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PILR α and PILR β have a siglec fold and provide the basis of binding to sialic acid

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Paired immunoglobulin-like type 2 receptor α (PILR α) and β (PILR β) belong to the PILR family and are related to innate immune regulation in various species. Despite their high sequence identity, PILR α and PILR β are shown to have variant sialic acid (SA) binding avidities. To explore the molecular basis of this interaction, we solved the crystal structures of PILR α and PILR β at resolutions of 1.6 Å and 2.2 Å, respectively. Both molecules adopt a typical siglec fold but use a hydrophobic bond to substitute the siglec-specific disulfide linkage for protein stabilization. We further used HSV-1 glycoprotein B (gB) as a representative molecule to study the PILR–SA interaction. Deploying site-directed mutagenesis, we demonstrated that three residues (Y2, R95, and W108) presented on the surface of PILR α form the SA binding site equivalent to those in siglecs but are arranged in a unique linear mode. PILR β differs from PILR α in one of these three residues (L108), explaining its inability to engage gB. Mutation of L108 to tryptophan in PILR β restored the gB-binding capacity. We further solved the structure of this PILR β mutant complexed with SA, which reveals the atomic details mediating PILR/SA recognition. In comparison with

processes. In addition to the natural host ligands, PILR α is also hijacked by some viruses, such as HSV-1 (10) and porcine pseudorabies virus (11), for cell entry. The viral surface glycoprotein B (gB) is shown to recognize PILR α and mediate the virus infection (10, 11). Elucidation of the mechanisms underlying these ligand–receptor interactions is important in understanding PILR-involved physiological processes. Current knowledge on these interactions, however, only indicates the involvement of sialic acid (SA) moieties residing on the ligand surface in PILR engagement (7, 8, 12). This character drew parallels between PILRs and siglecs, a family of SA-binding Ig-type lectins (13). Nevertheless, PILRs, unlike siglec molecules, are of low SA-binding avidity and fail to bind to single SA sugars in a glycan microarray (14). The molecular basis of the PILR–SA interaction is an interesting, yet unresolved, issue.

In this study, we first solved both PILR α and PILR β structures, demonstrating that they have siglec-like folds but maintain protein stability by hydrophobic interactions, different from siglecs, which have disulfide bonds. We also developed a Bia-

the free PILR structures, amino acid Y2 oriented variably in the complex structure, thereby disrupting the linear arrangement of PILR residues Y2, R95, and W108. In conclusion, our study provides significant implications for the PILR-SA interaction and paves the way for understanding PILR-related ligand binding.

There are two members in the paired immunoglobulin-like type 2 receptor (PILR) family: PILR α and PILR β (1). Both are expressed as a monomeric transmembrane protein with a single V-set Ig-like (IgV) extracellular domain (2). In the cytoplasmic tail, PILR α bears two immunoreceptor tyrosine-based inhibitory motifs that deliver inhibitory signals by recruiting SHP-1 and SHP-2, whereas PILR β binds to the DAP-12 molecule bearing a tyrosine-based activation motif (ITAM) for transduction of activating signals (3). Several studies in mice showed that the former is always related to the inhibition of the immune system, whereas the latter plays pivotal roles in activating natural killer (NK) cells and dendritic cells (DCs) and is involved in the mass production of inflammatory factors during infection (4). In addition, a recent report also demonstrated that PILR α could function to regulate neutrophil infiltration via activation of integrins during inflammation (5). Reminiscent of these immune-modulation functions, both receptors are largely expressed on cells of the immune system, especially those of the myeloid lineage such as monocytes, DCs, and macrophages (6, 7). PILR β is also abundantly expressed on NK cells (6).

To exert their regulatory functions, the PILR receptors require engagement of specific ligands via their extracellular domains. Mouse CD99 is the only identified ligand for PILR β to date (8). However, a set of host molecules, including mouse CD99 (8), PILR-associating neural protein (9), neuronal differentiation and proliferation factor-1 (NPDC1) (7), and collectin-12 (7), can recognize PILR α , implicating important roles of PILR α in diverse

core-based assay for quantitative calculations of the PILR-SA interaction based on HSV-1 gB protein. A triresidue motif consisting of Y2, R95, and W108 was identified as a key SA-binding site in PILR α , and a W108L substitution in the motif was

Significance

The paired immunoglobulin-like type 2 receptor α (PILR α) and β (PILR β) are important surface molecules which, upon ligand binding, can deliver opposing signals to modulate the host immune responses. In this study, we elucidated the molecular basis on the ligand binding of PILRs by systematic structural and functional assays. Both PILR α and PILR β show a typical siglec-like fold but exhibit variant binding avidities for sialic acid (SA). We further identified key residues responsible for SA binding and elucidated the atomic interaction details via a complex crystal structure. In conclusion, the SA recognition mechanism for the PILR receptors has been, for the first time to our knowledge, systematically investigated and clearly presented.

Author contributions: G.F.G. designed research; Q.L., H.W., Y.X., Q.W., Y.L., and Y.Z. performed research; C.Z. and Z.F. contributed new reagents/analytic tools; J.Q. collected data and solved structures; Q.L., G.L., J.Q., and J.Y. analyzed data; and G.L., J.Y., and G.F.G. wrote the paper.

The authors declare no conflict of interest.

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Data deposition: Crystallography, atomic coordinates, and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes **4NFB** for PILR α , **4NFC** for PILR β , and **4NFD** for the PILR β -mut/SA complex).

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shown to be responsible for the inability of PILR β to interact with gB. We further reported a complex structure of SA bound to a PILR β L108W mutant protein, thereby presenting the atomic details mediating the PILR–SA interaction.

Results

Both PILR α and PILR β Have a Siglec Fold. We first solved the structure of PILR α at 1.6 Å resolution. The final model, which was refined to $R_{\text{work}} = 0.1980$ and $R_{\text{free}} = 0.2249$ (Table S1), contains residues L1 to T119 (numbering based on the mature protein) of the native PILR α sequence (NM_013439.2). The content of the asymmetric unit of the crystal is a monomer. Consistent with the categorization of PILR α into the single IgV superfamily, the solved structure comprises two antiparallel β -sheets formed by strands ABDE and A'CC'FG (G, G'), exhibiting the characteristics of an IgV-like fold (Fig. 1A). Different from the classical IgV domain, however, the G strand of PILR α is kinked in the middle, splitting into two short strands (G and G') connected by a short linker. In addition, the IgV-specific disulfide bond is also missing in PILR α , with only a single cysteine residue (C94) situating on the F strand.

The structure of PILR β at 2.2 Å resolution was solved with an $R_{\text{work}} = 0.2192$ and an $R_{\text{free}} = 0.2575$ (Table S1). Two molecules related by a twofold axis are present in the asymmetric unit of the crystal. Overall, the two PILR β protomers are of the same structure with an rmsd of only about 0.07 Å for all of the C α pairs. Each molecule contains electron-density traceable residues from L1 to T119 (numbering based on the mature protein) of the native PILR β sequence (NM_178238.2), folding into an IgV-like structure composed of the ABDE and A'CC'FG(G,G') sheets (Fig. 1B). As expected, this observed fold is very similar to that of PILR α described above. Superimposition of the two structures revealed that all of the scaffold strands and a majority of the intervening loops are well aligned, except for the BC and CC' loops, which exhibit obvious conformational variance (Fig. S1).

Despite sharing low sequence identities (less than 25%) with siglecs (Fig. S2), PILR α and PILR β display significant structural similarities to the siglec molecules (Fig. 1A–C). For example, when superimposed, PILR α exhibits an rmsd of 1.3 Å over 91 C α pairs to sialoadhesin (PDB ID code 1QFO), an rmsd of 2.3 Å over 84 C α pairs to siglec-7 (PDB ID code 1O7V), and an rmsd of 2.6 Å over 96 C α pairs to siglec-5 (PDB ID code 2ZG2) (Fig. 1D). According to a previous study (15), a kink of the G strand (into short G and G' strands) was shown to be a conserved feature of siglecs, with which the G strand directly contacts the SA moiety and the GG' loop accounts for ligand specificity. PILR α and PILR β also contain this unique character. Hence, the PILR pairs strikingly adopt a typical siglec fold and preserve the structural features necessary for ligand interaction (Fig. 1A–D).

Compared with canonical siglecs, however, the conserved intrasheet disulphide bond linking strands B and E is absent in PILRs. The corresponding positions are replaced with a phenylalanine residue (F22 in both PILR α and PILR β) on strand B and a glycine residue (G77 in both PILR α and PILR β) on strand E, which form hydrophobic interactions to stabilize the protein

Fig. 1. Crystal structures of PILR α and PILR β reveal a siglec fold. (A–C) Structures of PILR α in magenta (A), PILR β in cyan (B), and siglec-7 (PDB ID code 1O7V) in blue (C) are depicted. F22 and G77, which form hydrophobic interactions in both PILR molecules, as well as C28 and C88, which form a disulfide bond in siglec-7, are shown in sticks. The β -strands in all structures are indicated with capitals. The terminal residues are marked in parentheses. (D) Superimposition of PILR α , PILR β , siglec-1 (PDB ID code 1QFO), siglec-5 (2ZG2), and siglec-7 (1O7V) shown in a C α ribbon representation and labeled in magenta, cyan, green, orange, and blue, respectively. The protein termini and the G, G' strands are indicated. (E–G) A special linear arrangement of the Y2/R95/W(L)108 triresidue in PILRs. According to previous studies, the SA binding of siglecs involves three sterically proximate residues: an arginine and two aromatic amino acids. These three residues and their equivalent amino acids in PILRs are overlaid and shown as sticks. (E) An overlay of siglec-1 (W2, R97, and W106 in green), siglec-5 (Y5, R103, and Y112 in orange), and siglec-7 (Y8, R106, and W114 in blue). (F) An overlay of PILR α (Y2, R95, and W108 in magenta) and PILR β (Y2, R95, and L108 in cyan). (G) An overlay of PILR α (Y2, R95, and W108 in magenta) and siglec-7 (Y8, R106, and W114 in blue). The steric arrangement of the triresidue is indicated with dotted lines.

fold (Fig. 1 A–C).

Steric Arrangement of the Y2/R95/W(L)108 Triresidue in PILRs. The observation of an overall siglec fold for the PILR pairs urged us to further compare PILRs with siglecs for the steric arrangement of those residues identified as the key elements engaging SA. These include a conserved arginine residue (R97 in siglec-1, R103 in siglec-5, and R106 in siglec-7, numbering based on the mature proteins) situated in the middle of the F strand and two amino acids of less conservation but invariably aromatic side chains (W2 and W106 in siglec-1, Y5 and Y112 in siglec-5, and Y8 and W114 in siglec-7) located in the A and G strands, respectively (15–18). In siglecs, these three residues are sterically in close proximity, similarly positioned on the molecule surface, and arranged at the three points of an obtuse triangle (Fig. 1E). According to the previously reported siglec-7 structure in complex with a sialylated ligand GT1b, the arginine residue plays an indispensable role in SA binding by forming salt bridges with the SA carboxylate group. The two aromatic amino acids further facilitate the ligand interaction by providing hydrophobic and weak H-bond interactions (19).

In PILRs, amino acids Y2 in strand A, R95 in strand F, and W(L)108 in strand G (a tryptophan in PILR α and a leucine in PILR β) are equivalent to the identified SA-binding triresidue of siglecs (Fig. S2). The three amino acids are spatially conserved between PILR α and PILR β (Fig. 1F) but exhibit obvious variance in steric arrangement in comparison with siglec proteins (Fig. 1G). A detailed structure overlay using PILR α and siglec-7 as the representative revealed a well-aligned arginine residue but large conformational variances for the tyrosine and tryptophan residues between the two molecules. In PILR α , W108 rotates its indole ring $\sim 90^\circ$ toward strand A, thereby occupying the position initially taken up by Y8 of siglec-7. Consequently, PILR α extends its Y2 residue away from the protein main body, leading to an

overall linear arrangement of the Y/R/W triresidue (Fig. 1 *F* and *G*).

PILR α , but Not PILR β , Binds HSV-1 gB Through SA. To further probe into the underlying molecular characters of PILR α and PILR β we analyzed the SA-binding features of both proteins by surface plasmon resonance (SPR) using Biacore technology. According to previous studies, SA alone is not sufficient for any detectable PILR engagement (7, 14). We therefore resorted to the viral ligand of HSV-1 gB for the binding studies. The trimeric ectodomain protein of HSV-1 gB was prepared from either mammalian 293T cells (gB-293T) or insect Sf9 cells (gB-Sf9), and purified to homogeneity (Fig. S3). As expected, a clear interaction was observed when PILR α flew over immobilized gB-293T. The binding kinetics revealed a fast-on/fast-off mode, showing an affinity of $\sim 7 \mu\text{M}$ (Fig. 2A and Fig. S4). This specific interaction was dependent on the SA moieties, removal of which by treatment with neuraminidase (NA) was shown to largely attenuate the binding response (Fig. 2E). This result is also in good accordance with a previous cell-based assay (12). Moreover, we also tested gB-Sf9 in the same experiment. To our knowledge, the *O*-glycosylation in mammalian cells is largely different from that of Sf9 cells (Fig. 2F), which lacks SA transferase and thereby could not decorate proteins with sialylated sugar tips (20). Accordingly, no detectable binding could be observed between gB-Sf9 and PILR α (Fig. 2E). In conclusion, these results clearly demonstrated that PILR α relies on SA binding for a full capacity of gB engagement and validated the current SPR system (with immobilized gB-293T) as an effective method for studying PILR-SA interactions.

We then tested whether PILR α binding to SA involves the aforementioned Y/R/W triresidue in a manner similar to that in siglecs. The amino acid arginine was mutated to alanine, tyrosine to leucine, and the tryptophan residue was replaced with a leu-

mutations abrogated the binding between PILR α and gB-293T, whereas the Y2L substitution leads to an approximately twofold decrease in the binding affinity ($\sim 14 \mu\text{M}$) (Fig. 2D). The mutagenesis data demonstrate the functional importance of these three residues in ligand recognition.

Despite a high sequence identity (88%) between PILR α and PILR β , the latter does not function as the HSV-1 receptor (21). Consistently, no obvious binding was detected between PILR β (up to $12.5 \mu\text{M}$) and gB-293T (Fig. 2G). We also tested by SPR the interaction between gB-293T and the PILR β L108W mutant (PILR β -mut). As expected, this mutant protein can be refolded (Fig. S5) and is observed to properly traffic to (Fig. S6) the cell surface as with wild-type PILR β . The mutation confers a significantly elevated binding avidity to PILR β , with a binding affinity of about $1 \mu\text{M}$ (Fig. 2H), confirming that L108 in PILR β is responsible for SA nonbinding. Nevertheless, this restored gB-binding capacity does not suffice for PILR β to mediate HSV-1 entry. Neither PILR β nor PILR β -mut was observed to facilitate the HSV-1 infection of CHO-K1 cells, in contrast to PILR α , which led to increased cell infection by the virus (Fig. S7).

Structural Basis of the PILR-SA Interaction. Despite great efforts, we failed to obtain any complex crystals of SA bound to PILR α . Instead, a cocrystal between PILR β -mut and SA was successfully obtained by cocrystallization. The final structure, with a single SA moiety and a single PILR molecule in the asymmetric unit, was determined to 1.7 \AA resolution and refined to $R_{\text{work}} = 0.1706$ and $R_{\text{free}} = 0.2117$ (Table S1). As expected, the L108W substitution did not change the overall structure of PILR β . Superimposition of the wild type and the mutant structures yielded an rmsd of about 0.42 for 104 C α pairs. The SA moiety is located on the lateral side of the IgV rear sheet in PILR β -mut, perching on top of the G strand (Fig. 3A). In comparison with the free PILR α

cine, as in PILR β , a gB nonbinder (21). The protein mutants were refolded and shown to behave similarly to wild-type PILR α in an analytical gel filtration assay (Fig. S5). In addition, none of the mutations interfered with the surface localization of PILR α (Fig. S6), indicating a proper folding of the PILR mutants. In the Biacore assay, both R95A (Fig. 2B) and W108L (Fig. 2C)

structure, the N-terminal loop and its residing Y2 residue oriented variantly in the complex structure. This resulted in a breaking of the linear arrangement of the Y/R/W triresidue as observed in the free PILR structures (Fig. S8), although it cannot be excluded that the variant conformation of Y2 in PILR β -mut is related to the L108W mutation itself.

Fig. 2. SPR assays characterizing HSV-1 gB binding to PILRs and their mutants. Gradient concentrations of the indicated PILR proteins were passed over gB [gB-293T, gB-Sf9, or gB-293T treated with neuraminidase (gB-NA)] immobilized on a CM5 sensor chip. The binding profiles are shown. (A) Wild-type PILR α to gB-293T. (B) PILR α R95A to gB-293T. (C) PILR α W108L to gB-293T. (D) PILR α Y2L to gB-293T. (E) Wild-type PILR α (3 μ M) to gB-293T (red), gB-NA (black), and gB-Sf9 (green). (F) A schematic model showing the difference in O-glycosylation between 293T mammalian and Sf9 insect cells. The terminal of the glycan modification is always occupied by an SA moiety in mammalian cells (*Left*) but by a galactose or mannose in insect cells (*Right*). (*Center*) The NA cleavage of terminal SA moieties. (G) Wild-type PILR β to gB-293T. (H) PILR β L108W to gB-293T.

Fig. 3. Structural basis of the PILR-SA interaction. (A) The complex structure of SA bound to PILR β -mut (L108W). The PILR protein is shown in surface, and the SA moiety in stick. Overall, SA is located on the lateral side of the PILR IgV rear sheet, which is highlighted as cartoons and labeled. (B) The atomic details mediating the PILR-SA interaction. The interface residues are shown as orange sticks and labeled and the SA moiety is presented in green. Dotted lines indicate H bonds or salt bridges.

We then characterized the atomic details at the binding interface. PILR β -mut clamps SA in a shallow pocket centered on the G strand. The SA moiety runs its sugar ring in parallel over the pocket surface and orients its carboxylate and glycerol groups downward facing the protein main body. This allows the formation of a bundle of hydrophilic interactions between PILR β -mut and SA to stabilize the binding. As for SA glycerol, this group was shown to simultaneously contact the main-chain groups of Q109 and the side chain of Y2 via H-bond interactions. The SA carboxylate group forms a salt bridge with the side chain of PILR residue R95 and simultaneously interacts with Q107 via an H bond (Fig. 3B).

The N-acetyl group of SA also contributes dramatically to the PILR-SA interaction. In the complex structure, the N-acetyl amide nitrogen was shown to form an H bond with the backbone oxygen of PILR Q107. In addition, the SA N-acetyl further appropriately positions its tip methyl group toward an apolar

ligand-receptor interaction requires the presence of SA moieties on the ligand surface, drawing parallels between PILRs and siglecs (7, 14). In this study, we report the high-resolution structures of both PILR α and PILR β , further demonstrating their structural similarities to siglecs. Nevertheless, PILR proteins, unlike siglec molecules, do not exhibit detectable binding for single SA sugar, and their SA-engaging avidity can only been seen in comparative assays with sialylated and desialylated ligands (e.g., HSV-1 gB) (8, 12). How PILRs interact with SA is an interesting issue. Despite great efforts, we were unable to obtain a complex crystal between PILR α and SA. Nevertheless, we successfully turned PILR β into a gB binder by a single L108W mutation mimicking the PILR α and managed to solve the complex structure between SA and this mutant protein. The revealed binding mode shows that residues Y2, W28, R95, Q107, W108, and Q109 are involved in the SA engagement. These amino acids are exactly the same between PILR α and PILR β -mut (Fig. S2). Further taking into account the extremely high structural similarity between these two molecules, we infer that PILR α likely uses a binding mode similar to that of PILR β -mut for SA interaction.

It is interesting that the Y/R/W triresidue is arranged in a linear mode in PILR α . In comparison with this ligand-free structure, an obvious orientation difference in the N-terminal loop and a dramatic conformational variance in the loop-residing tyrosine (Y2) residue are observed in the PILR β -mut-SA complex structure. This leads again to a triangular arrangement of the triresidue as in siglecs, although in a different manner as described in *Results*. Although it cannot be excluded that the L108W mutation may affect the steric positioning of Y2, this is unlikely the case because PILR α shares with PILR β -mut exactly the same amino acids for SA binding but arranges the tyrosine residue in an extended conformation. Therefore, the conservation in amino acids constituting the SA-binding site between

pocket formed by PILR residues Y2, W28, and W108, providing a strong hydrophobic interaction tying the sugar ligand with the protein receptor (Fig. 3B).

A Variant SA-Binding Mode in Comparison with Those Mediating Siglec-SA Interactions. We further compared our complex structure with previously reported structures of the siglec-SA complexes. Overall, the SA moiety adopts a similar orientation for protein engagement and occupies equivalent sites in the protein receptors. Nevertheless, the detailed atomic interactions in the binding site exhibit obvious differences between PILR β -mut and siglecs, especially for the two aromatic amino acids of the key-binding triresidue. In siglecs, these two amino acids orient the side chains in a parallel manner, stacking against each other via a strong π - π interaction. This π - π unit lays beneath the SA moiety and hydrophobically interacts mainly with SA glycerol. In turn, the SA molecule flips its tip hydroxide in the glycerol group for solvent exposure (Fig. 4A). In PILR β -mut, however, Y2 and W108 arranged their side chains almost perpendicularly. These two amino acids individually swung their side chains, relative to the corresponding residues in siglecs, $\sim 180^\circ$ along the CA-CB axis, leading to the formation of a hydrophobic concave suitable for accommodation of the SA N-acetyl methyl. Without the limitation from the apolar contact as observed in siglecs, the glycerol group of SA orients its tip hydroxide downward for an H-bond interaction after PILR engagement (also discussed above in *Results*). It is interesting that the Y/R/W triresidue is also positioned triangularly in the PILR β -mut-SA complex structure but mirroring the positioning in siglecs (Fig. 4B).

Discussion

The PILR pairs are important immune receptors that can deliver opposing cellular signals upon ligand recognition (1). This specific

PILR α and PILR β -mut seems to favor a possible rearrangement of the N-terminal loop (N loop) and Y2 as a ligand-induced event. Nevertheless, this remains hypothetical because of the lack of structures of either the PILR α -SA complex or the free PILR β -mut protein, which we have been unable to crystallize so far. Alternatively, it might be an intrinsic character of PILR α with an N loop of certain flexibility. Upon SA binding, the N loop is flipped and further stabilized by the bound SA moiety. In either case, we believe that the formation of an intact SA-binding site in PILR α requires a large shift in Y2 (Fig. 4C). In the free PILR α structure, this N loop cannot flip owing to the packing

Fig. 4. Comparison of the SA-binding mode between PILR β -mut and siglecs. Both PILR β -mut and siglecs use a key triresidue motif for SA engagement. The two aromatic residues of the motif, which rotate their side chains $\sim 180^\circ$ degrees in the PILR protein relative to the individual equivalent amino acids in siglecs, are marked. This leads the triangular arrangement of the triresidue to mirror each other between PILR β -mut and siglecs, which is highlighted at the bottom with solid-line triangles. (A) SA (yellow) bound to siglec-7 (cyan) as a representative. (B) SA (green) bound to PILR β -mut (orange). (C) A structural model between PILR α and SA. We believe that the formation of an intact SA-binding site in PILR α requires a large shift (marked with an arrow) in Y2. The observed tyrosine residue in the free PILR α structure is indicated with magenta sticks, and the modeled residue capable of engaging SA is shown as dashed gray sticks. The SA moiety is in green.

mode of the PILR α crystal. We believe the crystal pack obstructs Y2 shift and the PILR α /SA complex formation.

We also developed an effective SPR assay for a quantitative characterization of the PILR–SA interaction. The arginine and tryptophan amino acids of the triresidue are shown to be indispensable in the SA engagement of PILRs. In our complex structure, the arginine residue indeed plays a key role in binding by providing important salt-bridge contacts. Similar interactions have also been observed in siglecs (15, 17, 22). The tryptophan, however, only hydrophobically contacts the N-acetyl methyl of SA. Unlike its siglec counterpart, which is sandwiched between SA and the N-terminal tyrosine/tryptophan via π – π interactions (15, 17, 22), this PILR residue orients its indole ring perpendicularly relative to Y2 and thereby does not support a similar π – π contact as in siglecs. Nevertheless, strong hydrophobic interactions between these two amino acids can still be expected. We noted that Y2 oriented variantly in the ligand-free PILR α structure and in the SA-bound PILR β -mut structure, and W108 of PILR likely also plays a role in stabilizing Y2 in a conformation suitable for SA interaction. We therefore propose that PILR W108 contributes to the PILR–SA engagement via both a direct and an indirect way, explaining its indispensability in the binding of SA to PILRs. A leucine residue at this position, however, would not be of sufficient size and hydrophobicity to stabilize Y2, thereby precluding PILR β from interacting with SA.

Few PILR β ligands have thus far been identified, forming a sharp contrast to its paired partner PILR α , which can be recognized by multiple host and viral molecules (7–10). Mouse CD99 is a natural ligand for mouse PILR β ; specific binding of the two would deliver activating immune signals via the cytoplasmic ITAM motif of the PILR receptor (8). It should be noted that this mouse receptor contains a W108 residue rather than an L108 amino acid as in its human counterpart. Based on our functional and structural data in this study, human PILR β is

In either case, we believe the contact should be occurring on the accessible surface of the gB trimer [because we used a “crystal form” (26) of gB trimer in the binding study] and might be conserved between PILR α and PILR β (because we demonstrated that restoring the SA-binding avidity in PILR β could suffice for the receptor for gB engagement). Nevertheless, we showed in the virus infection assay that PILR β -mut, with a restored gB-binding capacity, was still unable to function as a coreceptor for HSV-1. This is consistent with a previous study (21) showing that PILR β with the L108W mutation, unlike PILR α , could not mediate the fusion of CHO-K1 cells expressing PILR with those expressing HSV-1 gD, gB, gH, and gL. The phenomenon favors a possible role of the cytoplasmic and transmembrane domains of PILR α in facilitating the cell entry of HSV-1. It also raises the possibility that PILR β -mut/SA binding is not reflecting the full features of PILR α –SA interaction or that the receptor specificity is provided by protein–protein contacts between PILR α and gB. The details on the PILR α –gB complex formation requires an atomic costructure, which should be pursued in the future.

Our structural and mutagenesis study delineated the basis for the variant SA-binding avidities between PILR α and PILR β . It is interesting that HSV-1 has evolved to use this difference to recognize an immuno-inhibitory receptor (PILR α) and to simultaneously avoid binding to its paired activation molecule (PILR β). Reminiscence of the common occurrence of HSV-1 latency in humans (27), it favors a role of PILR receptors in the HSV virulence. It is also noteworthy that our structures indicate a single SA-binding site in PILRs. The host SA-bearing PILR ligands, such as NPDC1 (7), should be able to compete with gB binding in HSV infection. These are interesting issues worth studying in the future.

Materials and Methods

Protein Preparation. The IgV-domain proteins of human PILR α , PILR β , and the indicated PILR mutants (PILR α Y2L, R95A, and W108L, as well as PILR β

unable to interact with SA. Accordingly, human CD99 lacks one of the two *O*-linked glycosylation sites present in mouse CD99 that are indispensable for PILR engagement (7). These observations do not favor a direct interaction between human CD99 and PILR β , raising questions about whether this human receptor can deliver activating signals similar to those in mice. Alternatively, human PILR β might use a new yet-unknown mechanism to interact with yet-unidentified molecules for immune activation. This interaction mode might not involve SA. Further studies are needed to settle the issue.

PILR α and PILR β exhibit a dramatic conformational difference in the CC' loop. It is notable that these two molecules differ only in a single amino acid (R47/G47) in this loop region. The residue, however, locates at the base of the CC' loop and is unlikely the reason for the observed conformation variance. We believe the variant CC' orientation in the PILR pairs possibly reflects a certain extent of flexibility in this loop. In siglecs, the same loop has been shown to be involved in the tuning of the ligand specificity, for which a flexible character would be a prerequisite (19). In accord with this notion, the flexible CC' loop of PILRs might exert similar functions as an element in fine tuning the ligand specificity.

Multiple host surface molecules have been shown to be exploited by HSV-1 for infection. A major category of the HSV-1 receptor includes herpes virus entry mediator and nectin-1, both of which are recognized by virus gD through specific protein-protein interactions (23–25). PILR α , however, relies on SA binding for the full capacity of gB engagement. Consistently, two *O*-glycosylation sites on gB were demonstrated in a previous study to be of vital importance in this interaction (12). Nevertheless, PILR α is not capable of binding single SA sugars, revealing the possibility of gB combining both SA recognition and amino acid interactions to engage PILR α . This residue-mediated contact could either be independent of or concert with SA-binding.

L108W) were individually expressed as inclusion bodies in *Escherichia coli* cells and then refolded as previously described (28). The refolded proteins were further purified by gel filtration chromatography.

The ectodomain protein of HSV-1 (strain F) gB with an engineered C-terminal 6 his tag was prepared either in Sf9 insect cells or 293T mammalian cells, followed by purification using an Ni-nitrilotriacetic acid HisTrap FF column (GE) and a gel filtration Superose 6 10/300 GL column (GE). The detailed protein-preparation procedures are given in [SI Materials and Methods](#).

Protein Characterization. The purified PILR and gB proteins were individually analyzed by an analytical gel filtration assay using calibrated Superdex 75 (10/300 GL) or Superose 6 (10/300 GL) columns. The pooled samples were further analyzed by SDS/PAGE or by Western blot.

Crystallization, Data Collection, and Structure Determination. The initial screening trials were set up with commercial crystallization kits (Hampton Research) using the sitting drop vapor diffusion method. Normally, 1 μ L protein was mixed with 1 μ L reservoir solution. The resultant drop was then sealed, equilibrating against 100 μ L reservoir solution at 4 or 18 °C. After optimization, diffractable crystals were obtained in 0.1 M sodium citrate tribasic dehydrate, pH 5.6, 20% (vol/vol) 2-propanol, and 20% (wt/vol) polyethylene glycol 4,000 for PILR α (4 mg/mL, 4 °C), in 3% (wt/vol) dextran sulfate sodium salt, 0.1 M Bicine, pH 8.5, and 15% (wt/vol) polyethylene glycol 20,000 for PILR β (8 mg/mL, 18 °C), and in 0.1 M Hepes sodium, pH 7.5, and 1.4 M sodium citrate tribasic dehydrate for the PILR β -mut-SA complex (5 mg/mL protein mixed in advance with 10 mM SA, 18 °C).

The crystals were first incubated in mother liquor containing 20% (vol/vol) glycerol for cryoprotection and then flash-cooled at 100 K. The heavy atom derivative data for PILR α were obtained from crystals transiently immersed (~30 s) in a cryoprotectant solution containing extra 1 M KI. All of the datasets were collected at the Shanghai Synchrotron Radiation Facility beamline BL17U. The data were processed and scaled using HKL2000 (29). The structure of PILR α was solved by single isomorphous replacement, and the structures of PILR β and PILR β -mut-SA complex were solved by molecular replacement method with the structure of PILR α as the search model using

