Spectroscopy of Glycosylated and Unglycosylated Peptide Antigens. High-resolution ¹H-NMR spectra were acquired with a Varian UNITY 500 MHz spectrometer, equipped with a 5-mm triple-resonance z-pulse field gradient probe. Spectra were recorded for 3 mM peptide and glycopeptide at 10 °C in 90% H₂O/10% D₂O pH 6.5 and 6.1, respectively, and processed and analyzed by using Varian software. Standard NMR pulse sequences were used for 2D double quantum filtered COSY, total correlation spectroscopy (100 msec mixing time), and rotating-frame Overhauser effect spectroscopy (300 msec mixing time) experiments. Water peak suppression was obtained by low-power irradiation of the H₂O during the relaxation delay (1.2 s). Proton resonance assignments were obtained by standard methods (27). Coupling constants (³J_{H_NH_α}) were measured directly from 1D and double quantum filtered COSY spectra.

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