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Structural and thermodynamic analyses reveal critical features of glycopeptide recognition by the human PILR α immune cell receptor

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Before entering host cells, herpes simplex virus-1 uses its envelope glycoprotein B to bind paired immunoglobulin-like type 2 receptor α (PILR α) on immune cells. PILR α belongs to the Siglec (sialic acid (SA)-binding immunoglobulin-like lectin)-like family, members of which bind SA. PILR α is the only Siglec member to recognize not only the sialylated O-linked sugar T antigen (sTn) but also its attached peptide region. We previously determined the crystal structure of PILR α complexed with the sTn-linked glycopeptide of glycoprotein B, revealing the simultaneous recognition of sTn and peptide by the receptor. However, the contribution of each glycopeptide component to PILR α binding was largely unclear. Here, we chemically synthesized glycopeptide derivatives and determined the thermodynamic parameters of their interaction with PILR α . We show that glycopeptides with different sugar units linking SA and peptides (*i.e.* “GlcNAc-type” and “deoxy-

GlcNAc-type” glycopeptides) have lower affinity and more enthalpy-driven binding than the wild type (*i.e.* GalNAc-type glycopeptide). The crystal structures of PILR α complexed with these glycopeptides highlighted the importance of stereochemical positioning of the O4 atom of the sugar moiety. These results provide insights both for understanding the unique O-glycosylated peptide recognition by the PILR α and for the rational design of herpes simplex virus-1 entry inhibitors.

Paired immunoglobulin-like type 2 receptors (PILRs)⁶ are surface proteins that are mainly expressed by immune cells, such as macrophages, dendritic cells, and granulocytes (1). PILRs form an archetypal paired receptor family, whose members typically include receptors with similar ectodomains but either inhibitory or stimulatory cytosolic signaling motifs. The inhibitory receptors such as PILR α incorporate an immunoreceptor tyrosine-based inhibition motif in the intracellular domain. By contrast, the activating receptors, such as PILR β , incorporate a positively charged amino acid in the transmembrane region that associates with the activating adaptor subunit, DAP12 (2, 3). The endogenous ligands for PILRs include murine CD99 (4), PILR-associating neural protein (5), neuronal differentiation and proliferation factor-1, and collectin-12 (6). PILR α has a higher affinity for these ligands than PILR β . Although the physiological effects of PILR binding to ligands remain largely unclear, *Pilra*^{−/−} mouse studies have shown increased neutrophil recruitment to inflammatory sites and increased susceptibility of the mice to endotoxin shock. Neutrophil recruitment in inflammatory responses is regulated by PILR α via modulation of integrin activation (7). PILR α is also the receptor for herpes simplex virus-1 (HSV-1), binding to its glycoprotein B (8). PILRs are sugar (sialic acid (SA))-binding

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This article contains supplemental Figs. S1–S5 and Table S1.

The atomic coordinates and structure factors (codes 5XOF and 5XO2) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

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⁶ The abbreviations used are: PILR, paired immunoglobulin-like type 2 receptor; HSV-1, herpes simplex virus-1; SA, sialic acid; ITC, isothermal titration calorimetry; Siglec, sialic acid-binding immunoglobulin-like lectin; r.m.s.d., root mean square deviation; sTn, sugar T antigen; Neu5Ac, N-acetylneuraminic acid; CLEC-2, C-type lectin-like receptor-2; Fmoc, N-(9-fluorenyl)methoxycarbonyl.

proteins and have part of a Siglec (sialic acid-binding immunoglobulin-like lectin)-like protein motif. It has been proposed that PILR α recognition of both endogenous and viral proteins is dependent on binding to both O-linked sugar T antigen (sTn) and peptide (9). The sTn antigen is produced by incomplete sugar modification, resulting in Neu5Ac(α 2-6)GalNAc and GalNAc linked to threonine residues. Other reports have shown that the sTn antigen is not usually expressed on healthy cell surfaces but is often found on cancer cells (10). It is thus important to elucidate the detailed binding mechanisms between PILR α and sTn peptide to better understand its role in immune cell responses to tumors and infection.

Recently, we reported the crystal structure of PILR α complexed with the GalNAc-type glycopeptide (9). This result indicated that PILR α simultaneously recognized both saccharide, especially the terminal SA, and peptide components. However, the precise contribution of each glycopeptide component to the strength and specificity of PILR α binding remains unclear. In this current study, we first determined the thermodynamic parameters necessary for the interaction between PILR α and GalNAc-type glycopeptide to better understand the mechanism for PILR α and glycopeptide interaction. This revealed that both the enthalpy and entropy contributed to PILR α binding to glycopeptides. Next, we synthesized glyco (and glyco analog)-peptide compound derivatives in which the sugar link between SA and O-linked Thr is GalNAc (wild type), GlcNAc, or deoxy-GlcNAc (with the hydroxyl group at position 4 replaced with hydrogen) and evaluated the affinity of their interactions with PILR α . The altered glycopeptides (hereafter GlcNAc-type glycopeptide and deoxy-GlcNAc-type glycopeptide) exhibited lower binding affinity than the wild-type GalNAc-type glycopeptide. To understand the detailed molecular mechanism of this reduction in binding affinity, we successfully determined the complex structures of PILR α with GlcNAc-type or deoxy-type glycopeptides by X-ray crystallography. Our results demonstrate the importance of each component of the glycopeptide for optimal stereochemical positioning of the sugar atoms that bind to PILR α . This study also further clarifies the molecular basis for the contribution of glycan and peptide binding to PILR α and provides novel insights for the rational design and development of HSV-1 entry inhibitors and immune checkpoint regulators.

Results

Thermodynamic properties of PILR α binding to HSV-1 gB GalNAc-type glycopeptide

Recently, we reported the crystal structure of PILR α complexed with a 7-mer GalNAc-type glycopeptide derived from the N-terminal region of HSV-1 gB (Gly⁵⁰–Pro⁵⁶) (Fig. 1*a*). The complex structure indicated that PILR α recognized terminal SA through a well-ordered network of hydrogen bonds and electrostatic interactions. Furthermore, a substantial conformation change in a region that contains hydrophobic amino acid residues, especially Phe⁷⁶ and His⁷⁷ in the CC' loop, was observed upon binding of proline and GalNAc of the glycopeptide to the receptor (9). To evaluate the physicochemical

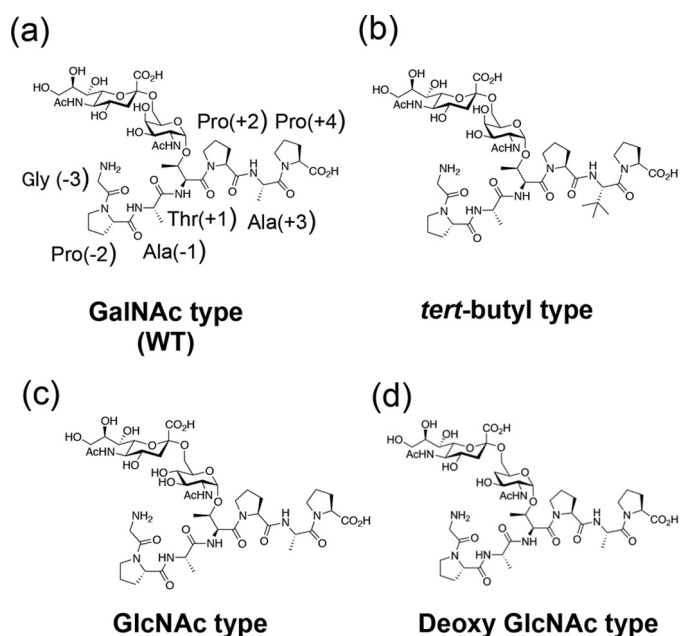


Figure 1. The synthetic and wild-type glycopeptides used in this study. *a*, GalNAc type (wild type); *b*, *tert*-butyl type; *c*, GlcNAc type; *d*, deoxy-GlcNAc type.

parameters of this interaction, we performed an isothermal titration calorimetry (ITC) experiment. The GalNAc-type glycopeptide (1 mM) was titrated into a 50 μ M PILR α solution. The dissociation constant (K_d) of the interaction is $6.7 \pm 1.6 \mu$ M, determined by two independent experiments and calculated to fit well with a 1:1 binding model (Fig. 2*a* and Table 1). This K_d value was similar to the affinity determined using the flexible N-terminal region of gB (amino acid residues 30–108) in surface plasmon resonance analysis ($\sim 2.8 \mu$ M) and competition assays (a few μ M) (9). We also titrated the SA, sTn-threonine, and GPATPAP peptide constituents of the GalNAc-type glycopeptide with PILR α , but none of them bound with detectable affinity (Fig. 3). These results supported the previous notion that simultaneous recognition of both sugar and peptide is necessary for binding to PILR α . This interaction is mainly enthalpy-driven with preferable entropy ($\Delta H = -5.8$ kcal/mol and $-T\Delta S = -1.2$ kcal/mol at 25 $^{\circ}$ C). The observation of an enthalpy–entropy-driven reaction is consistent with the fact that PILR α binds to the SA region of the GalNAc-type glycopeptide through hydrogen bonds and ionic interactions and to its peptide part through van der Waals interaction.

Thermodynamic property of the interactions of PILR α with *tert*-butyl-type glycopeptide

Our previous structural analysis indicated that Pro (+2) of the GalNAc-type glycopeptide, which is adjacent to Thr (+1) attached to O-glycan, interacts with Phe⁷⁶ and His⁷⁷ of PILR α . Further C-terminal amino acid residues, adjacent to the O-glycosylated threonine, form a turn structure that widely engages with the loop of PILR α . Increases in peptide chain flexibility can reduce the binding affinity of some protein–ligand interactions (11, 12). We thus sought to determine whether Ala (+3), which has a small side chain and presumably enhances flexibility,

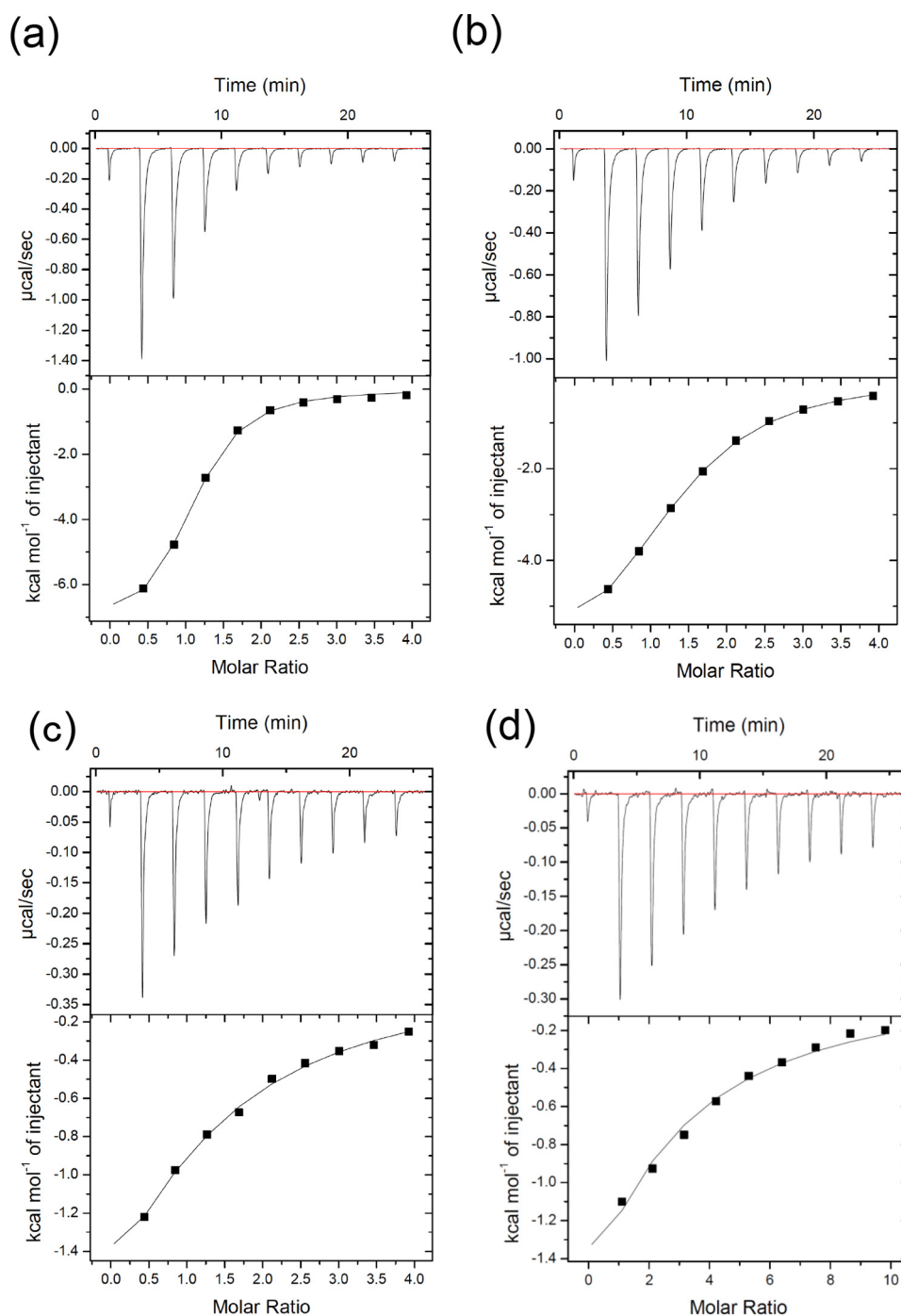


Figure 2. ITC measurements for PILR α binding to wild-type and synthetic glycopeptides. Upper panels show the representative titration thermograms, and lower panels show the data integration with fitted curves (1:1 binding model) of GalNAc-type (a), *tert*-butyl-type (b), GlcNAc-type (c), and deoxy-GlcNAc-type (d) glycopeptides with PILR α . The fitting error estimates were calculated using a 1:1 binding model and are as follows: a, $K_d = 5.1 \pm 0.1 \mu\text{M}$, $\Delta H = -7.2 \pm 0.0 \text{ kcal/mol}$; b, $K_d = 21.9 \pm 0.9 \mu\text{M}$, $\Delta H = -6.8 \pm 0.1 \text{ kcal/mol}$; c, $K_d = 97.1 \pm 5.4 \mu\text{M}$, $\Delta H = -4.0 \pm 0.1 \text{ kcal/mol}$; d, $K_d = 120.6 \pm 11.4 \mu\text{M}$, $\Delta H = -9.5 \pm 0.5 \text{ kcal/mol}$.

could be modified to reduce glycopeptide flexibility. Thus, a *tert*-butyl modification was introduced to the side chain of Ala (+3) (Fig. 1b). The ITC experiment using the bulky *tert*-butyl-modified Ala (+3) unnatural glycopeptide (*tert*-butyl-type glycopeptide) determined a K_d of $18 \mu\text{M}$ for the interaction with PILR α (Fig. 2b and Table 1). This indicated that the *tert*-butyl group modification of Ala (+3) did not result in the expected increase in binding but rather slightly decreased the affinity of glycopeptide for PILR α . Thermodynamic parameters for the

interaction indicated that it was enthalpy-driven with a small preferable entropy component ($\Delta H = -6.0 \pm 0.8 \text{ kcal/mol}$ and $-T\Delta S = -0.7 \pm 0.7 \text{ kcal/mol}$ at 25°C) (Fig. 2b and Table 1). These parameters were similar to those derived for interactions of PILR α with the wild-type peptide (Table 1). This result suggested that the addition of bulky side chains to the peptide region, which probably does not form a direct contact with PILR α , is not sufficient to change the binding characteristics.

Table 1
Thermodynamic parameters of PILR α binding

The values are means $\pm$ ranges derived from two independent experiments calculated using a 1:1 binding model. Representative binding data for one of the two experiments is shown in Fig. 2.

	Ligand			
	GalNAc type	<i>tert</i> -Butyl type	GlcNAc type	Deoxy-GlcNAc type
ΔG (kcal/mol)	-7.1 ± 0.2	-6.7 ± 0.0	-5.5 ± 0.0	-5.5 ± 0.2
$-T\Delta S$ (kcal/mol)	-1.2 ± 1.2	-0.7 ± 0.7	-1.4 ± 0.1	2.2 ± 1.9
ΔH (kcal/mol)	-5.8 ± 1.3	-6.0 ± 0.8	-4.1 ± 0.1	-7.7 ± 1.8
N	0.98	1.27		
K_A (M $^{-1}$)	1.6×10^5	6.1×10^4	10×10^3	12×10^3
K_d (μ M)	6.7	18	98	94

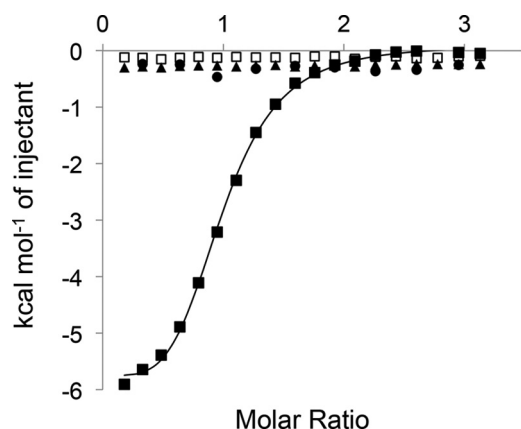


Figure 3. ITC measurements for PILR α binding to isolated components of the GalNAc-type glycopeptide. Titration isothermograms of PILR α with the different glycopeptide components are shown. ■, GalNAc-type glycopeptide; ▲, sTn-threonine; ●, GPATPAP; □, SA.

Effect of changing the GalNAc sugar moiety in the wild-type glycopeptide on the thermodynamic properties of binding interactions with PILR α

Our previous published crystal structure of the PILR α –GalNAc-type glycopeptide indicated that GalNAc does not significantly contribute to PILR α binding to glycopeptides. Thus, this region could be a potential target site for modification to alter the affinity of glycopeptide. To verify the role of GalNAc, we synthesized two glycopeptides with the substitution of either GlcNAc-type glycopeptide (GlcNAc type) (Fig. 1c) or deoxy-GlcNAc-type glycopeptide (deoxy type) (Fig. 1d) with GalNAc. Neither of these synthetic glycopeptides occurs naturally. GlcNAc-type and deoxy-type glycopeptides have a different orientation (equatorial) and a loss of the hydroxyl group at position 4 (4-OH), respectively. We determined the dissociation constants and thermodynamic parameters of the interactions of these altered peptides by ITC. Both unexpectedly and notably, a great reduction of the affinity of GlcNAc-type glycopeptide for PILR α was observed ($98 \pm 1.0 \mu$ M) (Fig. 2c and Table 1). Thermodynamic parameters of the interaction showed an enthalpy-driven reaction with a small favorable entropy effect ($\Delta H = -4.1$ kcal/mol and $-T\Delta S = -1.4$ kcal/mol at 25 °C). Furthermore, we also found that the K_d of the deoxy-type glycopeptide binding to PILR α ($94 \pm 26 \mu$ M) is weaker than that of the GalNAc-type (Fig. 2d and Table 1). Thermodynamic parameters for the interaction between PILR α and the deoxy-type glycopeptide showed a more unfavorable entropy effect ($\Delta H = -7.7$ kcal/mol and

$-T\Delta S = 2.2$ kcal/mol at 25 °C) than the GalNAc-type peptide. Although the hydroxyl group at position 4 of GalNAc is not directly involved in the interaction with PILR α , it nevertheless contributes to the interaction with PILR α with favorable entropy.

Crystallographic analyses of PILR α –GlcNAc-type and –deoxy-type glycopeptide complexes

To determine the molecular mechanism for the reduced affinity of GlcNAc-type and deoxy-type glycopeptides for PILR α , we performed X-ray crystallographic studies of the PILR α complexes with these two glycopeptides. Crystals of PILR α complexed with a 5-fold molar ratio excess of each GlcNAc-type glycopeptide were successfully obtained in 0.1 M Tris-HCl (pH 8.5) and 25% PEG6000. Crystals for the deoxy-GlcNAc-type complex were grown in 0.1 M MES monohydrate (pH 6.5) and 1.6 M magnesium sulfate heptahydrate. The diffraction data were collected at SPring-8 (Harima, Japan) with 1.96-Å resolution using the BL44XU beamlines for the GlcNAc-type complex and 2.2-Å resolution using the BL32XU beamlines for the deoxy-type complex. The crystallographic parameters are summarized in Table 2. Four complexes were observed in the asymmetric unit in the PILR α –GlcNAc-type glycopeptide complex that were essentially the same as the GalNAc type (root mean square deviation (r.m.s.d.) = 0.20–0.43 Å) (supplemental Fig. S1). Two chains were observed in the asymmetric unit in the deoxy-type complex; both bound glycopeptide without any significant changes (r.m.s.d. = 0.24 Å) (supplemental Fig. S2). In both complexes, the conformations of peptides between each chain were also hardly changed (PILR α complexed with GlcNAc-type or deoxy-GlcNAc-type glycopeptide is shown in supplemental Figs. S3 and S4, respectively). Thus, we use chain A in both complex structures as a representative chain hereafter.

Structural comparison of PILR α –GalNAc-type, –GlcNAc-type, and –deoxy-GlcNAc-type glycopeptide complexes

The crystal structures of PILR α complexes with the GlcNAc- and deoxy-GlcNAc-type glycopeptides and GalNAc type are shown in Fig. 4, a–c. The electron density maps clearly show that the synthetic GlcNAc-type and deoxy-GlcNAc-type glycopeptides exhibit the equatorial orientation and loss of the hydroxyl group, respectively, as shown in Fig. 4, d–f. The superimposition of the complex structures showed that GlcNAc type (light pink) and deoxy-GlcNAc type (yellow) are essentially the same, but in contrast, the GalNAc-type complex structure (green) does not fit well with the others (Fig. 4g). Next, we closely examined the binding sites and compared the molecular/atomic distances for each interaction. The key residues of PILR α for binding to GalNAc type are Tyr³³, Arg¹²⁶, Gln¹³⁸, and Gln¹⁴⁰ for binding to the sialic acid moiety; Phe⁷⁶ for binding to the peptide moiety; and His⁷⁷ for binding to both GalNAc and peptide. The superimposed structure of the interface and distances of essential interactions for the three complex structures are summarized in Fig. 5, a–d, and supplemental Table S1. Almost all of interactions with sialic acid are conserved. The carbonate group of the sialic acid residue interacts directly with

Table 2Crystallographic parameters for the PILR α -GlcNAc and -deoxy-GlcNAc-type glycopeptide complexes

	GlcNAc-type glycopeptide-PILR α complex	Deoxy-GlcNAc-type glycopeptide-PILR α complex
Data collection		
Space group	P21	C2
Cell dimensions		
<i>a</i> , <i>b</i> , <i>c</i> (Å)	54.66, 63.01, 78.6	81.28, 63.33, 55.07
α , β , γ (°)	90, 108.3, 90	90, 110.8, 90
Resolution (Å) ^a	50–1.96 (1.99–1.96)	50–2.2 (2.24–2.2)
<i>R</i> _{merge} ^a	0.105 (0.322)	0.054 (0.169)
<i>I</i> / σ (<i>I</i>) ^a	20.9 (5.5)	25.0 (8.4)
Completeness (%) ^a	99.4 (96.35)	99.9 (99.7)
Redundancy ^a	3.8 (3.8)	3.8 (3.6)
Total no. of observations ^a	137,800	51,035
Unique reflections ^a	36,271 (1,754)	13,393 (668)
Refinement		
No. of reflections (work/test)	32,742/1,670	12,752/673
Resolution (Å) ^a	40.05–1.963 (2.033–1.963)	40.14–2.201 (2.279–2.201)
<i>R</i> _{work} ^a	0.2145 (0.2671)	0.2163 (0.2352)
<i>R</i> _{free} ^a	0.2692 (0.3341)	0.2616 (0.3188)
No. of atoms		
Protein	4,116	2,058
Ligand	136	66
Water	258	49
B factors		
Protein	18.86	33.16
Ligand	16.85	25.46
Water	24.47	34.10
r.m.s.d.		
Bond lengths (Å)	0.009	0.008
Bond angles (°)	1.50	1.44
Ramachandran plot statistics		
Most favored (%)	97.35	95.53
Disallowed (%)	0	0.41

^a Statistics for the highest-resolution shell are shown in parentheses.

guanidine group of Arg¹²⁶ in all complexes (2.7 and 2.9 Å in PILR α -GalNAc type, 2.8 and 3.1 Å in PILR α -GlcNAc type, and 2.8 and 3.1 Å in PILR α -deoxy-GlcNAc type) (Fig. 5c and supplemental Table S1). The amide and carbonyl groups of the main chain of Gln¹³⁸ and Gln¹⁴⁰ interact with the hydroxyl group of the glycerol part and amide group of the *N*-acetyl group of SA, respectively, in all complexes. Tyr³³ also interacts with SA in a similar manner. These observations indicate that the interactions of PILR α with SA of the three different glycopeptides are almost unchanged. In contrast, the interactions of Phe⁷⁶ and His⁷⁷ with the different ligands are different between the different complexes. Phe⁷⁶ makes van der Waals contacts with the hydrophobic pyrrolidine rings of proline residues in the C-terminal part of the GalNAc-type sTn peptide (a distance of 3.4 Å; Fig. 5a, *white dotted line*). This distance increases to create weak contacts in the other complexes (3.8 Å in GlcNAc type and 4.1 Å in deoxy-GlcNAc type) (Fig. 5, *a* and *d*). Previous results also indicated that His⁷⁷ forms a hydrogen bond with the carboxyl group of Pro (+2) (Fig. 5a, *black dotted line*) and a van der Waals contact with GalNAc (Fig. 5a, *silver dotted line*) (9). Although these hydrogen bonds have similar distances in the three complexes (2.6 (GalNAc), 2.8 (GlcNAc), and 2.8 Å (deoxy-GlcNAc)), the distances between His⁷⁷ and the linker sugars are changed between the three complexes (Fig. 5, *b* and *d*). In the PILR α -GalNAc-type complex, the imidazole ring of His⁷⁷ forms a small contact distance with not only the 4-OH but also the whole hexose ring of the GalNAc sugar linker (supplemental Table S1). By contrast, the 4-OH hydroxyl group of GlcNAc does not face His⁷⁷ in the GlcNAc-type complex. There is no hydroxyl group on this

part of the sugar linker of the deoxy-GlcNAc-type glycopeptide. These structural differences reduce both the hydrophobic Phe⁷⁶-proline and His⁷⁷-linker sugar interactions, which contributes to the loss of the entropy effect observed in the ITC measurements.

Discussion

Our previous study showed that the affinity between PILR α and the N-terminal region (amino acids 30–108) of gB is 2.3 μ M and that the glycopeptide part, GPAT(sTn)PAP (amino acids 50–56), of this region is essential and was successfully cocrySTALLIZED with PILR α (9). In this study, we consistently determined the dissociation constant of the PILR α binding of GalNAc-type peptide (wild type), GPAT(sTn)PAP, to be 6.7 μ M by ITC. We also characterized the thermodynamic properties of PILR α binding to synthetic peptides. The wild-type GalNAc-type glycopeptide has entropy–enthalpy-driven binding to PILR α . While we solved the crystal structures of PILR α (Protein Data Bank code 3WUZ) and its complex with the glycopeptide (Protein Data Bank code 3WV0) (9), Lu *et al.* concurrently reported the crystal structures of unliganded PILR α (Protein Data Bank code 4NFB) and PILR β (Protein Data Bank code 4NFC) (13). Comparison of these structures indicates that the CC' loop of the PILR α s undergoes dramatic rearrangements upon glycopeptide binding. Interestingly, however, the CC' loop of the unliganded PILR β maintains a conformation similar to that of liganded PILR α (supplemental Fig. S5). As the sequence is highly conserved between PILR α and PILR β in this CC' loop-binding region, these data imply that the CC' loops of PILRs intrinsically have large flexibility with the predominant conformations selected for upon glycopep-

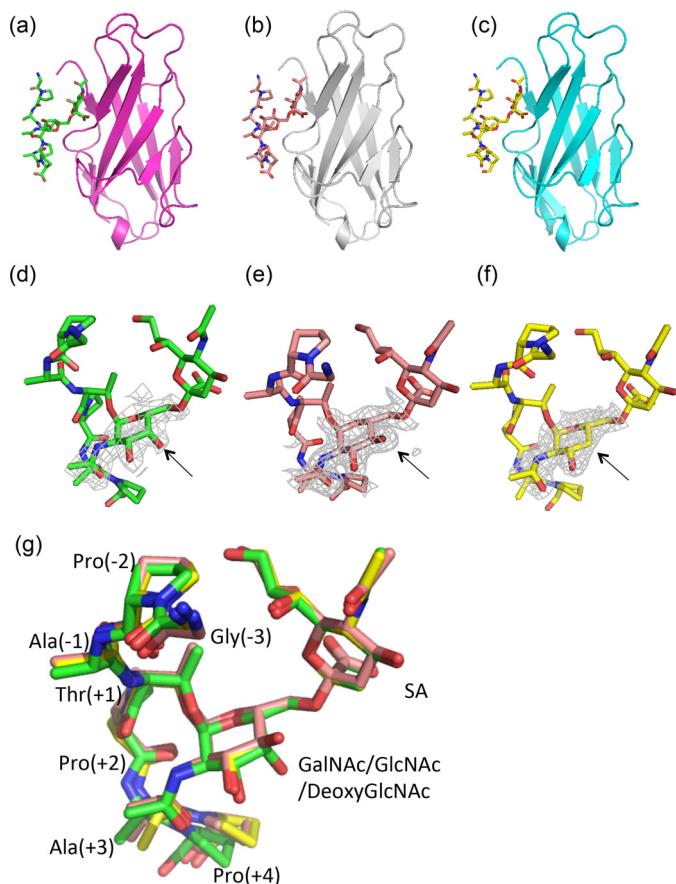


Figure 4. Crystallographic analysis of PILR α complexed with GlcNAc-type and deoxy-GlcNAc-type glycopeptides. *a–c*, the superimposed complex structures of GalNAc-type glycopeptide (green)–PILR α (light purple) (from Protein Data Bank code 3VW0) (9) (*a*), GlcNAc-type glycopeptide (light pink)–PILR α (gray) (*b*), and deoxy-GlcNAc-type glycopeptide (yellow)–PILR α (light blue) (*c*) are shown. *d–f*, the structures of GalNAc-type (*d*), GlcNAc-type (*e*), and deoxy-GlcNAc-type (*f*) glycopeptides with composite omit map ($2F_o - F_c$ map) on the linker sugar are shown. The electron density map is contoured at 1.0σ , and resolutions are 2.3, 2.0, and 2.2 Å, respectively. Arrows indicate the altered stereochemistry or loss of the sugar hydroxyl group. *g*, the structures of the glycopeptides superimposed at sialic acid in the complex with PILR α were extracted from the complex structures. The color of each glycopeptide is the same as in *d–f*.

tide binding. Indeed, this is consistent with our ITC experiments, which revealed no significant entropic loss upon glycopeptide complex formation.

Next, we introduced modifications of both peptide and sugar regions to determine their roles in binding and whether modification of linker residues could be developed for further rational inhibitor design. The *tert*-butyl modification of Ala (+3), aimed at reducing excessive flexibility in the peptide region, did not produce any significant changes in binding characteristics. It is possible that this modification may not reduce the flexibility sufficiently to affect binding and further modifications, such as introduction of a double bond in the peptide backbone, may further determine the contribution of peptide to binding. Conversely, the slight modification of a 4-OH group in the linker sugar GalNAc markedly reduced binding affinity, revealing the importance of the linker sugar for the formation of a well-ordered binding network with PILR α . Comparison of the crystal structures of the different complexes clearly reveals that the direction or absence of 4-OH in the linker sugars slightly alters

the network of binding interactions. This impacts a wide area of the interface, causing the C-terminal peptide part far from the linker sugar to be more distant from the protein surface of the CC' loop, resulting in less hydrophobic interactions between PILR α and either GlcNAc-type or deoxy-GlcNAc-type glycopeptides compared with the GalNAc-type glycopeptide. These changes in interactions contribute to the unfavorable entropic effect with the modified glycopeptides. This suggests that the sugar characteristics are finely tuned for the binding. Together these observations support that the global interaction network with both glycan and peptide parts of glycopeptides should be considered for designing better inhibitors for PILR α .

Proline residues at the adjacent C-terminal part of glycosylated threonine have been previously proposed to be important for receptor binding (9). In this study, we showed that sTn-threonine could not bind PILR α and verified the necessity of additional residues, including adjacent proline(s), for receptor interactions. The frequent appearance of a proline residue at an O-linked glycosylation site (14) may facilitate simultaneous recognition of sialic acid and proline(s), which would be beneficial for immune surveillance by PILR α . In contrast, the recent structures of sialic acid-containing glycopeptide recognition proteins, such as C-type lectin-like receptor-2 (CLEC-2), reveal a different binding mechanism (15). The complex structure of CLEC-2 with glycopeptide, derived from the ligand podoplanin, revealed the importance of terminal sialic acid and aspartic acid and glutamic acid at the N-terminal part of the attached peptide for receptor binding. Both the proline residue in the C-terminal region, adjacent to the glycosylated threonine, and the GalNAc of the glycopeptide are not involved in the interaction with CLEC-2. These results demonstrate that, although both PILR α and CLEC-2 can be categorized as sTn peptide-recognition proteins, they have different mechanisms for binding to ligands.

In summary, we synthesized various kinds of glycopeptides with modifications of either peptide or sugar moieties and determined both their binding affinities and their crystal structures with PILR α . Although PILR α exhibits essentially the same recognition of the different glycopeptides, slight modifications of the linker sugar cause significant changes in a wide area of the binding interface, resulting in a reduction of binding affinity. The findings of the present study provide insights into the mechanism of glycoprotein recognition by PILR α and contribute to the rational drug design of HSV-1 entry inhibitors.

Materials and methods

Synthesis of glycopeptides

The method for synthesizing the glycopeptides in Fig. 1 will be described in a separate publication⁷ (16). In brief, sialyl Tn-glycopeptides were synthesized by solid-phase peptide synthesis based on the Fmoc strategy using protected sialyl Tn antigen building blocks. The requisite disaccharide-threonine building blocks were prepared from FmocThrOBn (where

⁷ K. Kakita, N. Hanari, M. Anada, H. Nambu, A. Furukawa, J. Sakamoto, K. Kuroki, T. Saitoh, S. Matsunaga, K. Maenaka, and S. Hashimoto, manuscript in preparation.

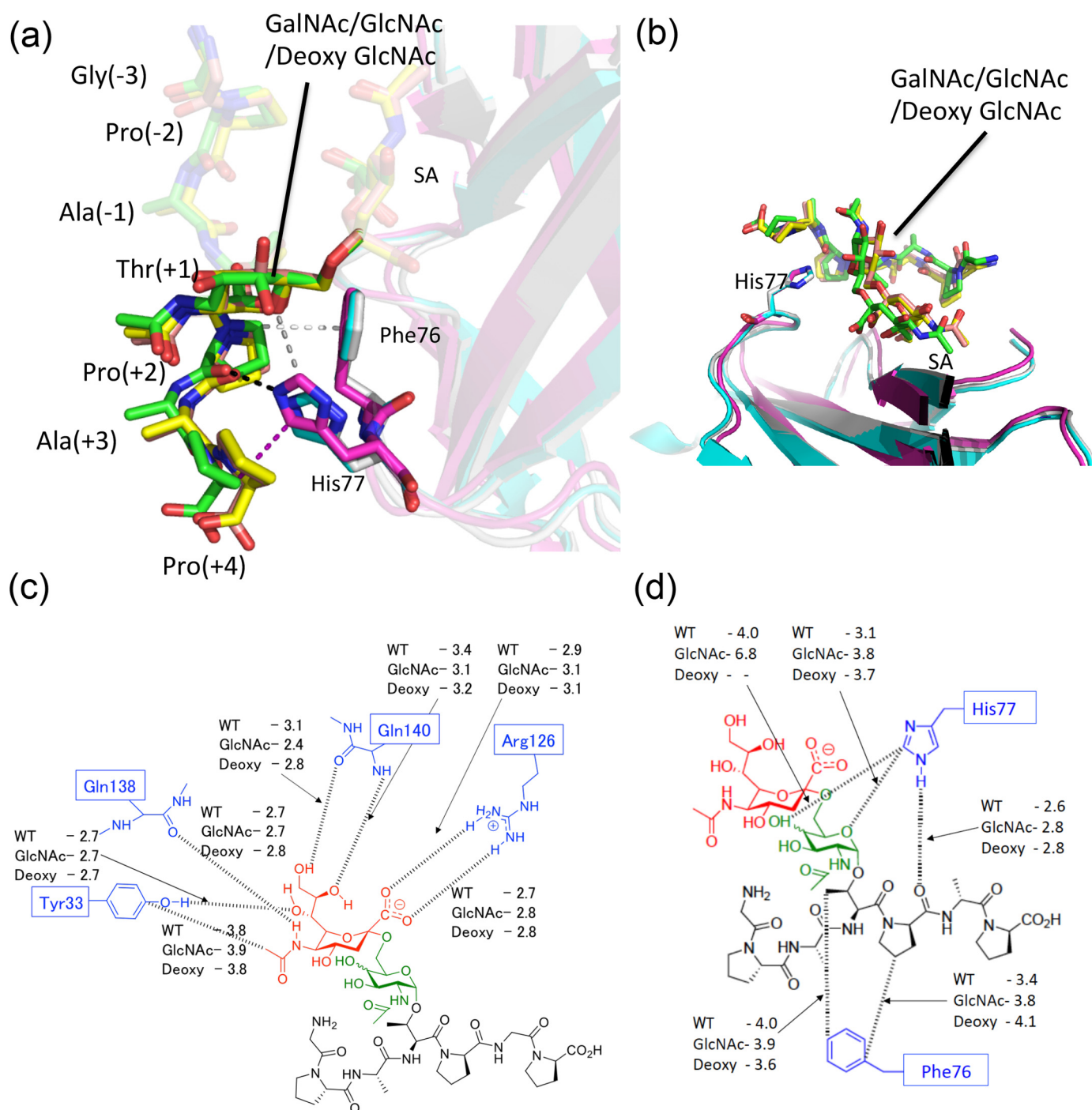


Figure 5. Close-up view of the interfaces of PILR α with glycopeptides. *a* and *b*, close-up view of the interactions between Phe⁷⁶ and His⁷⁷ from PILR α and the linker sugar and peptide part of the different glycopeptides are shown. The superimposed complex structures of PILR α (light purple)–GalNAc-type (green) (from Protein Data Bank code 3VW0), PILR α (gray)–GlcNAc-type (light pink), and PILR α (light blue)–deoxy-GlcNAc-type glycopeptide (yellow) are shown. Superimpositions of the three complexes showing amino acid residues that are involved in sialic acid binding (*a*) and the imidazole ring of His⁷⁷ (*b*) of PILR α are shown. Dashed lines represent interactions with PILR α –GalNAc-type glycopeptide. *c* and *d*, a schematic representation of the simultaneous recognition of glycopeptide (interactions around sialic acid are shown in *a*) and linker sugar and peptide (shown in *b*) region by PILR α . SA (red), GalNAc (green), the peptide region (black), and PILR α residues (blue) are shown. The distances of interactions in each complex are shown. The unit of distance represented is Å.

Bn is benzyl) via glycosylation with glycosyl diphenyl phosphates followed by triflic acid-promoted coupling with sialyl diethyl phosphite (16).⁷

Expression and purification of PILR α

Expression and purification of PILR α were performed as reported previously (17). Briefly, the extracellular domain of

PILR α (residues 32–150) was cloned into pGMT7 vector (17). The resulting plasmid was transformed into *Escherichia coli* Rosetta2 (DE3) (Millipore), and the PILR α protein was expressed as inclusion bodies. The inclusion bodies were washed, solubilized with guanidine buffer, and refolded using the standard dilution method (18). The refolded PILR α was purified by gel filtration chromatography followed by Resource

S column chromatography (GE Healthcare). Purified PILR α was preserved and maintained in buffer containing 20 mM succinate (pH 5.0) and 100 mM NaCl.

Isothermal titration calorimetry

The thermodynamic parameters for the binding of PILR α and glycopeptides were determined by ITC (Macrocal iTC200, Malvern). The cell and syringe were filled with 50 μ M PILR α and 1 mM ligands (GalNAc type and *tert*-butyl type) in HBS-P buffer (10 mM HEPES, 150 mM NaCl, and 0.05% (v/v) surfactant P20), respectively. Each experiment consisted of a single 2- μ l injection of the different ligands into the PILR α solution. For studying the binding of lower-affinity glycopeptides GlcNAc-type glycopeptide (50 μ M PILR α and 1 mM ligand) and deoxy-GlcNAc-type glycopeptide (20 μ M PILR α and 1 mM ligand), each experiment consisted of a single 4- μ l injection of ligands into the PILR α solution to overcome the low heat generated by weak interactions, adopting previously described methods (19, 20). Data analysis was performed with the software package Origin 5.0. The integrated heats generated in the experiments were fitted using a single-site binding model.

Crystallization of PILR α complexed with glycopeptide

The crystallization of PILR α complexed with glycopeptides was performed following procedures similar to those described previously for the PILR α -gB sTn glycopeptide complex (9, 17). The sitting drop consisted of a mixture of 0.2 μ l of PILR α complex solution (40 μ M) including GlcNAc-type glycopeptide or deoxy-GlcNAc-type glycopeptide (200 μ M) (20 mM succinate (pH 5.0) and 100 mM NaCl) and 0.2 μ l of reservoir solution. The crystallization well included 90 μ l of reservoir buffer at 293 K. Crystals of the PILR α -GlcNAc-type glycopeptide and -deoxy-GlcNAc-type glycopeptide complexes were obtained using PEGs Suite Number 45 buffer (0.1 M Tris-HCl (pH 8.5) and 25% (w/v) PEG6000) and Crystal Screen 2 Number 20 buffer (0.1 M MES monohydrate (pH 6.5) and 1.6 M magnesium sulfate heptahydrate) as reservoir solutions, respectively.

Data collection and structure determination

A diffraction data set was collected in SPring-8 (Harima, Japan) at 100 K on beamline BL44XU for the PILR α -GlcNAc-type glycopeptide complex and BL32XU for the PILR α -deoxy-GlcNAc-type glycopeptide complex. The structure was phased by molecular replacement using Molrep in the CCP4 package (21) using the GalNAc-type glycopeptide-binding PILR α (Protein Data Bank code 3WV0) structure as a search probe. The crystals of PILR α -GlcNAc-type glycopeptide belonged to the space group $P2_1$ with unit cell dimensions of $a = 54.66$ Å, $b = 63.01$ Å, $c = 78.60$ Å, $\alpha = \gamma = 90^\circ$, and $\beta = 108.34^\circ$ and contained four molecules per asymmetric unit. The crystals of PILR α -deoxy-GlcNAc-type glycopeptide belonged to the space group C2 with unit cell dimensions of $a = 81.28$ Å, $b = 63.33$ Å, $c = 55.07$ Å, $\alpha = \gamma = 90^\circ$, and $\beta = 110.08^\circ$ and contained two molecules per asymmetric unit. Further crystallographic refinement was carried out with Refmac, Phenix, and CNS and alternated with manual rebuilding using the interactive graphics program Coot. The final models of the two PILR α -GlcNAc-type glycopeptide and -deoxy-GlcNAc-type

glycopeptide complexes were refined to an R_{free} factor of 26.9% and an R factor of 21.5% in PILR α -GlcNAc-type glycopeptide and an R_{free} factor of 26.2% and an R factor of 21.6% in PILR α -deoxy-GlcNAc-type glycopeptide. Detailed crystallographic statistics are shown in Table 2. The coordinates for the refined PILR α -GlcNAc and -deoxy-type glycopeptide complex structures have been deposited in the Protein Data Bank (codes 5XOF and 5XO2, respectively).

Author contributions—A. F. and K. M. designed the study and wrote the paper. A. F., T. Y., and T. O. performed the X-ray study. A. F., T. Y., M. I., J. S., N. M., F. O., T. S., T. N., and Ki. K. performed the ITC study. Ko. K., N. H., H. N., S. M., M. A., and S. H. synthesized glycopeptides. A. F., A. H., and K. M. discussed the results. All authors analyzed the results and approved the final version of the manuscript.

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Structural and thermodynamic analyses reveal critical features of glycopeptide recognition by the human PILR α immune cell receptor

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