Recognition of N-Glycoforms in Human Chorionic Gonadotropin by Monoclonal Antibodies and Their Interaction Motifs*

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The glycosylation of human chorionic gonadotropin (hCG) plays an important role in reproductive tumors. Detecting hCG N-glycosylation alteration may significantly improve the diagnostic accuracy and sensitivity of related cancers. However, developing an immunoassay directly against the N-linked oligosaccharides is unlikely because of the heterogeneity and low immunogenicity of carbohydrates. Here, we report a hydrogen/deuterium exchange and MS approach to investigate the effect of N-glycosylation on the binding of antibodies against different hCG glycoforms. Hyperglycosylated hCG was purified from the urine of invasive mole patients, and the structure of its N-linked oligosaccharides was confirmed to be more branched by MS. The binding kinetics of the anti-hCG antibodies MCA329 and MCA1024 against hCG and hyperglycosylated hCG were compared using biolayer interferometry. The binding affinity of MCA1024 changed significantly in response to the alteration of hCG N-linked oligosaccharides. Hydrogen/deuterium exchange-MS reveals that the peptide β65–83 of the hCG β subunit is the epitope for MCA1024. Site-specific N-glycosylation analysis suggests that N-linked oligosaccharides at Asn-13 and Asn-30 on the β subunit affect the binding affinity of MCA1024. These results prove that some antibodies are sensitive to the structural change of N-linked oligosaccharides, whereas others are not affected by N-glycosylation. It is promising to improve glycoprotein biomarker-based cancer diagnostics by developing combined immunoassays that can determine the level of protein and measure the degree of N-glycosylation simultaneously.

Early diagnosis is critical in cancer treatment. Both therapy efficacy and survival rate can be significantly improved if specific and sensitive cancer biomarkers are available to facilitate the diagnosis and prognosis (1, 2). As of 2013, the Food and Drug Administration (FDA) has approved approximately 15 biomarkers for monitoring drug response, performing surveillance, or monitoring the recurrence of cancers (3). The majority of these biomarkers are glycoproteins (4). The abnormal glycosylation of proteins has been revealed to participate in the occurrence and development of many diseases, including various cancers (5, 6). For example, aberrant prostate specific antigen glycosylation is associated with prostate cancer (7). The fucosylation in alpha-fetoprotein N-linked oligosaccharides has been proven as an indicator for hepatocellular carcinoma (8). Simultaneously monitoring the level of protein biomarkers and their glycosylation modifications may significantly improve the accuracy of diagnostics. Clinically, the concentration of protein biomarkers in patients’ serum or urine is usually measured by an in vitro immunoassay using mAbs (9, 10). However, it is difficult to develop mAbs that can specifically bind to aberrant oligosaccharide structures due to the low immunogenicity and the heterogeneity of carbohydrates.

hCG4 is a glycoprotein hormone mainly produced by the placental syncytiotrophoblast cells (11). It can also be secreted by several normal non-placental tissues and certain cancerous tumors (12). The hetero-dimer structure of hCG consists of an

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4 The abbreviations used are: hCG, human chorionic gonadotropin; hCG-deN, N-deglycosylated hCG; hCG-H, hyperglycosylated hCG; BLI, biolayer interferometry; ESI, electrospray; HDX, hydrogen/deuterium exchange; PGC, porous graphitized carbon; PNGase F, peptide-N-glycosidase F.
**HCG N-Glycoforms Bind mAbs Differently**

α subunit and a β subunit that are held together by non-cova-

tent hydrophobic and ionic interactions (13). The α subunit of

cGG consists of 92 amino acid residues with two N-glycosyla-

tion sites, Asn-52 and Asn-78. The β subunit consists of 145

amino acid residues with two N-glycosylation sites, Asn-13 and

Asn-30, and four O-glycosylation sites, Ser-121, Ser-127, Ser-

132, and Ser-138 (14). Additionally, Thr-54 of the α subunit is

occasionally O-glycosylated (15). The carbohydrate moiety ac-

counts for ~30% of the total molecular mass of HCG, which is

~36.7 kDa (16). HCG-H is a major glycosylation variant of HCG,

in which oligosaccharide chains become more complicated

than the normal form (17). HCG-H has been reported to contain

larger fucosylated sialyl-N-acetyllactosamine tri-antennary

oligosaccharides at the two N-glycosylation sites on the β sub-

unit and abnormal tri-antennary oligosaccharides with the α,1,3

antenna terminating in mannose at the two N-glycosylation

sites on the α subunit (18). In addition, HCG-H has a particu-

lar double molecular size O-linked oligosaccharide when com-

pared with the normal trisaccharide structures (19). The roles

of HCG-H include promoting the invasion and growth of cho-

riocarcinoma and reproductive cancer cells, as well as driving

malignancy in these cancers (20).

Extensive efforts have been made to develop mAbs that can
distinguish the level of HCG-H from the total HCG in human
serum or urine to improve the diagnostic specificity and accu-

racy for various cancers, including choriocarcinoma, placental
site trophoblastic tumors, and testicular germ cell tumors (21).

The mAb B152, recognizing core 2 type O-glycosylation at Ser-

132 of the HCG β subunit, has been developed to detect this

abnormal HCG glycosylation isomer in Down syndrome preg-
nancies and trophoblastic disease (22–24). The mAb CTP104

has been reported to recognize the O-linked oligosaccharides at

Ser-138 in the C-terminal portion of the HCG β subunit, bind-
ing to both the core 1 and the core 2 type glycoforms (22).

Efforts to develop an anti-HCG mAb that can specifically bind to
the aberrant N-linked oligosaccharides have been unsuccessful

due to the low immunogenicity of carbohydrates. However,
the more branched and elongated N-linked oligosaccharides may change the spatial structure of HCG-H and affect its bind-
ing affinity to certain mAbs, especially ones with epitopes adja-
cent to the N-glycosylation sites.

In this study, the HDX coordinated with electrospray (ESI)-MS epitope mapping technique was applied to discovering the spatial relationship between the N-glycosylation sites of HCG and epitopes of antibodies with different binding affinities to normal and aberrant HCG N-glycoforms. We demonstrated that the binding affinity of mAb MCA1024 to HCG was signif-

icantly affected by the N-glycosylation patterns, whereas mAb

MCA329 exhibited no obvious changes in binding affinity to
HCG with different N-linked oligosaccharides. The structural

motif of the distinct HCG antigen-antibody interaction patterns in relation to N-glycosylation was discussed. These findings have important implications for monitoring hyper-N-glycosyl-
ated HCG and diagnosing choriocarcinoma, germ cell tumors, and other diseases in which alterations to HCG N-glycosylation may be involved.

**Experimental Procedures**

**Materials**—HCG purified from the urine of pregnant women
(purity >98%) was purchased from United States Biological

(Billerica, MA). The recombinant HCG β subunit expressed in

Pichia pastoris was purchased from Sigma-Aldrich. Peptide-N-
glycosidase F (PNGase F) was from ProZyme (San Leandro, CA).

Pepsin from porcine gastric mucosa and Trypsin Gold were purchased from Promega (Madison, WI). The porous graphitized carbon (PGC) solid-phase extraction columns were purchased from Alltech (Columbia, MD). The anti-HCG mAbs, MCA329 and MCA1024, were purchased from AbDSerotec. CNBr-activated Sepharose 4B was purchased from GE Health-
care. Acetonitrile (HPLC grade), methanol (HPLC grade),
water (HPLC grade), and formic acid (certified ACS grade) were purchased from Fisher Scientific. Vinylpyridine, D₂O (purity >99.9%), and tris(2-carboxyethyl) phosphate hydro-
chloride (purity ≥98%) were purchased from Sigma-Aldrich.

Ultra-centrifugal membranes were obtained from Millipore

(Billerica, MA). The protein silver stain kit was purchased from
Cwbiootech. All other reagents and chemicals were of the high-
est quality available.

**Purification of HCG-H from Urine Samples of Patients**—

HCG-H was purified from four female patients between the ages

of 23 and 40 years who were clinically diagnosed with invasive

mole. Early morning urine (~400–600 ml from each patient) was collected in sterile wide-mouthed containers and stored in

an ice bath. Na₂SO₄ was immediately added to the urine samples to a final concentration of 0.5 g/liter. The samples were clarified using filter paper to remove precipitates and further filtered using a disposable filter unit with 0.22-μm pore size. The protein fraction containing HCG was separated by centrifugation with ultra-centrifugal filtration units (molecular weight cut-off 10 kDa) and then purified by immuno-affinity chromatogra-

phy, following the protocol described by Valmu et al. (25).

Briefly, the mAb was immobilized onto the CNBr-activated

Sepharose 4B according to the manufacturer’s instructions.
The column was equilibrated with 50 mM sodium phosphate
(pH 7.4). The sample was applied to the affinity column and equilibrated at room temperature for 2 h. The column was washed with 3 volumes of 10 mM ammonium acetate (pH 4.5), and the bound protein was then eluted with 10 ml of 3 M acetic acid. The eluate was immediately neutralized with NH₄OH aqueous solution, and the buffer was changed to PBS using ultra-centrifugal filtration units (molecular weight cut-off 10 kDa).

**SDS-PAGE Analysis**—SDS-PAGE was performed using 12% (w/v) separating gel and 5% (w/v) stacking gel according to the protocol described by Laemmli (26). Each sample (~1 μg) was dissolved with 8 μl of water and then mixed with 2 μl of loading buffer containing 2% (v/v) β-mercaptoethanol as a reducing agent. The samples were boiled for 2 min. Electrophoresis was performed on a Mini-PROTEAN Tetra system (Bio-Rad). The initial voltage was kept at 80 V until the samples entered into the separating gel. Then, it was increased to a constant voltage of 120 V. The bands were visualized by silver stain.

**N-Linked Oligosaccharide Profiling of HCG Glycoforms**—The N-linked oligosaccharides of HCG samples from normal preg-
nant women and invasive mole patients were released by PNGase F. First, 15 μg of each sample was mixed with 2.5 μl of denaturation buffer, 2.5 μl of Nonidet P-40, and 2 μl of PNGase F solution (5 units/ml). The mixture was incubated at 37 °C for 72 h. The released N-linked oligosaccharides were recovered using PGC columns and dried by vacuum centrifuge (Eppendorf, Hamburg, Germany). The N-linked oligosaccharide samples were then reconstituted with 10 μl of 10 mM NH₄HCO₃ solution.

LC-MS analysis was performed on an 1100 capillary LC system (Agilent) coupled with an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Scientific). A Hypersil Hypercarb Kapp PGC column (Thermo Scientific, 0.32 mm × 100 mm, 5 μm) was used to separate the N-linked oligosaccharides. Mobile phase A was 10 mM NH₄HCO₃ in 2% aqueous acetonitrile, and mobile phase B was 10 mM NH₄HCO₃ in 85% aqueous acetonitrile. A step gradient of 0–60% B, 0–60 min; 60% B, 60–75 min; 60–100% B, 75–90 min; and 100% B, 90–105 min was used. The flow rate was 6 μl/min. The column temperature was maintained at 35 °C. The mass spectrometer was operated in the positive ion mode with the following parameters: sheath gas flow rate, 15 arbitrary units; auxiliary gas flow rate, 12 arbitrary units; spray voltage, 4.5 kV; capillary temperature, 275 °C; and S-lens level, 69%. The mass acquisition range was set at 500–2000, and the resolution of the orbitrap was set at 60,000 (m/z = 400).

Kinetic Analysis of the Interaction between hCG Glycoforms and Anti-hCG mAbs—The binding affinity of hCG glycoforms against anti-hCG mAbs was measured using an Octet-RED 96 biolayer interferometer (FortèBio). MCA329 and MCA1024 were biotinylated and then immobilized on the streptavidin-coated biosensors according to the manufacturer’s instructions. The biosensors were pre-wetted with PBS buffer. A 60-s washing step was performed, followed by the association step, which was performed for 60–150 s depending on the situation. Finally, the dissociation step was performed for 300 s. Data were generated and processed by the Octet-RED User Software (version 3.1).

Epitope Mapping Using HDX-ESI-MS—The recombinant hCG β subunit was used to determine the epitopes of MCA329 and MCA1024. The stock solutions of hCG β subunit without mAbs or with equivalent moles of mAbs were prepared at the concentration of 40 μg in 50 mM sodium phosphate buffer (pH 7.8). A 5-μl aliquot from a stock solution was mixed with 45 μl of 50 mM sodium phosphate in D₂O (pH meter reading 7.8) to initiate each HDX period. A 5-μl aliquot of hCG β subunit solution was diluted with 45 μl of 50 mM sodium phosphate in water (pH 7.8) as an unexchanged control. All HDX reactions were performed at 20 °C for 10 min and then quenched by adding 25 μl of 200 mM tris(2-carboxyethyl) phosphine hydrochloride solution in 1% formic acid. Then, 25 μl of 1 μg/μl pepsin solution in 1% formic acid was added immediately, and proteolysis was performed at 20 °C for 5 min.

On-line ESI-MS analysis was performed on a Prominence LC-20A system (Shimadzu, Kyoto, Japan) coupled with an LTQ-Orbitrap Velos Pro mass spectrometer (Thermo Scientific). The proteolytic products were injected onto a Hypersil ODS2-C₁₈ column (Dalian Elite Analytical, 4.6 mm × 20 mm, 5 μm) and eluted with a step gradient of 5% mobile phase B for 1.5 min; from 5 to 45% B over a period of 6.5 min; from 45 to 85% B over a period of 0.5 min; and 85% B for 1.5 min. Mobile phases A and B were 1% formic acid in 2 and 98% aqueous acetonitrile, respectively. The flow rate was 0.6 ml/min, and one-sixth of the fluids flew into the MS interface, controlled by a post-column splitter. The operation was maintained at 0 °C to prevent the back exchange of deuterium to hydrogen. ESI-MS analysis was set in the positive ion mode with the following parameters: source voltage at 4.5 kV, sheath gas flow rate at 12 arbitrary units, auxiliary gas flow at 3 arbitrary units, capillary temperature at 275 °C, and S-lens level at 69.8%. The mass acquisition range was set at 350–1500, and the resolution of the orbitrap was set at 100,000 (m/z = 400).

Data Processing—A database containing all theoretical pep- sin-digested hCG β subunit peptides was generated. The experimental monoisotopic m/z values corresponding to all peptides in unexchanged form were input into the database to look for matches. The deuterium levels of each assigned peptide were then determined using the HX-Express software (27).

Site-specific N-Glycosylation Analysis—hCG, hCG-H #2, and hCG-H #3 were reduced and alkylated according to the method described by Toll et al. (28). Briefly, samples (~10 μg) were denatured with 6 M guanidine hydrochloride, 0.5 M Tris-HCl, and 2.75 mM EDTA and reduced with 0.1 M dithiothreitol for 2 h at 37 °C. The samples were then alkylated by 0.04 M vinylpyridine for 30 min at 37 °C. The buffer was subsequently changed to 25 mM NH₄HCO₃ using ultra-centrifugal filtration units (molecular weight cut-off 3 kDa). Trypsin digestion was performed at 37 °C overnight with a 25:1 protein-to-enzyme ratio.

The digests were analyzed using a Thermo LTQ-Orbitrap Velos Pro mass spectrometer coupled to an Eksigent NanoLC Ultra 2D pump (SCIEX). The peptides (~1 μg) were desalted on a 0.3 × 5-mm C₁₈ trap column (Chemicals Evaluation and Research Institute, Tokyo, Japan) for 3 min and then separated on a 75-μm inner diameter nanoLC column packed with 20 cm of 1.9-μm, 120-Å ReproSil-Pur C₁₈-AQ resin (Dr. Maisch GmbH HPLC, Ammerbuch, Germany). Mobile phase A was 0.1% formic acid in 2% aqueous acetonitrile, and mobile phase B was 0.1% formic acid in 98% aqueous acetonitrile. A step gradient of 2% B, 0–5 min; 2–8% B, 5–20 min; 8–22% B, 20–75 min; 22–32% B, 75–90 min; and 32–85% B, 90–95 min was used. The flow rate was 300 nl/min. The mass spectrometer was operated in the positive ion mode with the following parameter settings: spray voltage, 2.0 kV; capillary temperature, 275 °C; and S-lens level, 55%. The mass acquisition range was set at 350–2000, and the resolution of the orbitrap for MS analysis was set at 60,000 (m/z = 400). The top 10 precursors were selected for MS² analysis by linear ion trap mass analyzer using collision induced dissociation.

N-Deglycosylation of hCG-H in Native Condition—For this purpose, 60 μg of hCG-H #1 (~1 μg/μl) was digested with 1.5 μl of PNGase F solution at room temperature for 5 h.

Results

Purification of hCG-H from Urine of Cancer Patients—hCG-H is the abnormal glycosylated variant of hCG, which can be found in the blood and urine of patients with a variety of reproductive cancers. Invasive mole disease is a type of repro-
hCG N-Glycoforms Bind mAbs Differently

**FIGURE 1. SDS-PAGE analysis of hCG glycoforms.** The purity and glycosylation degree of hCG-H purified from urine of invasive mole patients were assessed by SDS-PAGE. The normal hCG from urine of pregnant women was analyzed in parallel. Unstained protein molecular mass marker (Thermo Scientific catalogue number SM0431) was used as the molecular mass marker (MW).

Productive cancer, and the patients are usually treated with chemotherapy. In this study, hCG-H samples were purified from the urine of invasive mole patients. The patients were diagnosed with invasive mole disease based on pathology tests showing placental villi. The villi had extensive stromal edema with central cisterns, and the trophoblast proliferated circumferentially. Three hCG-H samples were prepared. hCG-H #1 was prepared from the pooled urine of patient 1 and patient 2. hCG-H #2 and #3 were prepared from the urine of patient 3 and patient 4, respectively. The purity and glycosylation were evaluated by reducing SDS-PAGE, using the regular hCG from urine of normal pregnant women as a reference. As shown in Fig. 1, the hCG showed two major bands representing the α subunit (the lower band) and the β subunit (the upper band). The β subunit of hCG-H exhibited the same molecular masses but smeared to a much broader degree than in hCG, indicating that the glycosylation pattern of hCG-H was more complicated than the normal form.

**Characterization of N-Linked Oligosaccharides of hCG and hCG-H.** It has been reported that hCG-H possesses a different N-glycosylation profile from normal hCG, as the percentage of more complicated tri- and tetra-antennary N-linked oligosaccharides is significantly larger (29). The overall N-linked oligosaccharide composition of hCG-H #1 was analyzed by LC-MS and compared with normal hCG. A total of 19 N-linked oligosaccharides were detected from hCG and hCG-H #1, eluting at approximately from 30.0 to 50.0 min on the PGC column. The structures of the N-linked oligosaccharides of hCG have been reported previously (28). We used high-resolution MS to confirm the identities of these oligosaccharides and sorted them into bi-, tri-, and tetra-antennary structures. The LC-MS data from 30.0 to 50.0 min were averaged to one MS spectrum, and the monoisotopic peak intensity of each assignable ion was recorded. The proportion of each specific oligosaccharide was then calculated using the sum of signals of its monoisotopic peak ions related to the 19 N-linked oligosaccharides. The N-glycosylation profiles of hCG-H #1 and hCG were compared by plotting the logarithms (Log2) of the ratios of the proportion of each oligosaccharide in hCG-H to the proportion of each oligosaccharide in hCG (Fig. 2). Although the 19 N-linked oligosaccharides were detected in both hCG and hCG-H #1, their relative abundances were significantly different. The proportions of 9 N-linked oligosaccharides in hCG-H #1 were more than 2-fold larger than in hCG. Most of them belong to the complicated tri- and tetra-antennary structures. The result showed that the hCG-H #1 purified from the cancer patients was hyperglycosylated, which was consistent with previous studies (25).

**BLI Measurements of hCG Glycoforms and Anti-hCG mAb Interaction—Binding kinetics and affinity are key characteristics for evaluating antigen-antibody interaction. Label-free techniques, such as SPR and BLI, provide rapid and real-time approaches to characterizing protein-protein interaction. The BLI technique has become increasingly popular because the samples can be easily recovered after measurement, which is of particularly vital importance when limited amounts of biological samples are available, as in the case of the biomarker glycoproteins purified from the fluids of cancer patients.**

A number of biosensors have been screened in preliminary studies, including Anti-Mouse Fc Capture sensor, protein A sensor, and streptavidin sensor. The Anti-Mouse Fc Capture and protein A sensors capture mAbs through the Fc portions and require no derivatization of the mAbs, but significant nonspecific adsorption was observed at the initial baseline measurement step. Minimal nonspecific interaction was achieved using biotinylated mAbs bound to the streptavidin sensor.

There are many commercial available anti-hCG mAbs against more than 17 different epitopes. The β2 and β4 epitopes are located at the β loop 3 region of hCG, where there are three N-glycosylation sites (Asn-52 of α subunit, and Asn-13 and Asn-30 of β subunit) nearby. MCA329 against the β2 epitope and MCA1024 against the β4 epitope were selected to interact with different hCG glycoforms. Both mAbs were biotinylated, immobilized onto streptavidin biosensors, and used to assay hCG and hCG-Hs (Fig. 3). The affinity constants (Kd) were
calculated using the FortéBio Octet QK software and are presented in Table 1. The binding affinities of MCA329 showed no obvious change from hCG (\(K_D\) value 0.64 nM) to hCG-Hs (averaged \(K_D\) value 1.08 nM). In contrast, the \(K_D\) value of MCA1024 increased dramatically when the N-linked oligosaccharides of hCG were altered.

**Epitope Mapping by HDX-MS—**The HDX-MS approach is very useful in investigating the peptide domains that participate in protein-protein interaction in aqueous solution. hCG, FSH, luteinizing hormone, and thyroid-stimulating hormone all belong to the hetero-dimeric glycoprotein hormone family. They share identical \(\alpha\)-subunits but are distinguished from each other by the \(\beta\) subunits. The HDX-MS analysis of the hCG-mAb complex was unsuccessful, mainly due to the resistance of the intact hCG to the protease. Instead, the recombinant hCG \(\beta\) subunit was used because both epitopes of MCA329 and MCA1024 are located at the \(\beta\) subunit.

To optimize the HDX-MS experiment, the pepsin digestion of the hCG \(\beta\) subunit in the absence of mAb was performed first. The peptide sequence coverage of 86.9% was achieved after the incubation, digestion, and MS conditions were carefully adjusted (Fig. 4). The entire \(\beta\) loop 3 region was recovered within the 12 mapped peptides. The deuterium exchange of hCG backbone amides in the presence of mAbs was then performed in the same environment. The proteolytic peptides were identified using in-house developed software, and the deuterium exchange rate was calculated using the HX-Express software. The \(\Delta\)Deuterium exchange rate of each peptide was expressed using equation 1

\[
\Delta\text{Deuterium exchange rate (\%)} = \frac{D_{\text{Complex}} - D_{\text{Control}}}{H_{\text{backbone amides}}} \times 100
\]

(Eq. 1)

where \(D_{\text{Complex}}\) and \(D_{\text{Control}}\) are the deuterium uptake values of each peptide derived from antigen-antibody complexes and the antigen-only control, respectively. \(H_{\text{backbone amides}}\) is the total amount of backbone amides of the corresponding peptide. The \(\Delta\)Deuterium exchange rates of all 12 peptides were plotted in Fig. 5. The majority of the peptides exhibited very small changes between the absence and presence of mAbs, as their \(\Delta\)Deuterium exchange rates remain close to the horizontal axis. However, \(\beta65-77\), \(\beta78-83\), and \(\beta116-133\) showed a substantial \(\Delta\)Deuterium exchange rate decrease when the hCG \(\beta\) subunit was incubated with MCA329. In the case of MCA1024, the \(\Delta\)Deuterium exchange rates of \(\beta65-77\) and \(\beta78-83\) dropped significantly.
**hCG N-Glycoforms Bind mAbs Differently**

**Site-specific N-Glycosylation Analysis**—The BLI experiment showed that the binding affinity of anti-hCG mAb MCA329 and MCA1024 responded differently to hCG glycoforms. The HDX-MS experiment revealed that although the two mAbs shared the sequences $\beta_{65-77}$ and $\beta_{78-83}$ as parts of their epitopes, they bound to hCG from different directions. Three $N$-glycosylation sites, Asn-52 on the $\alpha$ subunit and Asn-13 and Asn-30 on the $\beta$ subunit, were spatially adjacent to the epitope peptides (30). Site-specific analysis revealed that these three sites possessed different $N$-glycosylation patterns. Asn-52 was not hyperglycosylated, whereas Asn-13 and Asn-30 had more tetra-antennary oligosaccharides than normal hCG (Fig. 6).

**Confirmation of the Involvement of N-Linked Oligosaccharides in the Interaction between hCG and Anti-hCG mAbs**—To confirm that the $N$-linked oligosaccharides played a critical role in the interaction between hCG and anti-hCG mAbs, $N$-deglycosylated hCG-H (hCG-deN) was prepared by cleaving its $N$-linked oligosaccharides using PNGase F under mild conditions. Kinetic measurement of the interaction between hCG-deN and the two mAbs, MCA329 and MCA1024, was performed using the same procedure described under “Kinetic Analysis of the Interaction between hCG Glycoforms and Anti-hCG mAbs.” The results are presented in Fig. 7, and all $K_D$ values are summarized in Table 1. The $K_D$ values of MCA329 against three different hCG glycoforms, hCG, hCG-H #1, and hCG-deN, were barely altered. In contrast, the binding affinity of MCA1024 was affected dramatically by the $N$-linked oligosaccharides. Its $K_D$ values were 0.001 nM against hCG and 0.28 nM against hCG-H #1. However, when the $N$-linked oligosaccharides were removed from hCG-H #1, the $K_D$ value decreased back to the equivalent level of normal glycosylated hCG.

**Discussion**

We have described herein the distinct responses of two anti-hCG mAbs to the different glycoforms of hCG. The binding affinities of mAbs MCA329 and MCA1024 to normal hCG, hCG-H, and hCG-deN were measured by BLI technology. Interestingly, the $K_D$ value increased significantly from hCG to hCG-H for MCA1024. After the removal of $N$-linked oligosaccharides from hCG-H, the $K_D$ value decreased accordingly. Meanwhile, MCA329 showed almost no change in its $K_D$ when bound to normal hCG, hCG-H, and hCG-deN. To elucidate the mechanism of the two different binding behaviors of MCA329 and MCA1024, their interacting epitopes were determined using HDX-MS technology.

Seventeen different epitopes on hCG have been described as $\alpha_1$ to $\alpha_6$, $\beta_1$ to $\beta_7$, and $c_1$ to $c_4$ based on a mAb panel (31). The MCA329 was against the $\beta_2$ epitope, whereas MCA1024 was against the $\beta_4$ epitope, according to the manufacturer’s specifications. However, the exact amino acid sequences comprising these epitopes were not yet clear. Early studies based on mutating specific amino acid residues showed that substitutions of Arg-68, Arg-74, Gly-75, and Val-78 completely abolished the binding of mAbs against the $\beta_2$ and $\beta_4$ epitopes. Moreover, the single substitution of Glu for Arg-68 also abated the binding affinity of mAbs against the $\beta_2$ and $\beta_4$ epitopes (32). These 4 amino acid residues most likely contribute to the epitope clusters $\beta_2$ and $\beta_4$, which is consistent with our HDX-MS results. The deuterium uptake of peptide $\beta_{65-77}$, containing Arg-68, Arg-74, and Gly-75, and peptide $\beta_{78-83}$, containing Val-78, decreased when MCA329 and MCA1024 were present.

MCA329 and MCA1024 shared the epitopic sequence $\beta_{65-83}$, but the deuterium uptake of peptide $\beta_{116-133}$ decreased only when MCA329 was present. Previous studies showed that the nicked form of the hCG $\beta$ subunit lacking the sequence $\beta_{109-145}$ could still bind to mAbs against the $\beta_2$ epitope (34), suggesting that peptide $\beta_{116-133}$ was not a part of the epitope for MCA329. The peptide $\beta_{116-133}$ was located at the C terminus of hCG $\beta$ subunit and was assumed to be a random,
FIGURE 6. Site-specific $N$-glycosylation analysis of hCG and two hCG-H samples. Site-specific $N$-linked oligosaccharides of hCG, hCG-H #2, and hCG-H #3 were characterized by C$_{18}$ reverse phase nanoLC coupled with nanoESI-MS. A, the total ion chromatogram of hCG-H #2. Thirteen glycopeptides linked by tetra-antennary oligosaccharides were detected. B, the mass spectra of tetra-antennary $N$-linked oligosaccharide-attached glycopeptides in hCG-H #2. The glycopeptides were named by their attachment position and structures. For example, $\beta_{13}-N_{4,4,F}$ represents the $N$-linked glycopeptide with a tetra-antennary, mono-sialylated, fucosylated $N$-glycan linked to the Asn-13 residue of $\beta$ subunit. C, the log$_{2}$(Ratio$_{hCG-H/hCG}$) was plotted. The tetra-antennary oligosaccharides linked to Asn-13 and Asn-30 of the $\beta$ subunit in hCG-H #2 and hCG-H #3 increased significantly when compared with hCG. In contrast, the relative amount of tetra-antennary oligosaccharides linked to Asn-52 of the $\alpha$ subunit decreased.
non-folded component (13, 30, 33). It is likely that MCA329 hindered the exposure of the random C-terminal tail to the deuterium solution when binding to hCG. Meanwhile, MCA1024 approached the antigen from a different direction and did not interfere in the movement of the C-terminal tail.

The three-dimensional structure of hCG has been partially generated by different research groups using x-ray crystallography (13, 30, 33). Unfortunately, they were unable to crystallize natural hCG due to the heterogeneity of the eight carbohydrate side-chains. The four O-linked oligosaccharides accompanying the 34 amino acid residues (8112–145) from the C-terminal of the hCG β subunit had to be removed to successfully crystallize the complicated glycoprotein. The x-ray crystallography structure of hCG revealed that three N-glycosylation sites were spatially close to the epitope loop 65–83 (30). Asn-52 of the hCG α subunit was on one side of the loop, whereas Asn-13 and Asn-30 of the β subunit were on the other side. The α subunit is common among the hetero-dimeric glycoprotein hormone family, whereas the β subunit is unique to hCG. Site-specific analysis also revealed that only the two N-glycosylation sites on the β subunit were hyperglycosylated. MCA1024 was assumed to bind the epitope loop from the same side as the Asn-13 and Asn-30 linked oligosaccharides, as its binding affinity decreased significantly when the N-linked oligosaccharides became more complicated (Fig. 5). The antigen-antibody interaction might have been impeded by either steric hindrance from the more branched antennas of the N-linked oligosaccharides at Asn-13 and Asn-30 or stronger static repulsion of the sialic acid residues at the end of each antenna. This assumption was proved by the restoration of the binding affinity of MCA1024 after the N-linked oligosaccharides were removed from hCG-H.

The structural motif of hCG antigen-antibody interaction and the influence of N-linked oligosaccharides on the binding affinities of hCG to different mAbs have not previously been studied in depth. In most cases, such as x-ray crystallography analysis, the heterogeneous carbohydrate moiety of hCG must be removed first. In this study, we demonstrated the use of HDX-MS to reveal the binding domains of two different mAbs on hCG in aqueous solution. We found that MCA1024 was sensitive to changes in hCG glycosylation status. In other words, it was able to recognize different glycoforms of hCG. The aberrant modification of hCG glycosylation is a critical indicator of many placental and germ cell-originated trophoblastic tumors. Clinically, an immunoassay that can sensitively, accurately, and rapidly monitor the status of patients’ hCG glycosylation is in high demand. Based on the findings described herein, it is conceptually feasible to develop an immunoassay combining two distinct anti-hCG mAbs. For example, MCA329 can be used to determine the level of total hCG regardless of glycosylation. Simultaneously, MCA1024 can be used to measure the degree of N-glycosylation modification. Moreover, the change in N-glycosylation is a common feature in the development and metastasis of many cancers, such as lung cancer, liver cancer, breast cancer, prostatic cancer, and so on. Thus, the methods described herein may broaden the application of many existing biomarkers for malignancy and improve their diagnostic power. The recognition of N-glycosylation alterations of specific glycoprotein biomarkers for these cancers using the strategy developed in this study is currently under investigation.

Author Contributions—Lianli Chi and Q. Z. designed the study. D. L. performed all LC-MS and HDX-ESI-MS experiments. P. Z. selected the patients and prepared samples. F. L. performed the BLI experiment. Lequan Chi designed the data processing programs. D. Z. contributed to Fig. 5.

References

1. Heuvers, M. E., Hegmans, J. P., Stricker, B. H., and Aerts J. G. (2012) Improving lung cancer survival; time to move on. BMC Pulm. Med. 12, 77
2. Ford, P. J., and Farah, C. S. (2013) Early detection and diagnosis of oral cancer: strategies for improvement. J. Cancer Policy 1, e2–e7
3. Barh, D., Carpi, A., Verma, M., and Gunduz, M., eds (2014) Cancer Biomarkers: Minimal and Noninvasive Early Diagnosis and Prognosis, pp. 3–17, CRC Press, Inc., Boca Raton, FL
4. Pan, S., Chen, R., Aebersold, R., and Brentnall, T. A. (2011) Mass spectrometry based glycoproteomics: from a proteomics perspective. Mol. Cell. Proteomics 10, R110.003251
5. Tuccillo, F. M., de Laurentiis, A., Palmieri, C., Fiume, G., Bonelli, P., Borrelli, A., Tassone, P., Scala, L., Buonaguro, F. M., Quinto, I., and Scala, G. (2014) Aberrant glycosylation as biomarker for cancer: focus on CD43. Biomed. Res. Int. 2014, 742831
6. Durand, G., and Seta, N. (2000) Protein glycosylation and diseases: blood and urinary oligosaccharides as markers for diagnosis and therapeutic monitoring. Clin. Chem. 46, 795–805
7. Gilgumn, S., Conroy, P. J., Saldova, R., Rudd, P. M., and O’Kennedy, R. J. (2013) Aberrant PSA glycosylation: a sweet predictor of prostate cancer. Nat. Rev. Urol. 10, 99–107
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8. Ajdukiewicz, A. B., Kelleher, P. C., Krawitt, E. L., Walters, C. J., Mason, P. B., Koff, B. S., and Belanger, L. (1993) α-Fetoprotein glycosylation is abnormal in some hepatocellular carcinoma, including white patients with a normal α-fetoprotein concentration. Cancer Lett. 74, 43–50

9. Vashist, S. K., Marion Schneider, E., Lam, E., Hrapovic, S., and Luong, J. H. (2014) One-step antibody immobilization-based rapid and highly-sensitive sandwich ELISA procedure for potential in vitro diagnostics. Sci. Rep. 4, 4407

10. Findlay, J. W., Smith, W. C., Lee, J. W., Nordblom, G. D., Das, I., DeSilva, B. S., Khan, M. N., and Bowsher, R. R. (2000) Validation of immunoassays for bioanalysis: a pharmaceutical industry perspective. J. Pharm. Biomed. Anal. 21, 1249–1273

11. Yang, M., Lei, Z. M., and Rao, Ch. V. (2003) The central role of human chorionic gonadotropin in cancer. Mol. Cell Endocrinol. 21, 9239–9243

12. Cole, L. A. (2012) hCG, five independent molecules. Clin. Chim. Acta 413, 48–65

13. Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., and Hendrickson, W. A. (1994) Structure of human chorionic gonadotropin at 2.6 Å resolution from MAD analysis of the selenomethionyl protein. Structure 2, 545–558

14. de Medeiros, S. F., and Norman, R. J. (2009) Human choriogonadotropin: a variant with a normal and aberrantly glycosylated form. Mol. Endocrinol. 23, 15–32

15. Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., and Isaacs, N. W. (1994) Crystal structure of human chorionic gonadotropin. Nature 369, 455–461

16. Berger, P., Schwarz, S., Stopf, G., Wölk, G., and Mann, K. (1993) Variants of human chorionic gonadotropin from pregnant women and tumor patients recognized by monoclonal antibodies. J. Clin. Endocrinol. Metab. 77, 347–351

17. Jackson, A. M., Klonisch, T., Lapthorn, A. J., Berger, P., Isaacs, N. W., Delves, P. J., Lund, T., and Roitt, I. M. (1996) Identification and selective destruction of shared epitopes in human chorionic gonadotropin β subunit. J. Reprod. Immunol. 31, 21–36

18. Cole, L. A., and Khanian, S. A. (2007) Hyperglycosylated hCG: a variant with separate biological functions to regular hCG. Mol. Cell. Endocrinol. 260–262, 228–236

19. Cole, L. A. (2010) Biological functions of hCG and hCG-related molecules. Reprod. Biol. Endocrinol. 8, 102

20. Cole, L. A., and Butler, S. (2012) Hyperglycosylated hCG, hCGβ and Hyperglycosylated hCGβ: interchangeable cancer promoters. Mol. Cell Endocrinol. 349, 232–238

21. Stenman, U. H., Althans, H., and Hotakainen, K. (2004) Human chorionic gonadotropin in cancer. Clin. Biochem. 37, 549–561

22. Birken, S., Krachevsky, A., O’Connor, J., Schlatterer, J., Cole, L., Kardana, A., and Canfield, R. (1999) Development and characterization of antibodies to a nicked and hyperglycosylated form of hCG from a choriocarcinoma patient: generation of antibodies that differentiate between pregnancy hCG and choriocarcinoma hCG. Endocrine 10, 137–144

23. Birken, S., Yershova, O., Myers, R. V., Bernard, M. P., and Moyle, W. (2003) Analysis of human choriongonadotropin core 2 α-glycan isoforms. Mol. Cell Endocrinol. 204, 21–30

24. Birken, S. (2005) Specific measurement of O-linked core 2 sugar-containing isoforms of hyperglycosylated human chorionic gonadotropin by antibody b152. Tumour. Biol. 26, 131–141

25. Valmu, L., Althans, H., Hotakainen, K., Birken, S., and Stenman, U. H. (2006) Site-specific glycan analysis of human chorionic gonadotropin β-subunit from malignancies and pregnancy by liquid chromatography-electrospray mass spectrometry. Glycobiology 16, 1207–1218

26. Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685

27. Weis, D. D., Eng, J. R., and Kass, I. J. (2006) Semi-automated data processing of hydrogen exchange mass spectra using HX-Express. J. Am. Soc. Mass Spectrom. 17, 1700–1703

28. Toll, H., Bergh, P., Hofmann, A., Hildebrandt, A., Oberacher, H., Lenhof, H. P. (2006) Structure of human chorionic gonadotropin revealed by liquid chromatography-mass spectrometry and bioinformatics. Electrophoresis 27, 2734–2746

29. Elliott, M. M., Kardana, A., Lustbader, J. W., and Cole, L. A. (1997) Carbohydrate and peptide structure of the α- and β-subunits of human chorionic gonadotropin from normal and aberrant pregnancy and choriocarcinoma. Endocrine 7, 15–32