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Antibodies that recognize bisected complex *N*-glycans on cell surface glycoproteins can be made in mice lacking *N*-acetylglucosaminyltransferase III

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The bisecting GlcNAc is transferred to complex or hybrid *N*-glycans by the action of *N*-acetylglucosaminyltransferase III (GlcNAc-TIII) encoded by the *Mgat3* gene. CHO cells expressing mouse GlcNAc-TIII were shown by matrix-assisted laser desorption ionization (MALDI) mass spectrometry to produce mainly complex *N*-glycans with the predicted extra (bisecting) GlcNAc. In order to probe biological functions of the bisecting GlcNAc, antibodies that recognize this residue in the context of complex cell surface glycoconjugates were sought. The LEC10 gain-of-function Chinese hamster ovary (CHO) cell mutant that expresses GlcNAc-TIII and complex *N*-glycans with the bisecting GlcNAc was used to immunize *Mgat3*$^{+/+}$ and *Mgat3*$^{-/-}$ mice. ELISA of whole sera showed that polyclonal antibodies that bound specifically to LEC10 cells were obtained solely from *Mgat3*$^{-/-}$ mice. Fluorescence-activated cell cytometry of different CHO glycosylation mutants and western blotting after glycosidase treatments were used to show that anti-LEC10 cell antisera from *Mgat3*$^{-/-}$ mice recognize cellular glycoproteins with complex *N*-glycans containing both a bisecting GlcNAc and Gal residues. The polyclonal antibody specificity was similar to that of the lectin E-PHA. IgM-depleted serum containing IgG and IgA antibodies retained full binding activity. Therefore *Mgat3*$^{-/-}$ mice but not wild type mice can be used effectively to produce polyclonal antibodies that specifically recognize glycoproteins bearing complex *N*-glycans with a bisecting GlcNAc.
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Abbreviations: **Chinese hamster ovary, CHO; enzyme-linked immunosorbent assay; ELISA; *N*-acetylglucosaminyltransferase III, GlcNAc-TIII; polyethyleneglycol, PEG; fluorescence activated cell scanning, FACS; phosphate-buffered saline, PBS; erythroagglutinin from *Phaseolus vulgaris*, E-PHA; Tris-buffered saline, TBS; monoclonal antibody, mAb; HRP; horse radish peroxidase; alkaline phosphatase, AP; matrix-assisted laser desorption ionization-time of flight, MALDI-TOF; polyvinylidene difluoride, PVDF; PI, preimmune.**

Introduction

N-acetylglucosaminyltransferase III (GlcNAc-TIII) is encoded by the *Mgat3* gene in the mouse [1,2] and catalyzes the addition of the bisecting GlcNAc to complex and hybrid *N*-glycans [3]. The presence of the bisecting GlcNAc residue in complex *N*-glycans increases their affinity for the erythroagglutinin from *P. vulgaris* (E-PHA) [4,5] but reduces their affinity for Gal-binding lectins, particularly ricin [6]. Functional consequences of the bisecting GlcNAc are evident in cells expressing the *Mgat3* gene. Thus, LEC10 CHO cells, which express GlcNAc-TIII due to a gain-of-function mutation, are ∼15 times more resistant to the toxicity of ricin and ∼10 times more sensitive to the toxicity of E-PHA than CHO cells [7,8]. In mice over-expressing an *Mgat3* transgene in hepatocytes, a reduction in secretion of apolipoprotein and lipids from liver is observed [9]. GlcNAc-TIII activity is induced during the development of liver tumors in the rat [10–12] and GlcNAc-TIII is also upregulated in human hepatoma [13]. The progression of liver tumors

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is retarded in mice lacking GlcNAc-TIII [14,15] which appears to be due to the absence of the bisecting GlcNAc residue on N-glycans of glycoprotein(s) from a tissue other than liver [15].

Structural differences amongst oligosaccharides in glycoconjugates due to linkage or the presence of a single sugar residue like the bisecting GlcNAc have functional consequences. The ability to discriminate such differences is of interest for both experimental applications and diagnostics. Many blood cell carbohydrate antigens [16], glycolipids [17] and carbohydrate differentiation antigens such as the stage specific embryonic antigen SSEA-1 [18] give rise to monoclonal antibodies. Several monoclonal antibodies directed to the sugar chains of N-linked glycoproteins and mucins have been reported [19–23] and a monoclonal antibody to a glycan determinant present on many serum glycoproteins has also been obtained [24].

Antibodies to specific carbohydrates have been prepared using whole cells, pure glycoconjugates, oligosaccharide-carrier protein complexes and pyridylaminated glycans [20,23,25–27]. In the present study, LEC10 CHO cells that express N-glycans with a bisecting GlcNAc [7] were used to immunize wild type (*Mgat3*$^{+/+}$) mice and mice lacking N-acetylglucosaminytransferase III (*Mgat3*$^{-/-}$) in attempts to produce antibodies that recognize glycoproteins with bisected N-glycans. Sera from *Mgat3*$^{-/-}$ but not wild type mice contained antibodies that bound specifically to LEC10 cell glycoproteins. Western analysis after glycosidase treatments showed that the polyclonal antibody binding specificity was for complex N-glycans with a bisecting GlcNAc and Gal residues.

Materials and methods

Cell lines and cell culture

Pro$^-$5 CHO, Pro$^-$Lec1.3C [28], Pro$^-$Lec8.3D [29], Pro$^-$LEC10.3C [7], Pro$^-$LEC10.Lec8 [30] CHO cells and glycosylation mutants were used. CHO/GlcNAc-TIII and LEC10T transfectants overexpress GlcNAc-TIII from a mouse Mgat3 cDNA stably expressed in CHO or LEC10 CHO cells respectively [31]. All CHO cells were grown in suspension at 37°C in complete α medium (Life Technologies, Inc.) containing 10% fetal bovine serum (Life Technologies, Inc.).

Release of N-glycans and MALDI-TOF mass spectrometry

After washing in 20 mM Tris HCl pH 7.2, CHO and CHO/GlcNAc-TIII cell glycoproteins were extracted in 1.5% Triton X-100, and ~300 μg protein in 10 μl was added to wells of a 96-well MultiScreen-IP plate with polyvinylidene difluoride (PVDF) membrane (pore size 0.45 μm, Millipore). After being reduced and carboxymethylated as described [32], PNGase F (New England Biolabs) 0.5 μl (4 mU) was added to each well containing 50 μl of 10 mM Tris-acetate (pH 8.3) and the plate incubated at 37°C for 3 hr. Released N-glycans were treated with 0.1 volume of 150 mM acetic acid for 3 hr, dried, redissolved in 200 μl of deionized water and desalted on a 0.3 ml cation-exchange column (AG50W-X8 resin, hydrogen form, 100–200 mesh; Bio-Rad) in a Micro Bio-Spin column (Bio-Rad). After drying, samples were dissolved in 30 μl of deionized water. The matrix used in positive ion mode was prepared by dissolving 2 mg of 2,5-dihydroxybenzoic acid and 0.1 mg of 5-methoxysalicylic acid in 1 ml of ethanol/2 mM acqueous sodium chloride 1:1 (v/v). The mass spectrometer was a Voyager-DE^{TM} STR Biospectrometry Workstation (PerSeptive Biosystems, Framingham, MA) equipped with a delayed extraction. Spectra were acquired in the linear configuration (2 m flight path). The samples were irradiated with a pulsed nitrogen laser at 337 nm. After 500 ns delay, ions were accelerated with 20 kV. The grid wire and guide wire voltages were 95 and 0.2% of the accelerating voltage, respectively. For each spectrum, 100 scans were averaged. A two-point external calibration using oligosaccharide standards was used for mass assignment of the ions.

Immunization of mice with LEC10 CHO cells

Six week old female Balb/c *Mgat3*$^{+/+}$ mice and *Mgat3*^{T37T37} mice that carry an inactivate *Mgat3* gene due to a targeted neo gene insertion in the coding region [14,31] and termed *Mgat3*$^{-/-}$ mice herein for simplicity, were bled to obtain preimmune (PI) serum and injected intraperitoneallly with LEC10 cells (1×10^7), suspended in 0.3 ml of phosphate-buffered saline (PBS). Cells were injected every week thereafter for 5 weeks. At 6 weeks, cells (1×10^7) were mixed with Complete Freund's adjuvant (Pierce) (1:1; v/v), and injected intraperitoneallly. Mouse serum was obtained by orbital or tail bleeding at 5, 10, and 16 weeks after the first injection. Thimerisol was added to 0.05% (v/v) to sera before storage at 4°C.

ELISA assay

Binding of antibodies to parent and LEC10 CHO cells was determined by ELISA. Cells (4×10^4) were added to each well of a 96 well plate and cultured overnight. After washing 3 times with PBS containing 0.1% Tween 20 (PBS/T), cells were fixed on the plate with 0.5% glutaraldehyde in PBS (25°C for 1 h). Glutaraldehyde was inactivated by adding PBS containing 100 mM lysine and 10 mM ethanolamine (37°C for 1 h) and the plates were blocked with PBS containing 2% bovine serum albumin (PBS/BSA) at 37°C for 1 h. Absorbed mouse serum or culture supernatant (50 μl) was incubated with the fixed cells for 1 h at 37°C. After washing 3 times with PBS/T, goat anti-mouse IgG-alkaline phosphatase (IgG-AP) conjugate (Fisher), diluted 1:2000 with PBS/BSA, was added (50 μl/well) and incubated for 1 h at 37°C. After washing with PBS/T, AP activity was detected using p-nitrophenylphosphate (1 mg/ml; 50 μl/well) and absorbance was read at 405 nm after incubation for 20 min at 37°C.

Preparation of CHO-absorbed mouse serum

Whole serum was diluted 1/1000 with PBS/BSA containing 0.02% sodium azide. Diluted serum (0.3 ml) was resuspended with Pro^{-}5 parent CHO cells (1×10^7) in a 1.5 ml microcentrifuge tube. The suspension was stirred at 4°C for 7–12 h and the cells removed by centrifugation in a microfuge for 1 min. The supernatant was absorbed twice more with CHO cells in the same way and polyclonal antibodies were stored at 4°C.

Fluorescence-activated cytometry

Parent and mutant CHO cells were washed with PBS three times and resuspended at 1×10^6 cells/ml in PBS/BSA. Cells (0.2 ml) were incubated with 50 μl of CHO-absorbed mouse serum for 1 h at 4°C on a rotator. After washing with 3 ml PBS, the cells were resuspended with 0.2 ml PBS/BSA and 30 μl of goat anti-mouse IgG-fluorescein (0.1 μg/μl) were added to the cell suspension. The mixture was incubated for 1 h at 4°C on a rotator. The cells were washed with 3 ml PBS and resuspended in 0.3 ml PBS. The samples were analyzed on a Becton Dickson FACS Pstar Plus.

Western blot analysis

CHO cells (1×10^7 cells) were washed 3 times with 10 ml PBS containing 0.01 M $CaCl_2$, 0.01 M $MnCl_2$, 0.01 M $MgCl_2$, 0.02% azide. Following extraction in 1.5% Triton X-100 and protease inhibitor tablet for 10 min on ice, nuclei were removed by centrifugation. Glycerol was added to 20% by volume, and extracts were stored at −80°C. Protein was measured by DC protein dye reagent (Bio-Rad) using bovine serum albumin (Sigma) as standard. Cell extracts (50 μg protein) were electrophoresed in a 10% SDS-PAGE reducing gel. The gel was transferred to polyvinylidene difluoride membrane (Dupont, NEN) in Tris base, 192 mM glycine, and 5% methanol at 0.1 amps. After rinsing with Tris-buffered saline (TBS, pH 7.2), membrane was blocked with 5% nonfat milk in TBS with 0.01% Thimersol for 1 h at 37°C. The membrane was incubated with CHO-absorbed mouse sera for 1 h at 37°C or with biotinylated E-PHA (1 μg/ml; Vector) in TBS containing 0.05% NP-40 (TBS/N) and 5% non-fat milk. After washing for 30 min with several changes of TBS/N, membrane was incubated in a 1:5000 dilution of goat-anti-mouse IgM and IgG-horse radish peroxidase (IgG-HRP; Pierce) or in 0.2 μg/ml horse radish peroxidase-streptavidin (Vector) in TBS/N containing 5% non-fat milk for E-PHA for 1 h at 37°C. The blot was washed six times with TBS/N and twice with TBS before exposure to ECL reagent (Dupont, NEN) for 1 min and to X-ray film. Blots were stripped with stripping buffer (62.5 mM Tris-HCl buffer, pH 6.8 containing 2% SDS and 100 mM 2-mercaptoethanol) for 30 min at 50°C and reused as described.

Glycosidase digestions

For removal of N-glycans from CHO glycoproteins, 50 μg cell extract prepared as for western blotting was incubated for 10 min at 80°C in 0.5% SDS. After cooling to room temperature, PNGase F buffer (Oxford Glycosystems) and 1 unit PNGase F were added and the mixture was incubated for 9 h at 37°C. For glycosidase treatments, 50 μg cell extract was incubated with 50 mU of neuraminidase from *Clostridium perfringens* (Sigma), 5 mU of β-galactosidase from *Diplococcus pneumoniae* (Boehringer Mannheim), or 62 mU Jack bean N-acetylglucosamindase (Sigma) in 15 mM sodium cacodylate buffer, pH 5.5, either singly or together at 37°C under toluene. The enzymes were added three more times and the incubation continued for 7 days.

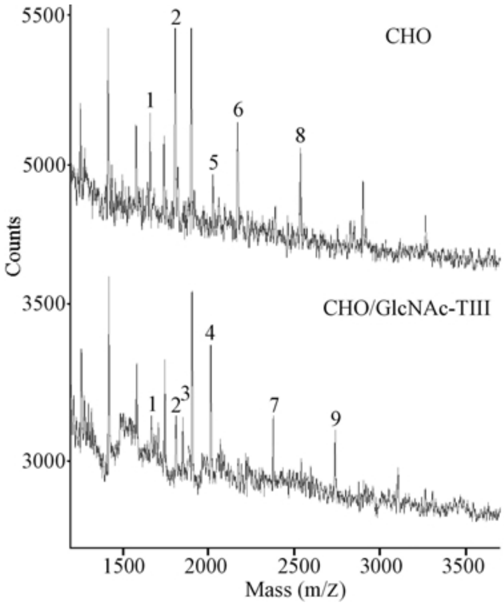
IgM depletion and isotyping

Isotyping of the CHO cell absorbed Ab17 was performed by ELISA using 50 μl of 1:500 diluted goat anti-mouse immunoglobulin standards (Southern Biotechnology). One ml of rat anti-mouse IgM beads (Dynal) was mixed with 1 ml of Ab17 at 37°C for 1 h on a rotator. The mixture was centrifuged and supernatant was used for western analysis. The beads were washed with 75 mM HEPES buffer, pH 7.2 and bound material was twice eluted with 0.5 ml 75 mM HEPES buffer, pH 7.2 containing 3.0 M $MgCl_2$ and 25% ethylene glycol. The combined eluates were dialyzed against PBS and used for western analysis.

Results

Complex N-glycans of CHO cells expressing GlcNAc-TIII

Previous radiolabeling and ^{1}H-NMR spectroscopy showed that the G glycoprotein from vesicular stomatitis virus produced in LEC10 cells carries complex biantennary N-glycans with the bisecting GlcNAc [7,30]. To examine the general population of complex N-glycans on glycoproteins from CHO cells expressing GlcNAc-TIII, extracts from CHO and CHO cells stably expressing a mouse cDNA encoding GlcNAc-TIII (CHO/GlcNAc-TIII) were treated with peptide N-glycosidase F (PNGase F) and the released N-glycans were analyzed by MALDI-TOF mass spectrometry (Figure 1). The major species of neutral complex N-glycans detected in positive ion mode are identified by numbers in the spectra, and the corresponding observed masses are given in the table along with the predicted N-glycan structure. It can be seen that biantennary N-glycans were present but reduced in amount in CHO/GlcNAc-TIII cells (peaks 1 and 2), whereas biantennary N-glycans with an extra (bisecting) GlcNAc (not detected in parent CHO) were prevalent (peaks 3 and 4). Similarly, tri-and tetra-antennary N-glycans were present in CHO (peaks 5, 6 and 8) and absent from CHO/GlcNAc-TIII cells. Instead GlcNAc-TIII expressing CHO cells had tri- and tetra-antennary N-glycans with an extra (bisecting) GlcNAc (peaks 4, 7 and 9). Therefore the majority of complex N-glycans produced by CHO cells expressing GlcNAc-TIII carry a bisecting GlcNAc as we observed previously for complex N-glycans from mouse kidney glycoproteins [31].



No.	Mass		Composition	Structure
	Calc.	Obs.		
1	1663.6	1664.1	$G_2Gn_2M_3Gn_2$	
2	1809.5	1810.3	$G_2Gn_2M_3Gn_2F$	
3	1850.7	1851.6	$GGn_3M_3Gn_2F$	
4	2012.7	2013.3	$G_2Gn_3M_3Gn_2F$	
5	2028.7	2028.8	$G_3Gn_3M_3Gn_2$	
6	2174.7	2175.2	$G_3Gn_3M_3Gn_2F$	
7	2377.9	2378.0	$G_3Gn_4M_3Gn_2F$	
8	2539.7	2540.4	$G_4Gn_4M_3Gn_2F$	
9	2742.9	2743.1	$G_4Gn_5M_3Gn_2F$	

●, galactose; ■, N-acetylglucosamine; ○, mannose; Δ, fucose

Figure 1. MALDI-TOF mass spectrometry of *N*-glycans from CHO and CHO/GlcNAc-TIII cells. Mass spectra of PNGase F released neutral *N*-glycans from CHO and CHO/GlcNAc-TIII cells are on the left. Major complex *N*-glycan species are identified by numbers on the spectra and in the table with calculated and observed masses of the deduced structures. Most CHO/GlcNAc-TIII complex *N*-glycans have an extra (bisecting) GlcNAc that is absent from CHO complex *N*-glycans.

Only *Mgat3*$^{-/-}$ mice produce antibodies that specifically recognize LEC10 CHO cells

A cohort of *Mgat3*$^{+/+}$ and *Mgat3*$^{-/-}$ mice were immunized with LEC10 CHO cells that express GlcNAc-TIII [7]. Sera were prepared by orbital bleeding before and at various times after immunization, and tested for binding to fixed CHO and LEC10 cells by ELISA. First bleed sera from *Mgat3*$^{+/+}$ or *Mgat3*$^{-/-}$ mice showed no difference in binding to CHO compared to LEC10 cells. Second bleed sera from wild type *Mgat3*$^{+/+}$ mice also did not show better binding to LEC10 versus CHO cells. By contrast, second bleed sera from all five mutant *Mgat3*$^{-/-}$ mice gave significantly more binding to LEC10 cells compared to CHO cells (not shown). In order to remove antibodies that bound to parent CHO cells, second bleed sera were diluted 1000-fold and incubated with CHO cells. After three rounds of absorption, sera were tested for binding to LEC10 and CHO cells. It can be seen from Figure 2 that wild type mice did not mount a significant immune response to LEC10 cells, whereas sera from all five *Mgat3*$^{-/-}$ mice gave a positive ELISA response at a dilution of 1:1000. The 3rd and 4th bleed sera from *Mgat3*$^{-/-}$ mice gave similar binding to LEC10 cells after CHO cell absorption. Attempts to induce *Mgat3*$^{+/+}$ mice to mount a LEC10 cell specific immune response were made using the immunosuppressant cyclophosphamide [33]. By selectively killing B cells that were responding to CHO cells prior to injection with LEC10 cells it was hoped to target the immune response to LEC10-specific structures. However, this was not observed to occur.

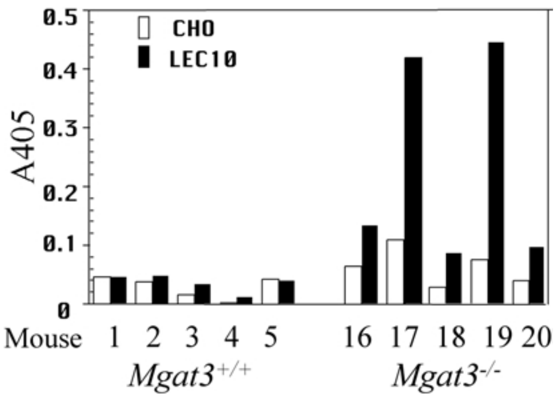


Figure 2. Polyclonal antibodies specific for LEC10 CHO cells. Five wild type (Balb/c) *Mgat3*$^{+/+}$ mice and 5 *Mgat3*$^{-/-}$ mice were immunized with LEC10 CHO cells as described in Methods. Sera obtained 10 weeks after the first injection were diluted 1000-fold and absorbed with CHO cells. An ELISA was performed with the absorbed sera to determine binding to fixed CHO and LEC10 cells, respectively. Bound polyclonal antibodies were detected with goat anti-mouse IgG-AP.

Polyclonal Ab17 binds only to CHO cells expressing *N*-glycans with a bisecting GlcNAc

Polyclonal serum from the third bleed of mouse #17 was diluted 1000-fold and absorbed with CHO cells. This serum (termed Ab17) was tested by FACS analysis for binding to CHO and LEC10 cells, as well as to CHO glycosylation mutants defective in *N*-glycan biosynthesis. CHO cells have glycoproteins with

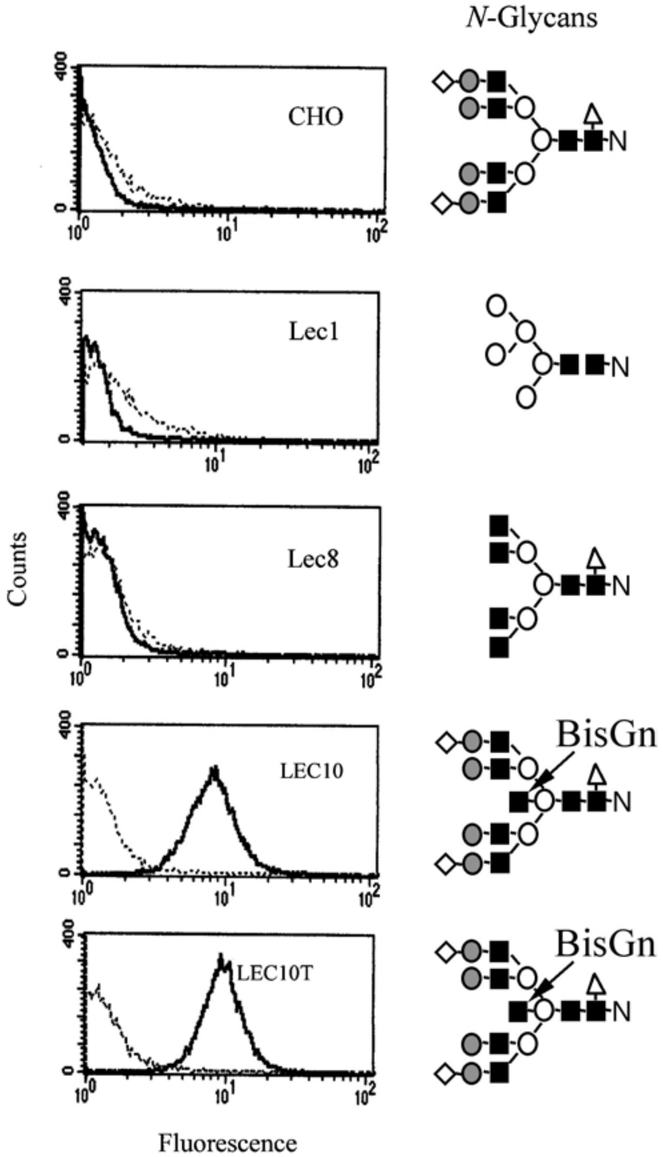


Figure 3. Polyclonal Ab17 binds specifically to CHO cells expressing GlcNAc-TIII. FACS analysis of parent CHO and various CHO glycosylation mutants was performed with absorbed serum from the third bleed of mouse #17 (Figure 2) and termed Ab17 (thick line), or absorbed preimmune serum (thin line). The *N*-glycans depicted represent the effect of the respective glycosylation mutation on complex *N*-glycan structure. Sialic acid (diamond); Gal (gray circle); GlcNAc (black square); Man (circle); Fuc (triangle).

sialylated and neutral complex *N*-glycans that do not contain the bisecting GlcNAc ([32] and Figure 1). LEC10 CHO mutants express GlcNAc-TIII [7] and LEC10T cells are LEC10 cells overexpressing GlcNAc-TIII from both their endogenous *Mgat3* gene and a transfected mouse Mgat3 cDNA controlled by the CMV promoter [31]. Both LEC10 and LEC10T cells carry bisected, complex *N*-glycans on glycoproteins. Lec1 CHO mutants lack *N*-acetylglucosaminyltransferase I activity [28] and do not make complex or hybrid *N*-glycans. Lec8 CHO mutants have a defective UDP-Gal transporter [29] and carry non-galactosylated *N*- and *O*-glycans on cell surface glycoproteins and glycolipids. The cytometry scans in Figure 3 show that Ab17 bound to LEC10 and LEC10T cells but not to CHO, Lec1, or Lec8 cells. Therefore polyclonal Ab17 recognizes cells with cell surface glycoproteins that possess bisected complex *N*-glycans (LEC10T), but it does not bind to wild type CHO cells with non-bisected complex *N*-glycans, nor to cells that lack Gal on complex *N*-glycans (Lec8), nor to cells expressing only oligomannosyl *N*-glycans on glycoproteins (Lec1).

Polyclonal Ab17 has a specificity similar to E-PHA and does not bind to protein

To investigate whether Ab17 recognizes only *N*-glycans, or may bind to other glycans or protein determinants, western blotting was performed with cell extracts from CHO and LEC10T cells. Several glycoprotein bands were detected in LEC10T extract that were absent from CHO extract (Figure 4). The same bands were also bound specifically by E-PHA under conditions in which E-PHA detects only complex *N*-glycans that contain the bisecting GlcNAc (Figure 4). Following treatment with PNGase F, the bands recognized by Ab17 and E-PHA were no longer present. PNGase-F removes *N*-glycans from glycoproteins by cleaving at the Asn-linked GlcNAc [34]. Therefore, Ab17 contains polyclonal antibodies specific for complex *N*-glycans with a bisecting GlcNAc that are also recognized by

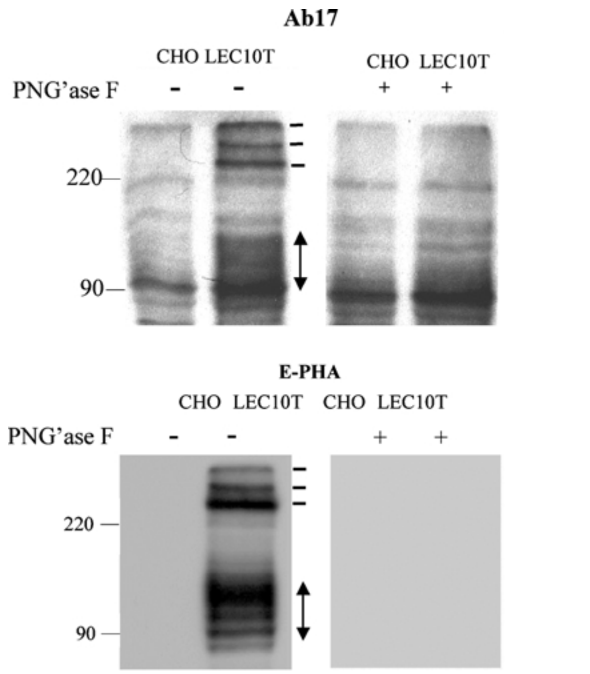


Figure 4. Polyclonal Ab17 binds to *N*-glycans. Cell extracts of CHO and LEC10T cells were treated with PNGase F and subjected to western blot analysis with Ab17 or E-PHA as described in Methods.

E-PHA, and neither Ab17 nor E-PHA bind after the removal of N-glycans by PNGase F treatment.

Polyclonal Ab17 requires both galactose and the bisecting GlcNAc for binding to N-glycans

The cohort of CHO cell glycoproteins recognized by the antibodies in Ab17 was similar to those bound by E-PHA. E-PHA binds with highest affinity to complex N-glycans containing the bisecting GlcNAc and Gal residues [6]. To determine if Ab17 has a similar requirement for Gal residues, binding was examined to LEC10.Lec8 cell extract. LEC10.Lec8 cells carry two glycosylation mutations—a gain-of-function *LEC10* mutation that activates expression of GlcNAc-TIII and a *lec8* mutation that inactivates the UDP-Gal Golgi transporter [30]. Thus glycoproteins carrying N-glycans with the bisecting GlcNAc will not be galactosylated in LEC10.Lec8 cells. It can be seen in Figure 5 that LEC10.Lec8 glycoproteins did not bind Ab17, whereas glycoproteins from LEC10T cells were bound by Ab17 as shown in Figure 4. Thus polyclonal Ab17 recognizes complex N-glycans that must contain both a bisecting GlcNAc (in LEC10T and LEC10.Lec8 but not in CHO) and Gal residues (not in LEC10.Lec8).

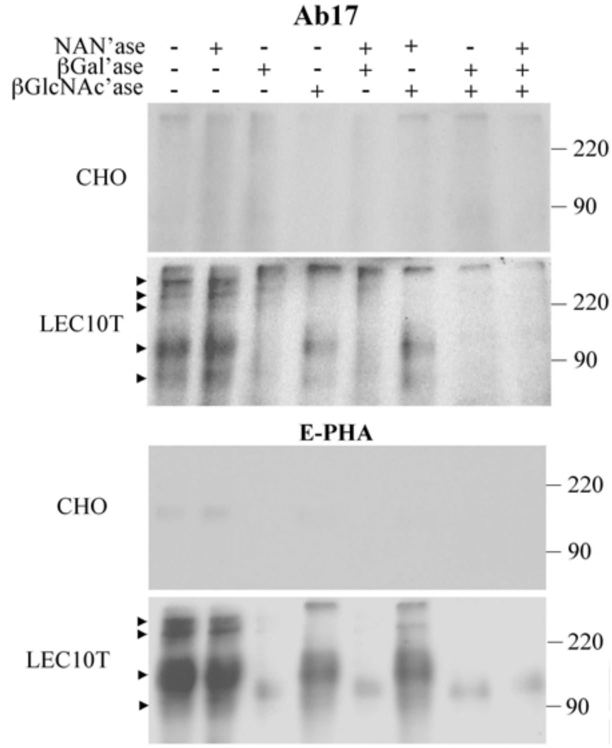


Figure 6. Polyclonal Ab17 binds to *N*-glycans with GlcNAc and Gal. Cell extracts of CHO and LEC10T were treated with various exoglycosidases, subjected to 10% SDS-PAGE, transferred and blotted with Ab17 or E-PHA. NAN'ase, neuraminidase from *C. perfringens*; βGal'ase, β-galactosidase from *D. pneumoniae*; βGlcNAc'ase, N-acetylglucosaminidase from Jack bean.

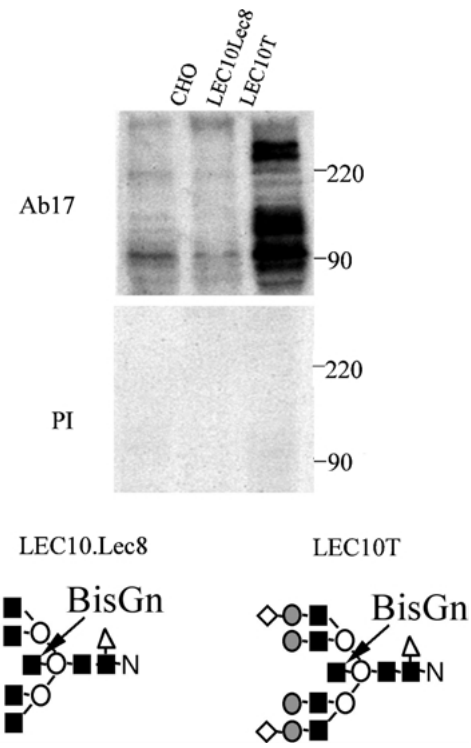


Figure 5. Polyclonal Ab17 binds to *N*-glycans with a bisecting GlcNAc and Gal. Immunoblotting of cell extracts from CHO, LEC10.Lec8, and LEC10T cells with Ab17 or preimmune (PI) serum was performed as described in Methods. The *N*-glycan structures depicted represent the effect of the respective glycosylation mutation in each mutant on complex *N*-glycan structure. Sialic acid (diamond); Gal (gray circle); GlcNAc (black square); Man (circle); Fuc (triangle).

The binding specificity of polyclonal Ab17 was further investigated and compared to that of E-PHA by glycosidase treatments of CHO and LEC10T extracts followed by western blot analysis (Figure 6). Neuraminidase treatment did not significantly alter binding of either Ab17 or E-PHA but β-galactosidase treatment markedly reduced the binding of both to LEC10T glycoproteins. β-N-acetylglucosaminidase treatment also reduced binding but somewhat less markedly. However, when the LEC10T extract was treated with both β-galactosidase and β-N-acetylglucosaminidase, no specific bands were detected by Ab17 or E-PHA. Therefore polyclonal Ab17, like E-PHA, recognizes the bisecting GlcNAc residue in the context of a complex, galactosylated N-glycan.

Polyclonal Ab17 was shown by isotype analysis to contain IgM, IgG and IgA antibodies. Many antibodies to sugars are IgM. To determine if the antibodies specific for bisected N-glycans in Ab17 were IgM, an aliquot of Ab17 was incubated with anti-mouse-IgM beads and the depleted serum, as well as bound and eluted IgM antibodies, were tested for binding to LEC10T glycoproteins by western analysis. The IgM-depleted serum retained all bisected N-glycan-specific binding activity and eluted IgM Ab17 antibodies did not bind to LEC10 glycoproteins (data not shown). Thus IgG (or IgA) antibodies in polyclonal Ab17 are responsible for recognizing complex N-glycans with a bisecting GlcNAc.

Discussion

N-glycans of glycoproteins are poorly immunogenic in mice and rats and thus there are few monoclonal or polyclonal antibodies to the core region of N-glycans. To improve immunogenicity, N-linked glycans have been derivatized with lipid or 2-aminopyridine [20,21]. Monoclonal antibody MT-5 was produced following immunization with pyridylaminated, galactosylated, bisected biantennary N-glycan antigen [20]. The reactivity of MT-5 is increased by β-galactosidase treatment and decreased upon subsequent treatment of antigen with β-N-acetylglucosaminidase. Thus MT-5 may be directed to an epitope that includes the bisecting GlcNAc residue, or it may simply recognize terminal GlcNAc residues, regardless of linkage. The monoclonal antibody, OMB4, was generated using neoglycolipids, which were derived by conjugation of asialo-, agalacto- and bisected biantennary N-glycans to phosphatidylethanolamine dipalmitoyl [21]. However, OMB4 was shown to preferentially recognize non-reducing terminal GlcNAc residues with no specificity for the bisecting GlcNAc. In this paper, we used *Mgat3*$^{-/-}$ mice that lack GlcNAc-TIII activity and do not add the bisecting GlcNAc to N-glycans [14,31] to produce polyclonal antibodies that recognize glycoproteins carrying bisected N-glycans. Other mice with a targeted gene mutation have been useful in producing antibodies against autologous antigens [36]. More recently, mice immunologically naive to complex gangliosides were used as hosts to raise high-affinity, anti-ganglioside IgG antibodies [37]. The immunogen used herein was LEC10 CHO cells which express GlcNAc-TIII due to the activation of the endogenous *Mgat3* gene, a quiescent gene in parent CHO cells (X. Yang and P. Stanley, unpublished observations). Polyclonal antibodies that recognize both Gal and the bisecting GlcNAc on N-glycans were obtained. These antibodies differ from the monoclonal antibodies MT-5 and OMB4 in that they absolutely require the presence of the bisecting GlcNAc for binding. Polyclonal Ab17 does not recognize oligomannosyl N-glycans or sialic acid or Gal in the absence of the bisecting GlcNAc. Ab17 specifically binds to LEC10 cells, which express the bisecting GlcNAc, but does not bind to the LEC10.Lec8 double mutant that has the bisecting GlcNAc but does not carry Gal on N-glycans [30]. Based on these binding properties and the effects of sequential glycosidase treatments on binding to glycoproteins from LEC10 cells (Figure 6), the specificity of polyclonal Ab17 for galactosylated, bisected complex N-glycans was shown to be very similar to that of the lectin E-PHA.

The properties of polyclonal Ab17 establish the fact that *Mgat3*$^{-/-}$ mice can be stimulated to produce antibodies against bisected N-glycan immunogens to which wild type mice (and presumably other mammals) are tolerant. Thus a range of antibodies recognizing the bisecting GlcNAc in the context of different N-glycan structures typically expressed on mature glycoproteins in a complex setting like the cell surface might be obtained. In addition, *Mgat3*$^{-/-}$ mice might produce antibodies, potentially of the IgG class, specific for the bisecting GlcNAc residue itself that would recognize the bisecting GlcNAc whenever it occurs in all N-glycans. Most monoclonal antibodies against N-glycans belong to the low-affinity IgM type, which may be technically difficult to work with and likely to show nonspecific cross-reactivity. However, glycans conjugated to biotinlylated diaminopyridine evoke an IgG immune response in mice [35]. Using this method, high affinity IgG monoclonal antibodies (mAb) directed against the core region of non-bisected complex N-glycans containing fucose on the Asn-GlcNAc have been generated [23].

Unfortunately, attempts to produce LEC10-specific monoclonal antibodies were not successful. In three separate experiments, spleen cells from mouse #17 or #19 (Figure 2) were fused with myeloma cells Ag8.653 or NSO or NSO/Bcl-2 that overexpress Bcl-2 and rescue self-reactive B cells that are anergic or undergoing apoptosis [38]. Hybridomas were obtained and several produced antibodies that bound to LEC10 cells at least 2-4-fold better than to CHO cells. However, clones obtained by limiting dilution invariably lost LEC10 binding specificity during subsequent culture. Attempts to obtain monoclonal antibodies from ascites following injection of positive hybridoma clones into SCID mice were also not successful (J. H. Lee, S. Buhl, M. Scharff and P. Stanley, unpublished observations). These difficulties may potentially be overcome by using different myeloma cells as fusion partners, by cloning onto a feeder layer of cells after fusion, or by purifying B cells away from potentially cytotoxic cells or factors present in spleen cell populations before fusion. Ultimately, it is hoped to obtain monoclonal antibodies from *Mgat3*$^{-/-}$ mice that will recognize specific aspects of bisected N-glycan structures.

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