Biophysical Characterization of O-Glycosylated CD99 Recognition by Paired Ig-like Type 2 Receptors*§

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Paired Ig-like type 2 receptors (PILRs) are one of the paired receptor families, which consist of two functionally opposite members, inhibitory (PILRα) and activating (PILRβ) receptors. PILRs are widely expressed in immune cells and recognize the sialylated O-glycosylated ligand CD99, which is expressed on activated T cells, to regulate immune responses. To date, their biophysical properties have not yet been examined. Here we report the affinity, kinetic, and thermodynamic analyses of PILR-CD99 interactions using surface plasmon resonance (SPR) together with site-directed mutagenesis. The SPR analysis clearly demonstrated that inhibitory PILRα can bind to CD99 with low affinity (Kd ~ 2.2 μM), but activating PILRβ binds with ~40 times lower affinity (Kd ~ 85 μM). In addition to our previous mutagenesis study (Wang, J., Shiratori, I., Saito, T., Lanier, L. L., and Arase, H. (2008) J. Immunol. 180, 1686–1693), the SPR analysis showed that PILRα can bind to each Ala mutant of the two CD99 O-glycosylated sites (Thr-45 and Thr-50) with similar binding affinity to wild-type CD99. This indicated that both residues act as independent and equivalent PILRα binding sites, consistent with the highly flexible structure of CD99. On the other hand, it is further confirmed that PILRβ can bind the T50A mutant, but not the T45A mutant, indicating a recognition difference between PILRα and PILRβ. Kinetic studies demonstrated that the PILR-CD99 interactions show fast dissociation rates, typical of cell-cell recognition receptors. Thermodynamic analyses revealed that the PILRα-CD99 interaction is enthalpically driven with a large entropy loss (ΔTS = 8.9 kcal·mol⁻¹), suggesting the reduction of flexibility upon complex formation. This is in contrast to the entropically driven binding of selectins to sugar-modified ligands involved in leukocyte rolling and infiltration, which may reflect their functional differences.

Immune cell surface receptors are involved in both activating and inhibitory signaling, and their balance determines the functional responses of immune cells (1–4). Some of these receptor families have members with very similar extracellular regions responsible for ligand recognition but with different functions, activation and inhibition, due to the amino acid sequences of the transmembrane and intracellular regions. These receptor families are called “paired receptor families” and include human killer cell immunoglobulin (Ig)-like receptors (KIRs/CD158), human Leukocyte Ig-like receptors (LILRs/LIRs/ILTs/CD85), CD94/NKG2, and mouse Ly49.

Paired Ig-like type 2 receptors (PILRs) are one of the paired receptor families and are composed of 1) inhibitory PILRα, which has the immune receptor tyrosine-based inhibitory motif that recruits a phosphatase to mediate the inhibition of immune responses, and 2) activating PILRβ, which has a positive lysine residue in the transmembrane region and associates with DAP12, harboring the immune receptor tyrosine-based activating motif (5–7). PILRs are expressed in immune cells, including natural killer cells, macrophages, and dendritic cells. Cellular-based assays revealed that PILRβ can induce the activation of natural killer cells and dendritic cells (7).

Our previous work identified the heavily O-linked glycosylated CD99 as a physiological PILR ligand (7). CD99 is a cell surface ligand on all leukocyte lineages, and it is especially highly expressed on activated T cells, where it may regulate the functional communication between PILR-expressing dendritic cell and CD99-expressing T cells. A recent report demonstrated that PILRs recognize two sialylated core1-type O-glycosylation sites of CD99 but both the core 2 branching and desialylation disturbed the PILR recognition (8). The extracellular region of PILR has one Ig-like domain (residue numbers 40–156) with the C-terminal stalk region. A Basic Local Alignment Search Tool (BLAST) analysis revealed that sialoadhesin (9), one of Siglec (sialic-acid-binding Ig-like lectins) receptors, which have an Ig fold and recognize sialic acid (Fig. 1), exhibits weak homology (~22% identity) with the Ig-like domain of PILRs, suggesting the possibility of a similar sugar binding mode between PILRs and Siglecs. On the other hand, the extracellular domains between PILRα and β are highly conserved (amino acid iden-

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§ The abbreviations used are: PILR, paired Ig-like type 2 receptor; SPR, surface plasmon resonance; MES, 4-morpholineethanesulfonic acid; BSA, bovine serum albumin.

The online version of this article (available at http://www.jbc.org) contains supplemental Figs. S1 and S2 and Tables S1 and S2.

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An immunoprecipitation experiment showed that the inhibitory PILRα can bind to CD99 more strongly than the activating PILRβ (7), suggesting the functional dominance of inhibitory PILRα, as in other paired receptor families, such as KIRs and CD94/NKG2.

A biophysical characterization of the properties of the PILR-CD99 interactions would yield important insights for understanding their physiological relevance. Here we report a comprehensive analysis of the kinetic and thermodynamic properties of the interactions by surface plasmon resonance and site-directed mutagenesis. We also compared the molecular mechanisms for the recognition of sugar-modified ligands by PILRs and selectins.

**EXPERIMENTAL PROCEDURES**

**Production of Soluble V-set Domains of PILRα and PILRβ—**

The DNAs encoding the V-set domains of the extracellular regions (residues 40–156) of PILRα and PILRβ were amplified from the cDNAs obtained as described previously (7). The resultant fragments were digested with the restriction enzymes NdeI and HindIII and were ligated into pGMT7, creating the plasmids pGMPILRα and pGMPILRβ encoding PILRα and PILRβ, respectively. With regards to PILRβ, the C48S mutation
was additionally introduced by QuikChange (Stratagene), to avoid the formation of the disulfide-linked dimer. The plasmids were transformed into the *Escherichia coli* strain BL21(DE3)/RIL, and the cells were cultured in a 1 liter of 2YT medium (1.6 % Bacto-Tryptone, 1% yeast extract, and 0.5 % NaCl) with 100 mg/liter ampicillin (Nacalai Tesque, Japan) at 310 K. When the $A_{600}$ reached 0.6, isopropyl β-d-thiogalactopyranoside (Nacalai Tesque, Japan) was added for induction at a final concentration of 1 mM. The cells were induced for 6 h, and were harvested by centrifugation. The recombinant proteins accumulated as insoluble aggregates in inclusion bodies. The cell pellet was suspended in resuspension buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl) and was centrifuged. The pellet was washed with Triton buffer (0.5% Triton X-100, 50 mM Tris-HCl, pH 8.0, 100 mM NaCl) and was centrifuged twice. The pellet was then suspended in resuspension buffer and was centrifuged to remove unnecessary detergent. The purified inclusion bodies were finally dissolved in guanidine buffer (6 M guanidine-HCl, 50 mM MES, NaOH, pH 6.5, 100 mM NaCl, 10 mM EDTA).

To refold the proteins, 20 mg of solubilized inclusion bodies was gradually diluted by the addition of refolding buffer (1 M L-arginine-HCl, 0.1 M Tris-HCl, pH 8.0, 2 mM EDTA) at 277 K. The resultant solution was stirred for 2 days and was concentrated to 5–10 ml by a VIVA FLOW system. The proteins were dissolved in HBS-EP buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 2 mM EDTA) at 277 K. The proteins were purified by gel filtration (HiLoad26/60 Superdex 75 pg, GE Healthcare).

**Production of Soluble CD99s**—The DNA fragments encoding the extracellular portions of CD99 and its mutants (CD99-T45A (Thr-45 → Ala), -T50A(Thr-50 → Ala), and -FM (full Ala mutations of all potential O-glycan sites)) were each cloned into the XhoI site of a modified pME18S expression vector, containing a mouse CD150 leader sequence at the N-terminal site and the Fc fragment of human IgG at the C-terminal site (8). Briefly, the proteins were produced in COS-7 cells by transient expression, using 293T cells. The CD99 fusion protein was purified by chromatography and the final products showed high purity. The supernatants were transformed into the *E. coli* standard state Gibbs energy change upon binding was obtained from Equation 1:

$$\Delta G = RT \ln K_d$$  

(Eq. 1)

where $K_d$ is dissociation constant expressed in units of mol·L$^{-1}$, and $R$ is the gas constant. The ΔG values of each data set were plotted against the temperatures, and were fitted with the non-linear van’t Hoff equation (Equation 2),

$$\Delta G = \Delta H - T\Delta S + \Delta C_p(T - 298.15) - \Delta C_p T \ln(T/298.15)$$  

(Eq. 2)

where $\Delta H$ and $\Delta S$ are the binding enthalpy and entropy at 298.15 K, respectively, and $\Delta C_p$ is the heat capacity, which is assumed to be temperature-independent.

**Glycan Array Binding Screening**—The Fc-fused proteins of PILRα and PILRβ were prepared as previously reported in Wang et al. (8). Briefly, the proteins were produced in COS-7 cells by transient expression, similarly to the preparation of CD99s. The supernatants were collected 72 h after the transfection, and the purification was performed by a protein A affinity column. The glycan array screening was supported by the international established group, The Consortium for Functional Glycomics (detailed on the their website). A huge library of natural and synthetic glycans with amino linkers is covalently attached onto glass microscope slides via amide linkages, resulting in a printed glycan array. The Fc-fused PILRs (200 μg/ml) were incubated with the glycan microarray (version 3.0), including >300 sugars (see supplemental Tables S1 and S2). Then the Alexa Fluor 488-labeled goat anti-human IgG antibody (Molecular Probes) was used for the detection of the fluorescence if the proper binding occurred.

**RESULTS**

**Expression and Purification of PILRs and CD99**—The V-set Ig domains (residues numbers 40–156) of mouse PILRα and PILRβ were expressed in *E. coli* as inclusion bodies and were refolded in vitro by a dilution method (see details under “Experimental Procedures”). The refolded proteins migrated as monomers and were purified on size-exclusion columns (Fig. 2), and the final products showed high purity.

According to Wang et al. (8), the ectodomain of mouse CD99 fused with the Fc fragment of human IgG at the C-terminal site was expressed by a transient expression system, using 293T cells. The CD99 fusion protein was purified by chromatography on a protein-A column. It migrated as a 47-kDa species under reduced SDS-PAGE, and was used without further purification for binding studies (data not shown).

**Differential Binding Activity Mediates CD99 Recognition by PILRs**—The CD99 binding activity of the PILR ectodomains was analyzed by SPR. Each PILR was simultaneously injected (Fig. 3, solid bar) onto four flow cells containing sensor surfaces to which wild-type CD99 (CD99-WT) or BSA (negative control) had been immobilized by direct amine coupling. A greater response was observed with the injection of the inhibitory PILRα onto CD99-WT in comparison with that onto BSA, indi-
cating specific binding to CD99-WT (Fig. 3A). In contrast, the activating PILR showed much lower binding responses to CD99-WT (Fig. 3B). Previous cellular-based assays revealed that the activating PILR regulates the CD99-transfected cells much less efficiently than the inhibitory PILR (7). Therefore, the present binding study supports the differential binding affinities of PILRα and PILRβ.

The affinities of PILR binding to CD99 were determined by an equilibrium binding analysis. A range of concentrations of PILRα or PILRβ were injected through flow cells with immobilized CD99-WT or BSA as a control. The binding response was calculated by subtracting the equilibrium response in the control flow cell from the response in each flow cell. Conventional (Fig. 4) and Scatchard (Fig. 4, inset) plots of these binding data indicated that the interaction conforms to a simple 1:1 Langmuir binding model. The dissociation constant (Kd) for the PILRα-CD99 interaction was 2.2 μM at 25 °C (298.15 K), whereas that for the PILRβ-CD99 interaction was much lower, 85 μM. The results are summarized in Table 1. The PILRα-CD99 interaction showed low affinity within the range of typical cell-cell recognition events, but the PILRβ-CD99 interaction was at the lowest edge of the range (Table 2).

Two Equivalent PILRα Binding O-Glycan Sites—As described above, the previous cellular-based binding study using CD99 mutants (8) demonstrated that PILRα and PILRβ recognize CD99 modified with sialylated O-linked sugar. Here, we further performed the glycan array screening provided by The Consortium for Functional Glycomics (detailed on their website). The Fc-fused PILRα and PILRβ did not show significant binding to any sugar molecules on the glycan microarray (version 3.0), including >300 sugars, although PILRα showed subtle binding to α-Neu5Ac and Neu5Gcα (supplemental Figs. S1 and S2 and Tables S1 and S2). Especially, sialylated sugars such as Neu5Acα2→3Gal1→3GalNAc and Neu5Acα2→6Gal1→3GalNAc were not recognized by PILRs. In addition, the cellular-based competition assay indicated that neither Neu5Ac, Neu5Acα2→3Gal, or Neu5Acα2→6Gal competes with the PILR-CD99 interaction.4 These results suggest that PILRs recognize more complicated structure rather than simple sugar molecules, which are sialylated O-linked sugars presumably together with a certain sugar modification and/or the peptide sequence specific to CD99.

Furthermore, Wang et al. (8) demonstrated that the two mutations, Thr-45 and Thr-50, of CD99 reduced the molecular

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4 J. Wang and H. Arase, unpublished observation.
Biophysical Characteristics of PILR-CD99 Interactions

The affinities of the PILR-CD99 interactions were determined by equilibrium binding experiments. A–C, ten 2-fold dilutions of PILRα (69–0.13 μM) were injected over the CD99s, and the responses were plotted against the concentrations of injected PILRα protein. A, binding to CD99-WT (closed squares). B, binding to CD99-T50A. C, binding to CD99-T50A. Inset, Scatchard plots of the same data are shown. The solid lines are linear fits. D and E, five 2-fold dilutions of PILRβ (69-5.9 μM, E: 79-4.9 μM) were injected over the CD99s. D, binding to CD99-WT (closed squares). E, binding to CD99-T50A. The coefficient determinations for the fittings are: B (PILRα-CD99-T45A), 0.969 (non-linear) and 0.956 (Scatchard); D (PILRβ-CD99-WT), 0.984 (non-linear); and E (PILRβ-CD99-T50A), 0.994 (non-linear). Errors for these fittings are reasonably small, <30% of \( K_d \) values.

**TABLE 1**

**Binding affinities of the interactions between PILRs and CD99s at 25 °C**

| Analyte | Immobilized | \( K_d \) \( \mu M \) |
|---------|-------------|-----------------------|
| PILRα   | CD99-WT     | 2.2 ± 0.03            |
|         | CD99-FM     | NB*                   |
|         | CD99-T45A   | 3.3 ± 0.2             |
|         | CD99-T50A   | 2.8 ± 0.02            |
| PILRβ   | CD99-WT     | 85 ± 15               |
|         | CD99-FM     | NB                    |
|         | CD99-T45A   | NB                    |
|         | CD99-T50A   | 154 ± 37              |

*NB, no detectable binding.*

weight to be essentially the same as the mutant whose potential O-glycosylation sites were all mutated to alanine, indicating that the two sites are only O-glycosylated. These two O-glyco-

sylation sites, Thr-45 and Thr-50, are relevant for PILR recognition; however, the extent of the roles of these sites in the binding activity has been unclear. Therefore, we performed SPR binding studies of the inhibitory PILRα to the CD99 mutants. As expected, no specific responses were observed in the flow cell of the CD99 mutant with Ala replacements at all potential O-glycosylation sites (full mutations, CD99-FM) upon the injection of either PILRα or PILRβ (Fig. 3), confirming the previous result (8) that the O-glycosylation is necessary for PILR recognition. On the other hand, the single Ala mutation at two O-glycosylation sites (T45A and T50A) did not cause any reduction of PILRα binding in comparison with that of the CD99-WT (Fig. 4, A–C, and Table 1). This indicated that the two O-glycan sites are independent and equivalent for the PILRα recognition. In contrast, the PILRβ bound the T50A mutant with similar affinity as the CD99-WT (Fig. 4, D and E), but did not bind to the T45A mutant (data not shown), supporting the previous observation (8) that these binding sites are not equivalent for PILRβ recognition.

**Binding Kinetics**—The equilibrium binding data for both PILRα-CD99-WT interactions indicated very fast kinetics (Fig. 3). The PILRβ-CD99-WT interaction shows quite low affinity with much faster kinetics, making it difficult to obtain the precise association and dissociation rates. Therefore, the detailed kinetic parameters of the PILRα-CD99-WT interaction were determined, by global fitting of the mono-exponential rate equations derived from the simple 1:1 Langmuir binding model to the SPR data. Fig. 5 illustrates the reasonable fitting to the data for PILRα binding to CD99-WT, which yielded the association and dissociation rate constants, summarized in Table 2. PILRα bound CD99 with typical association (\( k_{on} = 3.9 \times 10^5 \text{M}^{-1}\text{s}^{-1} \)) and fast dissociation (\( k_{off} = 1.2 \text{s}^{-1} \)) kinetics similar to those of the interactions of other cell-cell recognition receptors (Table 2). The amount of immobilized CD99-WT did not affect the kinetic parameters significantly (Table 2), indicating that mass transport and rebinding artifacts were not factors in this experiment. The good consistency between the kinetically derived \( K_d \) (Table 2) and the \( K_d \) determined by equilibrium binding (Table 1) provided further evidence that these kinetic parameters are correct.
Biophysical Characteristics of PILR-CD99 Interactions

TABLE 2

Kinetic parameters of the interactions between PILRα or PILRβ and CD99 at 25 °C

| Analyte                      | Immobilized CD99 | $k_{on}$ ($10^9$ M$^{-1}$ s$^{-1}$) | $k_{off}$ s$^{-1}$ | $K_d$,kin | $K_d$,eq | References |
|------------------------------|-------------------|-------------------------------------|--------------------|-----------|----------|-----------|
| PILRα                        | WT (700 RU)$^b$   | 3.9                                 | 1.2                | 3.0       | 2.2 ± 0.03| (17)      |
|                              | WT (3800 RU)      | 5.1                                 | 1.0                | 2.0       | 2.2 ± 0.03|           |
| PILRβ                        | WT (3800 RU)      |                                     |                    |           | 85 ± 15  |           |

Other protein-protein interactions

| Analyte                      | Immobilized CD99 | $k_{on}$ ($10^9$ M$^{-1}$ s$^{-1}$) | $k_{off}$ s$^{-1}$ | $K_d$,eq | References |
|------------------------------|-------------------|-------------------------------------|--------------------|----------|-----------|
| E-selectin                   | GlyCAM-1          | 0.48                                | 2.7                | 56       | (17)      |
| P-selectin                   | LILRB1D1D2        | 1.4                                 | 0.0028             | 0.0021   | (27)      |
| KIR2DL3                      | HLA-Cw7/DS11      | 2.1                                 | 1.1                | 5.2      | (28)      |
| CD8α                        | MHC class I       | 1 ≥ 1                               | ≥ 18               | ≥ 18     | (29)      |
| CD22                        | CD45              | 1.5                                 | 1.6                | 2.4      | (30)      |
| CD80                        | CTLa-4            | 9.4                                 | 0.43               | 0.46     | (30)      |
| CD80                        | CD28              | 6.6                                 | 1.6                | 2.4      | (30)      |
| FcRRIla.11b.III              | hFc1              | 3.8–4.4                             | 0.31–0.69          | 0.72–1.9 | (31)      |
| TCR                         | peptide-MHC      | 0.009–0.2                           | 0.01–0.1           | 1–90     | (10, 11)  |

$^a$ The values are means ± range derived from two experiments.

$^b$ RU, response unit(s).

FIGURE 5. Kinetic analysis of the PILRα-CD99 interaction. PILRα at the indicated concentrations, was injected (solid bar) over CD99-WT (700 response units (RU)). Rate equations derived from the 1:1 binding model (A + B → AB) were fitted to the association and dissociation phases of all six injections (global fitting). Residual errors from the fits are shown in the bottom panel.

Thermodynamic Properties of the PILRα-CD99 Interaction—Using the non-linear van’t Hoff analysis (Equation 2 under “Experimental Procedures”), a series of binding experiments at a range of temperatures can simultaneously provide the following thermodynamic parameters: the enthalpy change ($\Delta H$), the entropic change ($\Delta S$), and the heat capacity ($\Delta C_p$). Therefore, we determined the binding affinities of the PILRα-CD99-WT interaction at five temperatures (10, 15, 20, 25, and 30 °C). The reasonable fitting of the non-linear van’t Hoff equation to the data produced the thermodynamic parameters (Fig. 6 and Table 3). The quite favorable enthalpic changes ($\Delta H$ ~ −16.6 kcal·mol$^{-1}$), beyond the entropic loss ($\Delta S$ ~ −8.9 kcal·mol$^{-1}$), contribute to the binding energy ($\Delta G$ ~ 7.7 kcal·mol$^{-1}$) at 25 °C, and thus the PILRα-CD99-WT interaction is completely enthalpic-driven. On the other hand, $\Delta C_p$ is within the range of values measured for other protein-protein interactions (−0.44 ± 0.07 kcal·mol$^{-1}$·K$^{-1}$) (Table 3).

We also performed the non-linear van’t Hoff analyses for PILRα recognition of the T45A and T50A CD99 mutants (Fig. 6). Both mutants exhibited very similar enthalpically-driven thermodynamic parameters to the wild-type, thus confirming that Thr-45 and Thr-50 are both independent and equivalent for the PILRα recognition.

DISCUSSION

O-Glycosylation Sites—The present study clearly demonstrated that the O-glycans were indispensable for PILR recognition. This result agreed with the staining experiment with the CD99 mutant transfectants, which showed that non-O-glycosylated CD99 could not bind to either PILRα or PILRβ (8).

PILRα bound the CD99-WT and the CD99-T45A and -T50A mutants with similar affinities ($K_d$ ~ 2–4 μM), indicating that the two O-glycosylation sites, Thr-45 and Thr-50, were independent and equivalent for PILRα recognition. These sites are located very close together (only four amino acids apart), but the amino acid sequence around Thr-45 and Thr-50

Thermodynamic analysis of the PILRα-CD99 interaction

On the one hand, the previous cellular and present binding studies showed that PILRβ-CD99 binding is not affected by the T50A mutation but is abolished by the T45A mutation (8). The amino acid sequences around Thr-45 and Thr-50 are very similar, with the common motif PTPK (NMKPT$^{45}$PKAPT$^{50}$PKKPS, underlined), implying the duplicated sequence. Thus, the amino acid sequence at the N-terminal side, rather than that at the C-terminal side, of
and its attached polypeptide by PILR, will be necessary to clarify the functional characteristics. Taken together, these results indicate that both PILRs recognize the flexible O-glycosylated sites of CD99, even though there are differences in the peptide dependence. 

**Kinetic Characteristics**—PILRα bound to CD99 with a typical association rate constant ($k_{on} = 3.9 \times 10^5 \text{M}^{-1}\text{s}^{-1}$) and dissociated with a fast dissociation rate constant ($k_{off} = 1.2 \text{ s}^{-1}$). These kinetic parameters indicated that PILRα binds to CD99 with fast kinetics, within the range of typical cell-cell recognition events (Tables 1 and 2). PILRβ also showed fast binding kinetics, although the specific parameters were not determined. This indicates that the interactions between PILR and CD99 are unstable, and the fast dissociation rates promote the exchange of PILR-CD99 interactions, suggesting that the formation of appropriate multivalent complexes at the cell-cell interface is required for signaling. In a later section, we will discuss the functional consequences of the differences in the sugar-modified ligand recognition between PILRs and selectins.

**Thermodynamic Characteristics**—The binding of PILRα to CD99 was characterized by quite favorable enthalpic changes beyond the large entropic loss, in contrast to entropically and enthalpically driven binding observed in typical protein-protein interactions, including cell-surface receptor-ligand interactions, such as the KIR-MHC interaction (Fig. 7 and Table 3). Unfavorable entropic changes upon complex formation are mainly due to the reduction in the conformational flexibility and/or the trapping of water molecules at the binding interface. The enthalpically driven interactions include the TCR-MHC (10–15) and gp120-CD4 (16) interactions. These interactions exhibit slow $k_{on}$ values, suggesting that large, time-consuming conformational adjustments are necessary to achieve the proper binding state. However, the PILR-CD99 interaction exhibited a relatively fast association rate. Furthermore, the heat capacity change ($\Delta C_p$) is not very small, $\sim 440$ cal-mol$^{-1}$-K$^{-1}$, but it is much less than that in gp120-CD4 binding (1200–1800 cal-mol$^{-1}$-K$^{-1}$), indicating that only small conformational changes may be required. On the other hand, core 1 sugars of CD99 might be linear compared with the branched core 2 sugars and thus more flexible than the branched sugars like sialyl Lewis sugars and ligands of selectins, which would contribute to the entropy penalty of the CD99-PILR interaction. Thus, the PILRs probably capture the highly flexible structure of the O-glycan and its attached peptide of CD99 with some degree of conformational fixation and/or trapping of water molecules.

**Comparison with Selectin-Ligand Interactions**—The selectin family on leukocytes recognizes sugar-modified ligands on the vascular endothelium to facilitate leukocyte tethering and rolling. Binding studies revealed that the selectin-ligand interactions (E-selectin-ESL-1 (17), L-selectin-GlyCAM-1 (18), and P-selectin-PSGL-1 (19)) exhibit fast kinetics and are basically entropically driven (Tables 2 and 3, and Fig. 7). As described above, this is in sharp contrast to the enthalpically driven PILR-CD99 binding. The sugar-modified ligands have intrinsically high conformational entropy derived from the O-linked oligosaccharides, and their fixation upon protein binding contributes to unfavorable entropic changes, as observed in many sug-
Biophysical Characteristics of PILR-CD99 Interactions

| Analyte | Immobilized | References | \( \Delta G \) | \( \Delta H \) | \( -T\Delta S \) | \( \Delta C_p \) |
|---------|-------------|------------|-------------|-------------|-------------|-------------|
| PILRα   | CD99-WT*    | This study | -7.7 ± 0.03 | -16.6 ± 0.2 | 8.9 ± 0.2   | -0.44 ± 0.07 |
| PILRα   | CD99-T45A*  | This study | -7.5 ± 0.00 | -15.3 ± 0.2 | 7.8 ± 0.2   | -0.28 ± 0.03 |
| E-selectin | ES-1      | (17)       | -5.7        | -0.9        | -4.8        |             |
| FcγRlla, Iib | hFc1 | (31)       | -7.9 to -8.3 | -4.4 to -6.4 | -1.9 to -3.3 | -0.22 to -0.43 |
| KIR2DL3 | HLA-Cw7 | (28)       | -7.2        | -4.1        | -5.1        | -0.1        |
| CD22    | CD45       | (21)       | -5.1        | -10.1       | 5.0         | -0.08       |
| NK2G2D  | Rael       | (32)       | -8.6        | -5.2        | -3.4        |             |
| TCR     | MHC        | (10-15)    | -7.1 ± 0.6  | -14.6 ± 5.4 | 7.1 ± 5.7   | -0.62 ± 0.37 |

* The values are means ± range derived from two experiments.

The values for protein-protein interactions (excluding antibody-antigen interactions) are the mean ± S.D. of 30 distinct interactions taken from Stites (26).

ar-lectin interactions (20). Thus, the PILR-CD99 interactions presumably adopt such a recognition mode. On the other hand, the selectin-ligand recognition seems to avoid strong conformational fixation and/or entrapment of waters at the binding interface. It seems that fast kinetics and smaller conformational rearrangements upon selectin-ligand complex formation would be beneficial for the tethering and rolling of leukocytes, which move quickly in the bloodstream. In contrast, PILRs might utilize a more static binding mechanism to achieve proper signaling in a localized area, such as the lymph node, where PILR-expressing dendritic cell and CD99-expressing T cells interact.

Comparison with the Siglec Family—As described in the introduction, a BLAST analysis revealed that sialoadhesin exhibits weak homology (~22% identity) with PILRs (Fig. 1B). PILRs harbor the most important arginine residue for sialic acid recognition, conserved in Siglec family members. The mutation of the conserved arginine residue directly recognizing sialic acid (Arg-133 in PILRs/Arg-116 in sialoadhesin) abolished the PILR-CD99 interactions, suggesting that the PILRs have a similar sugar binding mode as the Siglecfs. However, PILR seems not to recognize simple sialylated sugars unlike Siglecfs. Furthermore, genome localization of PILR is not linked to that of Siglec family. Therefore, PILR seems to be a relative but not a member of Siglecfs.

CD22 is a member of the Siglec family and interacts with sialylated glycans on several molecules such as CD45, in an enthalpy-driven process (21), similarly to the PILR-CD99 interactions. However, PILRs do not recognize CD45-expressing CD4 T cells (8), and thus PILRs at least have recognition specificity different from CD22 even though sialylated sugars are involved in the ligand recognition by these receptors. It would be interesting to clarify the similarity and difference of these recognition systems by further investigation.

Functional Differences between PILRα and PILRβ—Previous studies of the recognition characteristics of paired receptor families clarified the tendency that the inhibitory receptor has a higher affinity than the activating one, such as CTLA4/CD28, KIRs, and CD94/NKG2 (22). The proposed physiological reason for this is that inhibitory receptor interactions should overcome activating ones for immune cells, such as T and natural killer cells, so that they are inactive toward normal cells unless the activation is truly necessary. The present binding study revealed that the PILR family also exhibits the same differential binding properties to regulate immune cells as the above-mentioned immune cell surface receptors.

The amino acid residue differences between PILRα and PILRβ are localized within the C terminus (Fig. 1A). As described above, based on an amino acid sequence alignment with other Siglecfs, these residues seem to constitute a part of the sugar recognition site (Fig. 1B). Thus, it is reasonable for these amino acid changes to significantly affect the binding affinity and specificity of PILRs to CD99.

There exists difference in recognition of the O-glycan mutated CD99s between PILRα and PILRβ. Single mutation at Thr-45 or Thr-50 did not affect the PILRα recognition but did for PILRβ binding. We have shown that both the single mutation (Thr-45 or Thr-50) reduced the killing activity of natural killer cells expressing PILRβ (8), indicating that the O-linked sugar modifications at Thr-45 and Thr-50 are regulating immune receptor tyrosine-based activating motif-mediated signals of PILRβ.

Weak binding affinity of PILRβ to CD99 was observed, and thus PILRβ may have other physiologically relevant roles. Arase et al. (23) proposed that some of the activating members of paired receptors may be reserved for surveying non-self ligands related to infectious diseases. For example, the mouse cytomegalovirus major histocompatibility complex class I-like molecule, m157, can bind to the activating Ly49 receptor, Ly49H, resulting in the acquirement of resistance to cytomegalovirus (24, 25). In a similar manner, PILRβ may have other ligands...
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derived from viruses, bacteria, etc. to achieve resistance to infectious diseases.

Functional Implication of O-Linked Sugar Modification of CD99—Activated lymphocytes express CD99. The intrinsic core 2 branching enzyme, core 2 \(\beta\)-1,6-\(N\)-acetylglucosaminyltransferase, expressed in B cells controls the generation of the B220 epitope on CD45. We have recently shown that PILRs recognize activated B220\(^-\) T cells but not activated B220\(^+\) B cells, even though both cells express CD99 (8). Because PILRs are expressed on antigen-presenting cells, this result suggested that O-linked sugar modifications generated by intrinsic core 2 \(\beta\)-1,6-\(N\)-acetylglucosaminyltransferase on lymphocytes control the PILR recognition, conferring the regulatory role of the CD99-PILR interactions on the innate and acquired immunity.

Conclusion—in this report, we showed that PILRs bind O-glycosylated CD99 with low affinities and fast kinetics, which are typical for cell-cell recognition receptors. The mutagenesis study revealed that PILRs recognize the flexible O-glycosylated sites of CD99, even though differential peptide dependence exists. The thermodynamic analysis showed unfavorable entropic changes upon PILR-CD99 complex formation, suggesting that PILRs capture the highly mobile CD99 structure with some degree of conformational fixation and/or trapping of water molecules. This is in contrast to the entropically driven selectin-ligand interactions, which are involved in the tethering and rolling of leukocytes in the bloodstream.

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