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An Essential Epitope of Anti-MUC1 Monoclonal Antibody KL-6 Revealed by Focused Glycopeptide Library

Naoki Ohyabu,[†,‡] Hiroshi Hinou,[†] Takahiko Matsushita,[†] Ryukou Izumi,[‡] Hiroki Shimizu,[#] Keiko Kawamoto,[§] Yoshito Numata,[§] Hiroko Togame,[‡] Hiroshi Takemoto,[‡] Hirosato Kondo,[§] and Shin-Ichiro Nishimura*[,†,#]

Division of Advanced Chemical Biology, Graduate School of Advanced Life Science, Frontier Research Center for Post-Genome Science and Technology, and Shionogi Innovation Center for Drug Discovery, Hokkaido University, N-21, W-11, Kita-ku, Sapporo 001-0021, Japan, Drug-Seeds Discovery Research Laboratory, National Institute of Advanced Industrial Science and Technology (AIST), 2-17-2-1 Tsukisamu-higashi, Toyohira-ku, Sapporo 062-8517, Japan, and Discovery Research Laboratories, Shionogi & Co., Ltd., 12-4, Sagisu 5-chome, Fukushima-ku, Osaka 541-0045, Japan

Received April 26, 2009; E-mail: shin@glyco.sci.hokudai.ac.jp

Abstract: Human serum Krebs von den Lungen-6 (KL-6) antigen, a high-molecular-weight glycoprotein classified as a polymorphic epithelial mucin (MUC1), is a biomarker of diseases such as interstitial pneumonia, lung adenocarcinoma, breast cancer, colorectal adenocarcinoma, and hepatocellular carcinoma. Anti-KL-6 monoclonal antibody (anti-KL-6 MAb) is therefore a potential diagnostic and therapeutic reagent. Although glycosylation at Thr/Ser residues of the tandem-repeating MUC1 peptides appears to determine the disease-associated antigenic structures of KL-6, an essential epitope structure recognized by anti-KL-6 MAb remains unclear. In the present study, a novel compound library of synthetic MUC1 glycopeptides allowed the first rapid and precise evaluation of the specific epitope structure of anti-KL-6 MAb by combined use of a tailored glycopeptides library and common ELISA protocol. We demonstrated that the minimal antigenic structure, an essential epitope, recognized by anti-KL-6 MAb is a heptapeptide sequence Pro-Asp-Thr-Arg-Pro-Ala-Pro (PDTRPAP), in which the Thr residue is modified by Neu5Acα2,3Galβ1,3GalNAcα (2,3-sialyl T antigen, core 1-type *O*-glycan). Anti-KL-6 MAb did not bind with other tumor-relevant antigens, such as GalNAcα (Tn), Neu5Acα2,6GalNAcα (STn), and Galβ1,3GalNAcα (T), except for Neu5Acα2,3Galβ1,3-(Neu5Acα2,6)GalNAcα (2,3/2,6-disialyl T). However, anti-KL-6 MAb could not differentiate the above minimal antigenic glycopeptide from some core 2-based glycopeptides involving this crucial epitope structure and showed a similar binding affinity toward these compounds, indicating that branching at the *O*-6 position of GalNAc residue does not influence the interaction of anti-KL-6 MAb with some MUC1 glycoproteins involving an essential epitope. Actually, anti-KL-6 MAb reacts with 2,3/2,6-disialyl T having a 2,3-sialyl T component. This is why anti-KL-6 MAb often reacts with various kinds of tumor-derived MUC1 glycoproteins as well as a clinically important MUC1 glycoprotein biomarker of interstitial pneumonia, namely KL-6, originally discovered as a circulating pulmonary adenocarcinoma-associated antigen. In other words, combined use of anti-KL-6 MAb and some probes that can differentiate the sugars substituted at the *O*-6 position of the GalNAc residue in MUC1 glycopeptides including the PDTRPAP sequence might be a promising diagnostic protocol for individual disease-specific biomarkers. It was also revealed that glycosylation at neighboring Thr/Ser residues outside the immunodominant PDTRPAP motif strongly influences the interaction between anti-KL-6 MAb and MUC1 glycopeptides involving the identified epitope. Our novel strategy will greatly facilitate the processes for the identification of the tumor-specific and strong epitopes of various known anti-MUC1 MAbs and allow for their practical application in the generation of improved antibody immunotherapeutics, diagnostics, and MUC1-based cancer vaccines.

Introduction

Human polymorphic epithelial mucin (MUC1) is a highly *O*-glycosylated transmembrane glycoprotein composed of three major characteristic domains, namely an *N*-terminus containing a hydrophobic signal sequence, a tandem repeating domain of precisely conserved 20-amino-acid residues, and a *C*-terminus containing a membrane spanning a region of 31-amino-acid residues and 60-amino-acid residues of cytoplasmic tail.[1−3]

[†] Division of Advanced Chemical Biology, Graduate School of Advanced Life Science, and Frontier Research Center for Post-Genome Science and Technology, Hokkaido University.
[‡] Shionogi Innovation Center for Drug Discovery, Hokkaido University.
[#] Drug-Seeds Discovery Research Laboratory, AIST.
[§] Discovery Research Laboratories, Shionogi & Co., Ltd.

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Intact MUC1 is therefore identified typically as a membrane glycoprotein distributing on the apical surface of normal epithelial cells. During carcinogenesis, however, the normal topology and polarity of epithelial cells are considered to alter markedly, and aberrantly *O*-glycosylated MUC1 is expressed on the whole-cell surfaces, thus leading to the shedding of cell surface MUC1 to the bloodstream.[4,5] As a result, a plethora of monoclonal antibodies (MAbs) generated against tumor-derived MUC1 have been reported and profiled as candidates of diagnostic tools for monitoring serum tumor markers.[6−9] In the mid-1990s, it had been proposed that the immunodominant MUC1 peptides were generally not glycosylated[10] and the tumor specificity of anti-MUC1 MAbs could be interpreted solely in terms of better access to peptide epitopes due to the reduced steric hindrance of highly branched *O*-glycans.[11−13] However, it has also been demonstrated that the sites and the composition of *O*-glycans of MUC1 produced by breast cancer cells differ significantly from those of normal cell lines.[4,14−16] Actually, it seems that binding patterns of many MAbs generated against tumor-derived MUC1 to the Pro-Asp-Thr-Arg (PDTR) motif are strongly influenced by *O*-glycosylation at its Thr residue.[17−20] Similarly, site-directed *O*-glycosylation at the Thr residue in the Val-Thr-Ser-Ala (VTSA) motif of the MUC1 tandem repeat may even improve binding affinity with some anti-MUC1 MAbs such as BW835 and MY.1E12.[21−23] These data suggest that naked MUC1 peptides might not correctly represent tumor epitopes. In addition, it is thought that glycosylation at sites other than an immunodominant glycosylation site covers or influences individual tumor-specific or tumor-associated glycopeptide epitope. Structurally well-defined and strong glycopeptide epitopes are of particular importance for humoral and cellular anti-tumor immune responses, since tumor-associated antigens usually vary in carcinomas of different organs. PankoMab,[17,18,24,25] a potent MAb (IgG1) developed by Goletz et al. from a desialylated human breast cancer source, is a distinguished anti-MUC1 MAb showing the highest glycosylation dependency and the strongest additive binding effect, such as antibody-dependent cell cytotoxicity (ADCC), compared with other known MUC1 MAbs. It is clear that successful development of carbohydrate-based cancer vaccines and antibody therapies requires new strategies for the characterization of more refined, tumor-specific MUC1 epitopes involving site and structure of *O*-glycosylation. Identification of organ-specific and strong glycopeptide epitopes would greatly accelerate the development of glycopeptide-based cancer vaccines as well as a new generation of tumor-specific MUC1 MAbs.[25−27]

Krebs von den Lugen-6 (KL-6), classified as a MUC1-derived glycoprotein antigen, is a sensitive serum biomarker for interstitial pneumonia, and anti-KL-6 MAb was established by Kohno et al.[28−30] Interstitial pneumonitis includes more than 100 pulmonary diseases in which aet al.lveolitis is the main manifestation of the affected lung. The serum level of KL-6 is known to elevate early in the disease patients with interstitial pneumonitis such as pulmonary fibrosis, hypersensitivity pneumonitis, sarcoidosis, and radiation pneumonitis. Since patients with non-interstitial lung disease do not show a significant elevation of KL-6, KL-6 is a benefitial serum marker for diagnosis and monitoring patients with interstitial pneumonitis. However, anti-KL-6 MAb is also known as a potential candidate of diagnostic reagent for lung, breast, colon, pancreas, ovary, and hepatocellular carcinomas, since high level expression of the serum KL-6 of patients with these cancers can be monitored by means of this MAb.[31−37] Although an essential epitope for anti-KL-6 MAb remains unclear, glycosylation by a sialylated carbohydrate at some Thr/Ser residue(s) of the tandem repeating peptides appears to determine the immunodominant motif of KL-6 and/or tumor relevant MUC1 glycoproteins because neuraminidase treatment destroyed binding of serum or tissue

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KL-6 with anti-KL-6 MAb.[28] Considering these features, we hypothesized that anti-KL-6 MAb may exhibit broad binding specificities toward various MUC-1 glycoproteins overexpressed and shed into the circulation during the progress of some cancers as well as interstitial pneumonia. Our interest is now focused on an essential epitope structure and profiling the binding specificity of anti-KL-6 MAb. We thought that our synthetic strategy to construct a robust MUC1-related glycopeptide library[38−42] would allow for identifying a minimal epitope structure recognized by anti-KL-6 MAb. This approach would greatly accelerate research toward the discovery of disease-specific glycopeptide epitopes and the development of novel MAbs showing much higher selectivity and stronger binding affinity than known MAbs.

Results and Discussion

Synthetic Glycopeptides and Tailored MUC1 Microplate. The identification of an essential epitope structure of anti-KL-6 MAb was performed by means of tailored enzyme-linked immunosorbent assay (ELISA) plates displaying synthetic MUC1 glycopeptides. Given that anti-KL-6 MAb strongly reacts with serum MUC1 antigens of various cancer patients as well as that of interstitial pneumonia,[31−37] we considered that the Thr residue within the PDTR, one of the most important motifs in the tandem repeating unit composed of 20 amino acids (HGVTSAPDTRPAPGSTAPPA),[9,17−20] may be glycosylated by some tumor-relevant *O*-glycans such as Tn (GalNAcα), 2,6-sialyl Tn (Neu5Acα2,6GalNAcα), T (Galβ1,3 GalNAcα), 2,3-sialyl T (Neu5Acα2,3Galβ1,3GalNAcα), 2,6-sialyl T [Galβ1,3(Neu5Acα2,6)GalNAcα], and 2,3/2,6-disialyl T [Neu5Acα2,3Galβ1,3(Neu5Acα2,6)GalNAcα] antigens.[23,43] To test this hypothesis, we designed and synthesized a focused compound library composed of 45 kinds of MUC1-related glycopeptides on the basis of a chemical and enzymatic glycopeptide synthesis protocol.[39,41,44−46] Figure 1 shows the major compounds (**1**−**27**) and outlines the completion assay used in the present study to determine the real epitope and binding specificity of anti-KL-6 MAb (see also Figure S1 in Supporting Information). Microwave-assisted solid-phase synthesis of compounds **1**, **2**, **4**, and **9** and other key intermediates (**28**−**45**) was performed on a Rink amide PEGA resin (Novabiochem, EMD Biosciences) using *N*-fluorenylmethoxycarbonyl (N^{α}-Fmoc)-protected amino acids and N^{α}-Fmoc-Thr derivatives bearing some core sugars, and subsequent sugar elongation was achieved by an established procedure.[39−42] To facilitate the immobilization of the synthetic glycopeptides on the surface of a streptavidin-coated multititer plate, the *N*-termini of seven typical MUC1 tandem repeating glycopeptides (**1**−**7**) were tagged with a biotin moiety through a PEG-type linker using N^{α}-FmocHN$(CH_2CH_2O)_2CH_2$NHCOCH_2CH_2COOH.[47]

ELISA Assay. A preliminary screening using an ELISA plate displaying compounds **1**−**7** elicited that anti-KL-6 MAb (Sanko Junyaku Co., Ltd.) reacts strongly with compounds **3**, **5**, and **6** in a similar manner, while this MAb did not show any significant affinity with other glycopeptides (Figure 2). However, anti-KL-6 MAb did not show any detectable affinity toward Tn, T, 2,6-sialyl Tn (data not shown), and 2,6-sialyl T antigens, respectively. The fact that anti-KL-6 MAb did not recognize compound **7**, having a 2,3-sialyl T antigen at Thr of the VTSA motif, indicating that tandem repeat MUC1 peptides involving the PDTR motif modified by at least 2,3-sialyl T antigenic moiety are needed for the interaction with anti-KL-6 MAb. Since three reactive compounds (**3**, **5**, and **6**) contain 2,3-sialyl T at the Thr residue of the PDTR motif as a shared core structure, it seems likely that anti-KL-6 MAb cannot differentiate glycopeptide **6**, bearing core 2-based common *O*-glycan at the immunodominant PDTR site, from tumor-relevant MUC1 glycopeptides **3** and **5** having 2,3-sialyl T and 2,3/2,6-disialyl T structure, respectively. These results motivated us to clarify the effect of substitution at the *O*-6 position of the GalNAc residue on the binding specificity of anti-KL-6 MAb.

Thus, we carried out competitive ELISA using anti-KL-6 MAb and a 96-well plate displaying compound **6** in the presence of core 2-based glycopeptides **8**−**12** as competitive inhibitors. As shown in Figure 3, compounds **8**, **10**, **11**, and **12** exhibited similar strong inhibitory effects on the binding of anti-KL-6 MAb with arrayed glycopeptide **6**, while compound **9**, having a core 2-based trisaccharide moiety [Galβ1,3(GlcNAcβ1,6)GalNAcα], did not exhibit any inhibitory effect. These results and the data shown in Figure 2 suggest that substitutions at the *O*-6 position of GalNAc residue by Neu5Acα2→, GlcNAcβ1→, Galβ1,4GlcNAcβ1→, and Neu5Acα2,3Galβ1,4GlcNAcβ1→ do not influence the binding profile of anti-KL-6 MAb toward any MUC1 tandem repeat glycopeptides when its PDTR motif is modified at least with some core 2-type branched oligosaccharides involving a 2,3-sialyl T moiety as a crucial antigenic component.

It is well documented that core 2-based mature *O*-glycans of MUC1 glycoproteins often alter aberrantly short *O*-glycans such as Tn, T, or 2,6-sialyl T antigen in addition to the 2,3-sialyl T antigen during oncogenesis.[23] Furthermore, in breast cancer and other epitherial tumors, major *O*-glycans attached to the MUC1 tandem repeat seem to be core 1- rather than core 2-based due to the reduced expression level of 1,6-*N*-acetyl-D-glucosaminyltransferase-1 (C2GlcNAcT-1) in comparison with that of 2,3-sialyltransferase-I (ST3GalT-I).[48,49] In normal lactating breast cells, however, C2GlcNAcT-1 functions as a dominant enzyme to produce various *N*-acetyllactosamine repeats at the *O*-6 position of the GalNAc residue. Considering the binding potency of anti-KL-6 MAb with some core 2-based *O*-glycans at PDTR region distributing abundantly in normal MUC1 glycoproteins, anti-KL-6 MAb does not meet a criterion for the specific probe to detect only tumor-relevant biomarkers.

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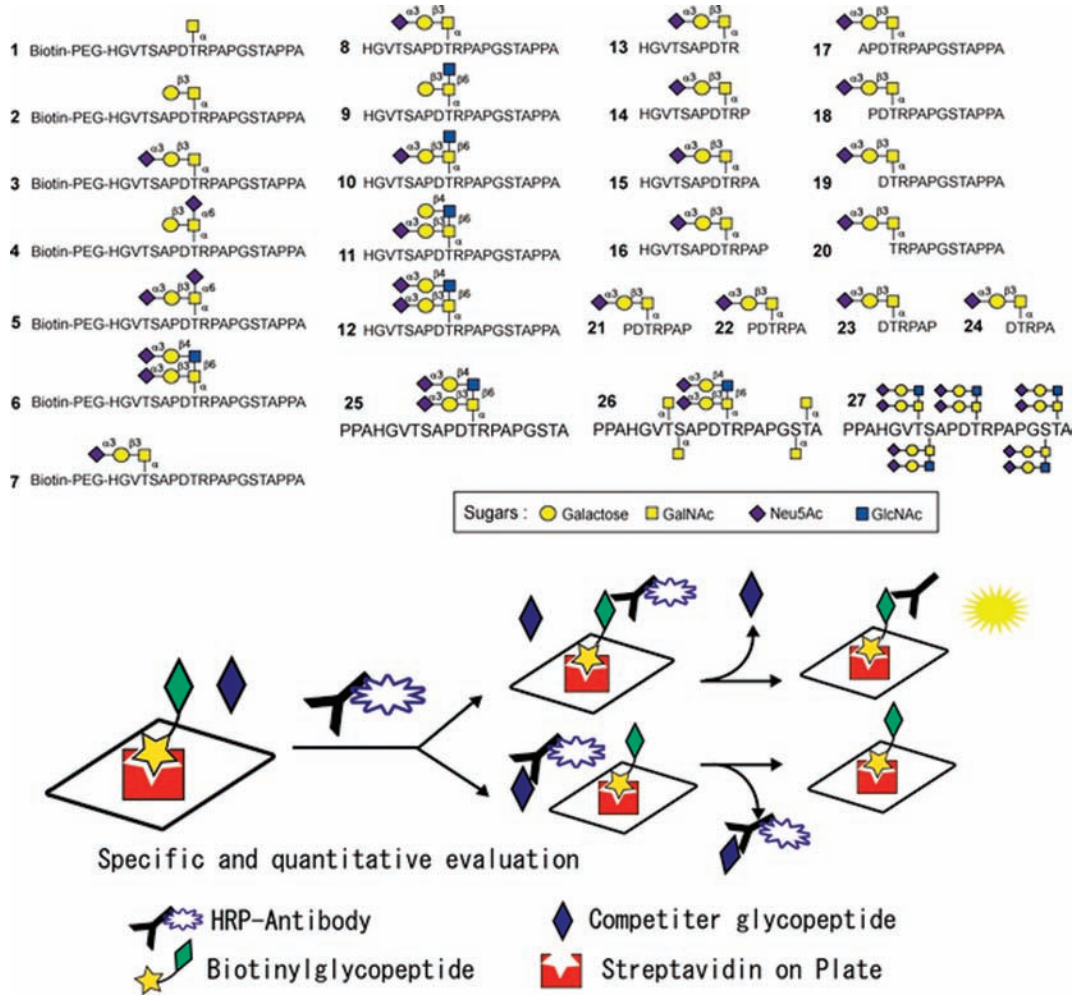

Figure 1. Major synthetic MUC1 glycopeptides and protocol of competition ELISA assay used in this study. Glycan structures in this Article are shown in the symbol nomenclature recommended by the Consortium for Functional Glycomics.

It should be emphasized that MAbs can differentiate a PDTR motif with 2,3-sialyl T antigen from other false positive candidates (such as MUC1 glycoproteins in which the PDTR regions are modified by 2,3/2,6-disialyl T or some core 2-based *O*-glycans involving 2,3-sialyl T antigen) and might be much more practically diagnostic probes for breast or some other tumor-specific biomarkers. On the other hand, it was demonstrated that the expression of C2GlcNAcT-1[50] in prostate carcinoma cells is strongly correlated with adhesion to type IV collagen and laminin, facilitating aggressive tumor growth and malignancy.[51] Therefore, it seems likely that some novel tumor epitopes can be identified in core 2-based *O*-glycans substituted at the PDTR motif.

Identification of an Essential Epitope. In order to fully assess an essential glycopeptide epitope for anti-KL-6 MAb, a minimal immunodominant peptide sequence in addition to the indispensable trisaccharide 2,3-sialyl T antigen, our interest was focused on evaluating the binding affinity of anti-KL-6 MAb with MUC1 glycopeptides **13**−**20**, in which the *N*- or *C*-terminal end is deleted sequentially. As indicated in Figure 4a, compounds **13**, **14**, **19**, and **20** did not exhibit any significant inhibitory effect on the interaction between anti-KL-6 MAb and an ELISA plate coated with the tandem repeat 20-mer glycopeptide **3**. Moreover, two 12-mer glycopeptides, **15** and **19**, showed only 35% and 3.5% cross activity compared with that of compound **8**, while compound **18** showed 76% cross activity. This suggests that the minimal epitope peptide sequence recognized by anti-KL-6 MAb may be heptapeptide Pro-Asp-Thr-Arg-Pro-Ala-Pro (PDTRPAP). To demonstrate this hypothesis, small glycopeptides **21**−**24** were prepared, and their cross activity was compared with that of 20-mer MUC1 glycopeptide **8**. As anticipated, the two proline residues in the tandem repeat, Pro-7 and Pro-13, proved to be crucial amino acids because compound **24** lost the cross activity and compounds **22** and **23** significantly reduced the inhibitory activity of the proposed glycopeptide epitope **21** (Figure 4b). We interpret these results as clear evidence that the minimal antigenic structure, an essential glycopeptide epitope, recognized by anti-KL-6 MAb is a heptapeptide sequence PDTRPAP, in which the Thr residue is modified by Neu5Acα2,3Galβ1,3GalNAcα (2,3-sialyl T antigen, core 1-type *O*-glycan, Figure 4c).

Influence of *O*-Glycosylation at Neighboring Thr/Ser Residues. Finally, our attention was directed toward the effect of *O*-glycosylation at neighboring Thr/Ser residues, other potential modification sites outside PDTRPAP, and on the interaction between this MAb and the site-specifically glycosylated PDTRPAP motif. To investigate this issue, we selected three plausible model

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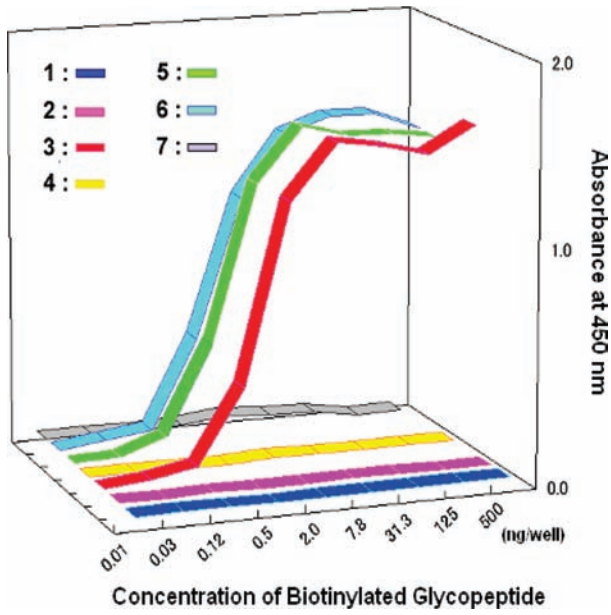

Figure 2. Interaction of anti-KL-6 MAb with compounds **1**−**7**. ELISA plates were coated with 100 μL/well of *N*-biotinylated glycopeptides **1**−**7**, serial diluted from 0.01 to 500 ng/well, and titers were determined by linear regression analysis, plotting dilution versus absorbance at 450 nm according to the modified protocol and under conditions recommended in the procedure for monitoring human serum KL-6 (EitestKL-6, Sanko Junyaku Co., Ltd.). We preliminarily screened the major glycopeptides listed in Figure S1 of the Supporting Information to establish an original ELISA assay system. Thus, compound **6**, a core 2-type major *O*-glycan involving a shared core structure among three reactive glycopeptides **3**, **5**, and **6**, was chosen as a tentative positive control used in the following "competition ELISA assay".

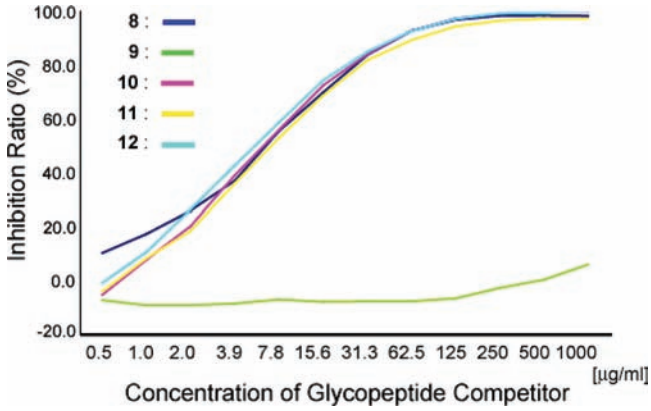

Figure 3. Effect of substitution at the *O*-6 position of the GalNAc residue on the interaction of anti-KL-6 MAb with compound **6**. Competition assay was carried out by using an ELISA plate coated with *N*-biotinylated glycopeptide **6**. The absorbance at 450 nm of this positive standard-coated ELISA plate ranged from 3.133 to 3.141, while that of the negative control (uncoated ELISA plate) ranged from 0.054 to 0.078. Inhibitory effects of compounds **8**−**12** on the interaction between anti-KL-6 MAb and glycopeptide **6** were represented as inhibition ratio (%).

compounds, **25**−**27**, in which the Thr residue at PDTRPAP moiety has a core 2-based *O*-glycan involving 2,3-sialyl T antigen, [Neu5Acα2,3Galβ1,3(Neu5Acα2,3Galβ1,4GlcNAcβ1,6)GalNAcα]. It was suggested that anti-KL-6 MAb does not recognize the immunodominant structure involved in compound **27**[41] when four other potential *O*-glycosylation sites are also covered by the same core 2-based *O*-glycans, namely a model of normal MUC1 glycopeptides fully *O*-glycosylated by five mature core 2-based hexasaccharide moieties (Figure 5). Interestingly, compound **26**, with four Tn (GalNAcα) antigenic sugars outside

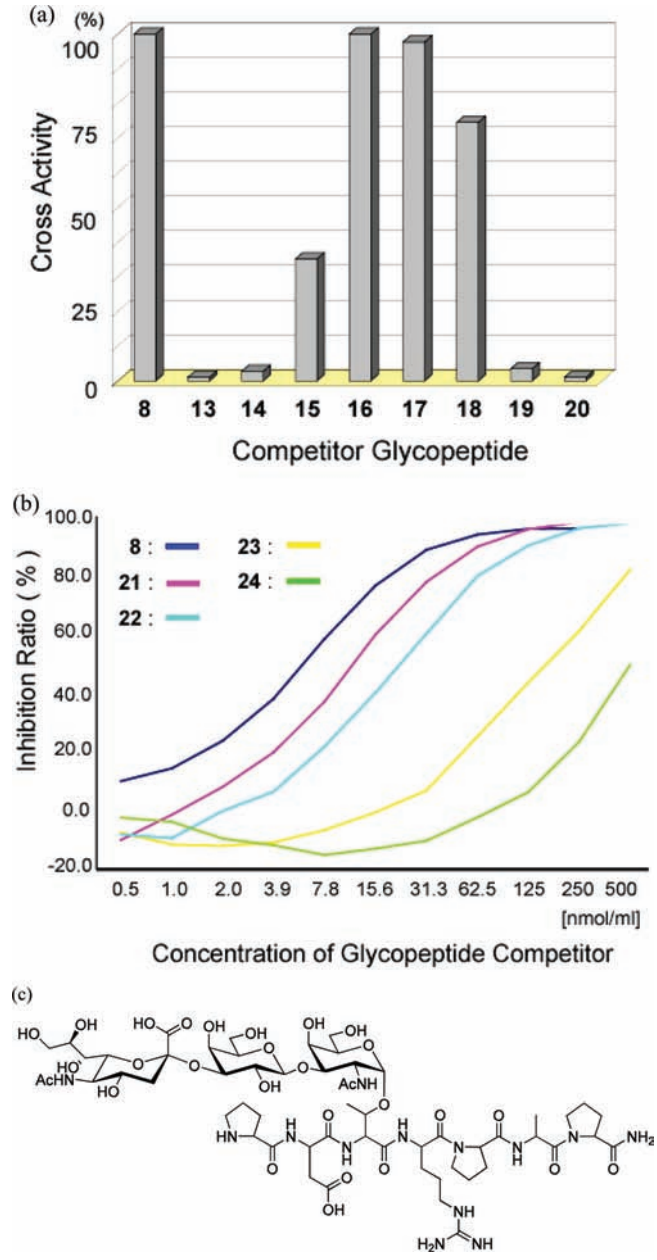

Figure 4. Identification of an essential glycopeptide epitope for anti-KL-6 MAb. ELISA plates were coated with *N*-biotinylated glycopeptide **3**. The absorbance at 450 nm of this positive standard-coated ELISA plate was a range from 2.968 to 3.541, while that of the negative control (uncoated ELISA plate) was a range from 0.051 to 0.054. (a) Binding affinity (shown as cross activity) of compounds **13**−**20** with anti-KL-6 MAb in comparison to that of compound **8**. (b) Determination of the minimal antigenic structure recognized by anti-KL-6 MAb. Competition assay using ELISA plate coated with *N*-biotinylated glycopeptide **3** and anti-KL-6 MAb was carried out in the presence of compounds **8**, **21**−**24**, and their inhibitory effects were represented as inhibition ratio (%). (c) Chemical structure of a minimal antigenic MUC1 glycopeptide **21** recognized by anti-KL-6 MAb.

the PDTRPAP region, HGVTSAPDTRPAPGSTAPPA, showed significantly higher inhibitory activity than compound **25**, indicating that attachment of aberrant GalNAc residues at neighboring Thr/Ser residues contributes to the formation of beneficial epitope conformation of this 7-mer MUC1 glycopeptide region. It seems likely that glycosylation by aberrantly short *O*-glycans such as Tn or T (Galβ1,3GalNAcα) antigens at VTSA and GSTA regions affects the conformational equi-

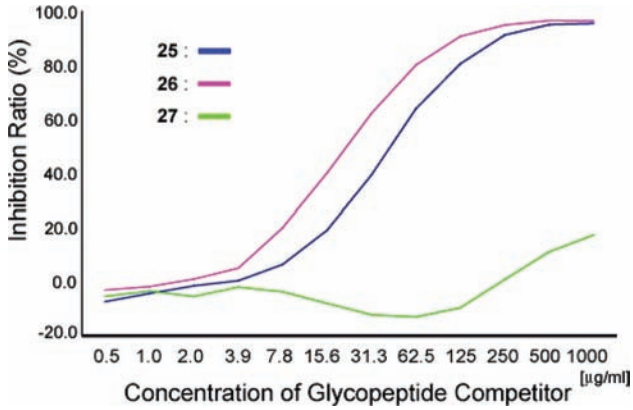

Figure 5. Effects of neighboring *O*-glycosylation on the interaction between anti-KL-6 MAb and a crucial glycopeptide epitope at the PDTRPAP site. ELISA plates were coated with *N*-biotinylated glycopeptide **6**. The absorbance at 450 nm of this positive standard-coated ELISA plate was a range from 3.133 to 3.141, while that of the negative control (uncoated ELISA plate) was a range from 0.054 to 0.078. Inhibitory effects of aberrantly *O*-glycosylated **26** and highly *O*-glycosylated MUC1 glycopeptide **27** were estimated by competition ELISA assay, comparing with compound **25**.

librium of this epitope heptapeptide region.[14,15,52−56] On the other hand, it was also independently documented that most MAbs generated by various breast cancer cell lines react with an epitope involving the PDTR motif, and the binding of these MAbs is often modulated directly at this sequence by the presence of particular *O*-glycans such as Tn, sialyl Tn, or T antigens.[11,17−20,43] Although other MUC1 MAbs such as SM-3[57] and MY.1E12[22] exhibited similar characteristics, our results also demonstrated that the glycoform at other potential *O*-glycosylation sites, such as VTSA and GSTA, greatly influences the binding profiles of anti-KL-6 MAb.

NMR study of the minimal antigenic glycopeptide **21** suggested highly converged β-turn-like structure at the PDTR sequence involving the GalNAcα1→ moiety, while other peptide regions and non-reducing disaccharide (sialylα2,3Galβ1→) appeared to be flexible (see Supporting Information, Tables S1−S4 and Figures S6, S7). It is also clear that attachment of highly bulky core 2-based hexasaccharide chains at four other *O*-glycosylation sites interrupts the access of MAbs to the minimal epitope moiety at PDTRPAP. However, it should be noted that further structural characterization using suitable synthetic MUC1 glycopeptides will allow for the precise analysis of the effects of the multiple *O*-glycosylation at four other potential sites on the epitope conformations as well as its antigenic property.

Conclusion

As summarized in Figure 6, we demonstrated for the first time that an essential epitope structure of anti-KL-6 MAb is

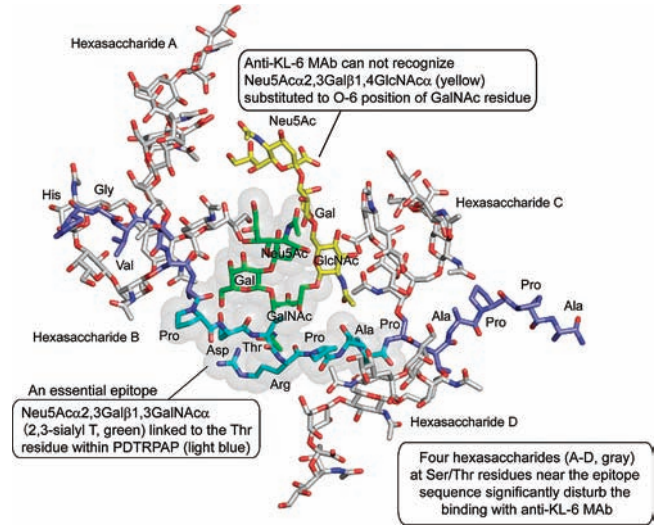

Figure 6. Summary of the essential epitope structure for anti-KL-6 MAb revealed by the synthetic MUC1 glycopeptide library.

the heptapeptide PDTRPAP sequence having 2,3-sialyl T antigen at the Thr residue. Anti-KL-6 MAb did not bind with other tumor-relevant antigens, such as GalNAcα (Tn), Neu5Acα2,6GalNAcα (STn), and Galβ1,3GalNAcα (T), except for Neu5Acα2,3Galβ1,3(Neu5Acα2,6)GalNAcα (2,3/2,6-disialyl T), involving the above minimal epitope structure. In addition, a robust synthetic glycopeptide library revealed that anti-KL-6 MAb could not differentiate core 1-based sialylT antigen at PDTRPAP sequence from some core 2-based glycopeptides having this crucial epitope structure and showed a similar binding affinity toward these core 2-based glycopeptides. These results indicated that branching at the *O*-6 position of the GalNAc residue does not influence the interaction of anti-KL-6 MAb with some MUC1 glycoproteins involving an essential epitope. It should be emphasized that human serum KL-6, a clinically important biomarker of interstitial pneumonia,[28−30] is a novel class of MUC1 glycoproteins containing this unique glycopeptide epitope. However, it has also been documented that anti-KL-6 MAb reacts with serum MUC1 glycoproteins derived from various cancers, such as lung adenocarcinoma, breast cancer, colorectal adenocarcinoma, and hepatocellular carcinoma as well as interstitial pneumonia.[31−37] We conclude herein that anti-KL-6 MAb exhibits broad binding specificities toward various MUC-1 glycoproteins overexpressed and shed into the circulatory system during the progress of many cancers as well as interstitial pneumonia, in which all MUC1 tandem repeats display at least 2,3-sialyl T, 2,3/2,6-disialyl T, or core 2-based *O*-glycans involving an essential epitope at the PDTR motif. Our results could suggest that the combined use of anti-KL-6 MAb with some MAbs such as BW835,[21] MY.1E12,[22] 5E5 and 2D9,[23] defining the epitopes of aberrantly *O*-glycosylated VTSA and GSTA regions, allows highly precise structural profiling of MUC1 glycoproteins and makes much more reliable disease-selective diagnosis possible. In addition, some MAbs that can identify the sugars substituted at the *O*-6 position of the GalNAc residue in core 2-based MUC1 glycopeptides including the PDTR region might become promising novel diagnostic probes for the immunodominant PDTR motif. Based on the essential epitope structure of anti-KL-6 MAb revealed by the present study, we succeeded in the generation of new class-specific MAbs that discriminate core 1- and core 2-based *O*-glycans of MUC1 glycopeptides containing the PDTRPAP sequence, and the potentials of these MAbs will be communicated shortly. It is

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our belief that high thoughput and precise epitope mapping of many known MUC1-related MAbs[9] by means of a robust synthetic glycopeptide library would greatly accelerate the development of practically available and highly potential new MAbs exhibiting much higher specificity against serum MUC1 glycoproteins relevant to the individual carcinomas of different organs.

Experimental Section

Materials. All commercially available solvents and reagents were used without purification. Rink amide PEGA resin[58,59] was purchased from Nova Biochem (now Merck Bioscience), and *N*-fluorenylmethoxycarbonyl (N^{α}-Fmoc)-amino acid derivatives except for glycosylated compounds were purchased from Nova Biochem (now Merck Bioscience) and Peptide Institute Inc. Biotin was purchased from Sigma-Aldrich. N^{α}-Fmoc-glycosylated amino acids[60−63] and polyethylene glycol (PEG)-linker (N^{α}-Fmoc $HN(CH_2CH_2O)_2CH_2NHCOCH_2CH_2COOH$)[47] were synthesized as described in previous reports. Solid-phase glycopeptide syntheses were performed on a commercial laboratory microwave reactor (IDX Corp. GreenMotif I). Recombinant human β1,4-galactosyltransferase (β1,4-GalT) was purchased from Toyobo, Ltd., and α2,3-(*N*)-sialyltransferase (α2,3-(*N*)-SiaT) and α2,3-(*O*)-sialyltransferase (α2,3-(*O*)-SiaT) were purchased from Calbiochem (Merck Bioscience). Uridine-5′-diphosphogalactose·2Na (UDP-Gal) and cytidine-5′-monophospho-*N*-acetylneuraminic acid·2Na (CMP-Neu5Ac) were purchased from Yamasa Co. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOFMS) data were recorded on Bruker Ultraflex II and Autoflex instruments, controlled by the Flexcontrol 1.2 software package as reported previously.[42,64] 2,5-Dihydroxybenzoic acid (DHB) was purchased from Wako Pure Chemical Industries, Ltd. Analytical and preparative reversed-phase high-performance liquid chromatography (HPLC) was performed on a Hitachi HPLC system equipped with an L-2130 intelligent pump and an L-2420 UV detector or equipped with an L-7100 intelligent pump and an L-7405 UV detector, and on a Shimadzu HPLC system equipped with an LC-20AD pump, an SPD-M20A diode array detector, and an LCMS-2010EV liquid chromatograph mass spectrometer using a reversed-phase C18 column. The analytical column used was an Inertsil ODS-3, 4.6 × 250 mm i.d., at a flow rate of 1.0 mL/min, and the preparative column was 10 × 250 mm i.d. (GL Science Inc.) Anti-KL-6 MAb was purchased from Sanko Junyaku Co., Ltd. (Tokyo, Japan; EITEST KL-6 kit). A 96-well microplate was purchased from Sumitomo Bakelite Co., Ltd. (S-BIO PrimeSuface ELISA plate) or Pierce (Reacti-Bind NeutrAvidin coated plate, high binding capacity (HBC) clear 96-well plate). Bovine serum albumin (BSA) was purchased from Sigma-Aldrich. Phosphate-buffered saline (PBS) tablets were purchased from Takara Bio. (dissolved 10 PBS tablets in distilled water to make a total volume of 1000 mL). 3,3′,5,5′-Tetramethylbenzidine (TMB) substrate solution was purchased from Kirkegaard & Perry Laboratories, Inc. The 96-well microplate reader was a Tecan Sunrise Thermo. Chromatography was monitored by absorption at 210 or 220 nm. Electrospray ionization mass spectrometry (HR-ESI-MS, JEOL JMS-700TZ) and amino acid analyses (Procise491 cLC, Applied Biosystems) were performed at the Center of Instrumental Analysis at Hokkaido University.

General Methods for Microwave-Assisted Solid-Phase Glycopeptide Synthesis. All glycopeptide intermediates were synthesized by our established procedures on a microwave reactor (Green Motif I, IDX Corp.)[38−42] with a Rink amide PEGA resin (Nova Biochem, EMD Biosciences) by means of N^{α}-Fmoc-protected amino acids, N^{α}-Fmoc-protected glycoamino acids, and 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethylaminium hexafluorophosphate (HBTU)[65] plus *N*-hydroxybenzotriazole (HOBt)[66] as the activating reagents. The following protected amino acids and glycoamino acids were used: N^{α}-Fmoc-Ala-OH, N^{α}-Fmoc-Arg(Pfb)-OH, N^{α}-Fmoc-Asp(O-*t*-Bu)-OH, N^{α}-Fmoc-Gly-OH, N^{α}-Fmoc-His(Trt)-OH, N^{α}-Fmoc-Pro-OH, N^{α}-Fmoc-Ser(*t*-Bu)-OH, N^{α}-Fmoc-Thr(*t*-Bu)-OH, N^{α}-Fmoc-Val-OH, N^{α}-Fmoc-Thr($AcO_3GalNAc\alpha$)-OH, N^{α}-Fmoc-Thr[$AcO_4Gal\beta1\rightarrow3(AcO_2GalNAc\alpha)$]-OH, N^{α}-Fmoc-Thr[$AcO_4Gal\beta1\rightarrow3(AcO_3GlcNAc\beta1\rightarrow6)GalNAc\alpha$]-OH, and N^{α}-Fmoc-Thr-[$AcO_4Gal\beta1\rightarrow3$(1-*O*-methyl $AcO_3Neu5Ac\alpha2\rightarrow6)GalNAc\alpha$]-OH [Pbf = N^{G}-(2,2,4,6,7-pentamethyldihyrobenzofuran-5-sulfonyl); Trt = trityl].[60−63] Coupling reactions of Fmoc-amino acids were agitated for 5 min and those of Fmoc-glycoamino acids for 20 min under microwave irradiation (0−40 W, 2450 Hz) at 50 °C. After the coupling reaction, unreacted amino groups on the resin were acetylated with a solution of acetic anhydride (Ac_2O) (4.75%, v/v), DIEA (2.25%, v/v), and HOBt (13 mM) in DMF for 5 min. N^{α}-Fmoc groups were cleaved by piperidine (20%, v/v) in DMF for 3 min under microwave irradiation. After completion of the synthesis, the resin having the protected glycopeptide was treated with trifluoroacetic acid (TFA)−water−triisopropylsilane (TIS, 95:2.5:2.5, v/v/v) at room temperature for 1 h and filtered. The solution was concentrated by streaming nitrogen gas and addition of ether and decanted. Subsequent purification of the crude product was carried out by preparative reverse-phase HPLC [column, Inertsil ODS-3 (10 × 250 mm); flow rate, 4.0 mL/min; elution buffer A, H_2O containing 0.1% TFA and buffer B, MeCN containing 0.1% TFA for glycopeptides having neutral sugars, or buffer A, H_2O containing 25 mM ammonium acetate and buffer B, MeCN containing 10% buffer A for glycopeptides containing sialic acid residue; composition of the solvent, 0−45 min in a linear gradient flow from (A/B) = (98/2) to (85/15) or (80/20); detection, UV at 220 nm]. Compounds **1**, **2**, **4**, **9**, and intermediates **28**−**45** were released from resin by treating with TFA−water−TIS (95:2.5:2.5, v/v/v) at room temperature for 1 h and purified by preparative RP-HPLC (Inertsil ODS-3 column). Details of the chemical synthesis and purification procedure and analytical data for these compounds are provided in the Supporting Information.

General Methods for the Enzymatic Modifications of Glycopeptides. Three optimized reaction conditions were employed according to the previous reports[38,44] as follows: (1) a solution of glycopeptide intermediate (1 mM), CMP-Neu5Ac (Yamasa Co., 5 mM), and α2,3-(*O*)-sialyltransferase (Merck Bioscience, 17.5 mU/mL) in 50 mM HEPES buffer (10 mM $MnCl_2$, 0.1% BSA, pH 7.0); (2) a solution of glycopeptide intermediate (1 mM), UDP-Gal (Yamasa Co., 5 mM), CMP-Neu5Ac (Yamasa Co., 5 mM), β1,4-galactosyltransferase (Toyobo Co., 100 mU/mL), and α2,3-(*O*)-sialyltransferase (Merck Bioscience, 17.5 mU/mL) in 50 mM HEPES buffer (10 mM $MnCl_2$, 0.1% BSA, pH 7.0); and (3) a solution of glycopeptide intermediate (1 mM), UDP-Gal (Yamasa Co., 5 mM), CMP-Neu5Ac (Yamasa Co., 5 mM), β1,4-galactosyltransferase (Toyobo Co., 100 mU/mL), α2,3-(*O*)-sialyltransferase (Merck Bioscience, 17.5 mU/mL), and α2,3-(*N*)-sialyltransferase (Merck Bioscience, 74 mU/mL) in 50 mM HEPES buffer (10 mM $MnCl_2$, 0.1% BSA, pH 7.0). All reactions were performed at 25 °C for 24 h. The reaction mixture was purified by RP-HPLC [column, Inertsil ODS-3 (10 × 250 mm); flow rate, 4.0 mL/min; elution buffer A, H_2O containing 25 mM ammonium acetate and buffer B, MeCN containing 10% buffer A; composition of the

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solvent, 0−45 min in a linear gradient flow from (A/B) = (98/2) to (85/15) or (80/20); detection, UV at 220 nm]. Details of the enzymatic reactions and purification procedure and analytical data for compounds **3**, **5**, **6**, **7**, **8**, and **10**−**27** are provided in the Supporting Information.

ELISA Assay. The microtiter plate [S-BIO PrimeSuface or ELISA plate, Sumitomo Bakelite Co., Ltd.; or Reacti-Bind NeutrAvidin-coated plate, Pierce; high binding capacity (HBC) clear 96-well plate] was coated with 100 μL/well of *N*-biotinylated glycopeptides **1**−**7**, serial diluted from 0.01 to 500 ng/well in buffer A, PBS containing 0.05% (v/v) Tween 20 (Sigma-Aldrich). Each well was washed three times with buffer A and blocked with buffer B, PBS containing 0.05% (v/v) Tween 20 and 0.1% (w/v) BSA (Sigma-Aldrich) at room temperature for 1 h. Anti-KL-6 MAb (EITEST KL-6 kit, Sanko Junyaku Co., Ltd., Tokyo, Japan) and buffer B were added at 100 μL/well. After incubation at room temperature for 1 h, the wells were washed three times with buffer A, and TMB substrate solution (Kirkegaard & Perry Laboratories, Inc.; TMB microwell peroxidase substrate, two-component system, 100 mL each) was added at 100 μL/well. After incubation for 30 min, the reaction was terminated with addition of 100 μL/well of stop solution (1 M H_3PO_4). The rates of reaction in each well were measured for absorbance at 450 nm. In addition to the key compounds **1**−**7** discussed mainly in this study, some glycopeptides having other popular antigenic *O*-glycans, such as core 3-based derivatives, core 6-based derivatives, and 2,6-sialyl Tn, were also employed for a preliminary screening, but they did not show any affinity with anti-KL-6 MAb (data not shown).

Competition ELISA Assay. The microtiter plate was coated with 100 μL/well of *N*-biotinylated glycopeptide **3** or **6** solution with buffer A (compound **3** for Figure 4, compound **6** for Figures 3 and 5). Each well was washed three times with buffer A and blocked with buffer B at room temperature for 1 h. The glycopeptide inhibitor (100 μL/well) and anti-KL-6 MAb (100 μL/well) were then added. Inhibitors were used at serial binary concentrations from 1000 to 0.5 μg/mL or from 500 to 0.2 nmol/mL, and anti-KL-6 MAb was diluted 1:60 with buffer B. After incubation at room temperature for overnight, the wells were washed three times, and TMB substrate solution (100 μL/well) was added. After 30 min, the reaction was terminated with addition of 100 μL/well of stop solution (1 M H_3PO_4). The rates of reaction in each well were measured for absorbance at 450 nm. The percentage of inhibition was calculated by comparing the binding of MAb with and without inhibitor: % of inhibition = (binding of MAb with inhibitor/binding of MAb without inhibitor) × 100. The 50% inhibition data from the ELISA plate reader were fed into a data analysis template in the program XL fit (CTC Laboratory Systems Corp., Japan): % cross-reactivity = [mass of glycopeptide at 50% inhibition/mass of competitor at 50% inhibition] × 100.[67]

NMR Study of Glycopeptide Epitope 21. The glycopeptide **21** was dissolved in 300 μL of either 10% D_2O in H_2O or 99.9% D_2O at 2.0 mM. The pH was adjusted to 5.0 (as indicated by pH meter) by addition of HCl and NaOH. Shigemi NMR sample tubes matched with D_2O (BMS-005B, 4.2 mm i.d., Shigemi Co., Ltd.) were used for NMR experiments. NMR spectra were collected at 300 K with a Bruker Avance 600 spectrometer at 600.03 MHz equipped with a cryo-probe for proton frequency. For the assignments and the structure determination of glycopeptides, two-dimensional homonuclear DQF-COSY,[68] TOCSY with MLEV-17 sequence,[69] and NOESY[70] spectra were recorded in the indirect dimension using States-TPPI phase cycling. Additionally, two-dimensional heteronuclear ^{13}C-edited HSQC and HSQC-TOCSY measurements were performed in echo−anti-echo mode for sensitivity enhancement. TOCSY experiments were applied for a spin-locking time of 60 ms, and NOESY experiments were carried out with mixing times of 100, 150, 200, and 400 ms. The water signal was suppressed by presaturation during the relaxation delay (1 s) and by a 3-9-19 WATERGATE pulse sequence with field gradient.[71] TOCSY and NOESY spectra were acquired with 2048 × 512 frequency data points and were zero-filled to yield 2048 × 2048 data matrices. DQF-COSY with 16384 × 512 frequency data points was also recorded and zero-filled to yield a 16384 × 16384 matrix in order to measure the coupling constants. Sweep widths of 8389.26 Hz were applied. Time domain data in both dimensions were multiplied by a sine-bell window function with a 90° phase shift prior to Fourier transformation. All NMR data were processed by NMRPipe software[72] and analyzed using the Sparky program.[73] Sequence-specific resonance assignments were achieved according to the standard methods for small proteins established by Wüthrich and co-workers.[74] Stereospecific assignments for methylene protons were carried out by analyzing the intensities of intra-residue NOE between amide and β protons. Fully assigned ^{1}H and ^{13}C chemical shifts of glycopeptide **21** and other NMR data are summarized in the Supporting Information.

Structure Calculations. Three-dimensional structures of glycopeptides **21** were calculated using the CNS 1.1[75] program with standard protocols for distance geometry-simulated annealing and refinement. Distance restraints for calculations were estimated from the cross-peak intensities in NOESY spectra with a mixing time of 150 ms. The estimated restraints were classified into four categories: strong (1.6−2.6 Å), medium (1.6−3.5 Å), weak (1.6−5.0 Å), and very weak (1.6−6.0 Å). In the first stage of structure determination, the structures of the glycopeptide were calculated using only inter-proton distance information. After validation that the distance restraints were fulfilled for the obtained structure, the restraints of the dihedral angles ϕ and $\chi 1$ were adopted for structure calculation. When the coupling constant $^3J_{HN\alpha}$ was more than 8.0 Hz and less than 6.0 Hz, the dihedral angle ϕ was constricted to $-120 \pm 30°$ and $-60 \pm 30°$, respectively. The conformation of the sugar rings was held fixed to the chair conformation. All analyses of rmsd values and the solution structures of glycopeptides were performed with PROCHECK[76] and MOLMOL[77] programs (see also the Supporting Information).

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Supporting Information Available: Synthesis and characterization of synthetic glycopeptides, Figures S1−S29, and Tables S1−S4. This material is available free of charge via the Internet at http://pubs.acs.org.

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