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*J Immunol* 2007; 178:369-377; doi: 10.4049/jimmunol.178.1.369

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Critical Residues at the Ly49 Natural Killer Receptor’s Homodimer Interface Determine Functional Recognition of m157, a Mouse Cytomegalovirus MHC Class I-Like Protein

Agnieszka Kielczewska,*† Hee-Seo Kim,*† Lewis L. Lanier,‡ Nazzareno Dimasi,§ and Silvia M. Vidal2*†¶

NK cell function is regulated by Ly49 receptors in mice and killer cell Ig-like receptors in humans. Although inhibitory Ly49 and killer cell Ig-like receptors predominantly ligate classical MHC class I molecules, recent studies suggest that their activating counterparts recognize infection. The quintessential example is resistance to the mouse CMV in C57BL/6 mice, which depends on the functional recognition of m157, a mouse CMV-encoded MHC class I-like molecule, by Ly49H, an activating NK cell receptor. We have taken advantage of the natural variation in closely related members of the Ly49C-like receptors and the availability of Ly49 crystal structures to understand the molecular determinants of the Ly49H-m157 interaction and to identify amino acid residues discriminating between m157 binding and nonbinding receptors. Using a site-directed mutagenesis approach, we have targeted residues conserved in receptors binding to m157 (Ly49H and Ly49I129) but different from receptors lacking m157 recognition (Ly49C, Ly49I166, and Ly49U). Wild-type and mutant receptors were transfected into reporter cells, and physical binding as well as functional activation by m157 was studied. Our findings suggested that the Ly49 MHC class I contact “site 2,” I226, may not be involved in m157 binding. In contrast, residue Y146 and G151, mapping at the receptor homodimer interface, are likely critical for functional recognition of the m157 glycoprotein. Our combined functional and three-dimensional modeling approach suggested that the architecture of the Ly49H dimer is crucial to accessing m157, but not MHC class I. These results link Ly49 homodimerization variability to the direct recognition of pathogen products.

Received for publication June 29, 2006. Accepted for publication October 9, 2006.

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† This work was supported by grants from the Canadian Institutes of Health Research and the Canadian Genetic Diseases Network. A.K. is a Canadian Institutes of Health Research Chair. L.L.L. is an American Cancer Society Research Professor and is funded by National Institutes of Health Grant AI068129.

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§ Abbreviations used in this paper: KIR, killer cell Ig-like receptor; CTLD, C-type lectin-like domain; MCMV, mouse CMV.

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N

atural killer cells are lymphocytes of the innate immune system specializing in the rapid recognition and killing of certain virus-infected and tumor cells (1). Thus, patients lacking an NK cell response, similarly to mouse models where NK cells or NK cell functions have been compromised, are exceptionally susceptible to infection with certain viruses (2–4). NK cell function is regulated by inhibitory receptors that recognize classical molecules of the class I MHC. These belong to the killer cell Ig-like receptor (KIR) family in humans and their functional analogues, Ly49 receptors, in mice (5). Signaling from inhibitory NK receptors recognizing self-MHC class I ligands provides a dominant signal (6), possibly to prevent an attack against normal healthy tissues. The activating NK receptors seem to be important for the recognition of transformed or pathogen-infected cells and are implicated in the resistance to viral infection (7–10). As we and others have shown, in C57BL/6 mice the activating receptor Ly49H provides innate resistance against MCMV (7, 8, 10), allowing characterization of the host-pathogen interaction.

Ly49H recognizes the MCMV m157 gene product, a cell surface glycoprotein with structural homology to MHC class I (11, 12), promoting NK cell killing and cytokine secretion (13). Resistance also depends on the DAP12 signaling chain, which associates with Ly49H (14). Remarkably, during MCMV infection of Ly49H-expressing mice, mutant viruses expressing m157 variants not recognized by the Ly49H receptor are positively selected (15), indicating a significant role of Ly49H in host defense against viral replication. The m157 protein is also recognized by an inhibitory receptor, Ly49I, expressed by NK cells in the 129 mouse strain (11), which has been proposed as a mechanism contributing to MCMV susceptibility and as a likely cause for the original evolution and selection of the m157 gene. Ly49H is a member of the highly polygenic and polymorphic Ly49 family (16), which appears to be rapidly evolving under selective pressure elicited by pathogens (17). These molecules are encoded in the NK gene complex, a region on mouse chromosome 6 with a cluster of genes preferentially expressed by NK cells (18). This complex has also been associated with the susceptibility to infection by the ectromelia virus (19) and the HSV (20), suggesting the presence of additional viral targets for recognition by Ly49 receptors.

Ly49 receptors are type 2 transmembrane proteins expressed as disulfide-linked homodimers (21–23). Inhibitory receptors such as Ly49A, Ly49C, Ly49G2, and Ly49I have ITIMs in their cytoplasmic tails that, upon phosphorylation, allow attenuation or damping of positive signals transmitted by other NK receptors (24).
Activating receptors, such as Ly49H, lack cytoplasmic ITIMs but contain a positively charged arginine residue in their transmembrane domain that mediates their association with the ITAM-bearing DAP12 protein (25). The extracellular domain of Ly49H is comprised of a flexible stalk and a C-type lectin-like domain (11, 12) and, presumably, a receptor or the Ly49I receptor in complex with their viral ligand I molecules (22, 29). More open conformation and is predicted to bind two MHC class I molecules (23). Similarly to Ly49C, the Ly49I129 homodimer presents with a dimerization mode of Ly49 receptors imposes physical constraints resulting in an open dimer conformation (23). This variation in their overall three-dimensional fold (29). Phylogenetic analysis at the nucleotide level of the CTLD suggested that Ly49 receptors fall mainly into Ly49A-like and Ly49C-like classes (30). Analysis of the crystal structures of the prototypical Ly49A and Ly49C or Ly49I receptors indicated differences in the structural characteristics of the formation of the homodimers rather than the monomer (29). Particularly, the functional binding site for the MHC class I molecule H2 has been mapped to a surface termed “site 2,” which is virtually superimposable in the Ly49A and Ly49C monomers (23). In contrast, in the Ly49A homodimer the α2 helices are side-by-side on the molecular interface, resulting in a closed dimer (21). Alternatively, in the Ly49C or Ly49I homodimers the two subunits are linked through their β-strand, β0, without involvement of the α2 helix in the dimer formation, resulting in an open dimer conformation (23). This variation in dimerization mode of Ly49 receptors imposes physical constraints on the binding of H2 ligands. The more closed Ly49A receptor engages its ligand asymmetrically, resulting in only one H2-D\\dagger molecule being bound to the Ly49A homodimer (21), whereas the Ly49C homodimer engages symmetrically two H2-K\\dagger molecules (23). Similarly to Ly49C, the Ly49H129 homodimer presents with a more open conformation and is predicted to bind two MHC class I molecules (22, 29).

<table>
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<tr>
<th>Primer Name</th>
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<th>Application</th>
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<td>Ly49H CTLD 5’ with Esp31 site</td>
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<tr>
<td>hp3’UBRI-R</td>
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<td>Ly49H 3’ end with Esp31 site</td>
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<tr>
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<td>Ly49H and U site-directed mutagenesis</td>
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<tr>
<td>V179L</td>
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<td>Site-directed mutagenesis V179L substitution</td>
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</tbody>
</table>

Table I. Nucleotide sequences of primers used for cloning and site-directed mutagenesis of chimeric Ly49 receptors

Cell lines and antibodies

Construction and maintenance of FLAG-DAP12 29T3 cells expressing a NH2-terminal FLAG-tagged DAP12 (32) and 2B4 reporter cells, which carry the FLAG-DAP12 and NFAT-GFP constructs (11), have been previously described. The production of the polyclonal Ab against the cytoplasmic domain of Ly49H has been previously reported (33). The 1F8 mAb (anti-Ly49C/D/H/U) was a gift from M. Bennett (University of Massachusetts Medical School, Worcester, MA). Anti-FLAG M2 mAb was obtained from Sigma-Aldrich. The m157-lg fusion protein was previously described (11). PE-conjugated goat anti-human IgG Fc and FITC-conjugated goat anti-rabbit IgG and goat anti-rabbit IgG were obtained from Jackson ImmunoResearch Laboratories.

Construction of chimeric Ly49 receptors and site-directed mutagenesis

The full-length cDNAs of Ly49H Cp71B16, Ly49F Cp71B16, and Ly49J Cp71B16 were cloned into the vector pBlueScript KS (MBI Fermentas). Ly49C Cp71B16 was a gift from F. Takei (University of British Columbia, Vancouver, Canada), Ly49A Cp71B16 was a gift from A. Makrigiannis (Institut de Recherches Cliniques de Montréal, Montréal, Quebec, Canada). For constructing all chimeric proteins of inhibitory Ly49 CTLD on an activating Ly49 backbone, the C-terminal CTLD domain of the inhibitory Ly49 receptors was amplified by PCR (see Table I for primer sequences) and fused via an Esp31 (MBI Fermentas) restriction site to the stalk, transmembrane region, and intracellular domain of the activating Ly49H. Esp31 cleaves downstream of its recognition site, allowing fusion of the domains. The chimeric receptor protein obtained with this approach acquires the ability to transmit activating signals upon ligation via the adapter protein DAP12. All cDNAs encoding the chimeric Ly49 receptors were subcloned into BamHI and NolI sites in the...
retroviral expression pMX-puro vector (34). Point mutations in the region encoding the CTLD of the Ly49 receptors were introduced by using an inverse PCR approach with Pfu polymerase (MBI Fermentas). Primers in opposite orientations were used to amplify the whole plasmid (Table I), and the PCR products were subsequently digested using the DpnI restriction enzyme to remove methylated DNA (MBI Fermentas). The resulting plasmids were transformed into competent bacteria (BD Clontech). All of the constructs were verified by sequencing.

**Transfections, reporter assays, and flow cytometry**

Chimeric and wild-type Ly49 receptors were retrovirally transduced into 293T NFAT-GFP reporter cells as previously described (11). Expression of the FLAG-DAP12 and Ly49 receptors on the surface of the 293T cells was verified by surface staining using anti-FLAG mAb (Sigma-Aldrich) and anti-Ly49 mAb 1F8, and cells were analyzed by flow cytometry. Following retroviral transduction, reporter cells expressing high levels of Ly49 were enriched by flow cytometric sorting. The NFAT-GFP reporter gene function was verified by culturing the reporter cells on anti-FLAG mAb-coated plates and using flow cytometric analysis for GFP expression. Ly49 GFP reporter cells and m157-transfected mouse Ba/F3 cells were constructed as described (11) and maintained in complete RPMI 1640 medium (supplemented 10% FBS, penicillin, streptomycin, and glutamine). For determination of the percentage of relative binding to the m157-Ig fusion protein, 293T cells expressing the chimeric Ly49 receptors were stained with m157-Ig followed by detection with PE-conjugated goat anti-human IgG1 (Jackson ImmunoResearch Laboratories). Relative binding of the Ly49 receptors to m157 was calculated by using the following formula: percentage of binding = (mean fluorescence intensity of m157-Ig-stained cells/MFI of IFN-γ-stimulated cells) × 100. For the GFP reporter assay, 293T GFP reporter cells were cocultured overnight with m157-Ba/F3 cells or parental Ba/F3 cells in 48-well plates at a 1:1 ratio. Activation of the NFAT-GFP reporter gene was detected by using a FACScalibur flow cytometer with Cell Quest software (BD Biosciences) and WinMDI (J. Trotter, The Scripps Research Institute).

**Construction of the Ly49H homology model**

Three-dimensional structures for the CTLDs of dimeric Ly49A (21), Ly49C (23), and Ly49I (22) are known at atomic resolution. A Ly49H structure has not been published; therefore, the Ly49H three-dimensional homology model from aa 142 to aa 262 was built by using the coordinates of the monomeric Ly49I protein (22) as template. This choice was suggested by a sequence alignment of the target and template with an overall identity of 91% in 120 overlapping residues. As a result, a Ly49H three-dimensional structure was computed after the template and target alignment with the program Modeller, version 6.2 (35). After the prediction of the interaction patches at the dimer interface with the program InterProTects (36), the dimeric form was built by applying a 2-fold symmetry as determined for the dimeric Ly49I crystal structure (22). The figures were prepared with MolScript (37), Render 3D (38), and PyMOL (39).

**Results**

**CTLD amino acid sequence alignment identifies critical residues for m157 of Ly49 C-like receptors**

To characterize the precise molecular details of recognition of the MCMV-encoded MHC class I-like molecule m157 by Ly49 receptors, we took advantage of natural amino acid sequence variation occurring in the CTLDs of five well-characterized members of the Ly49 C-like phylogenetic branch of the Ly49 family (30). These were the activating Ly49H<sup>57BL/J6</sup> (hereafter Ly49H) and Ly49I<sub>129</sub> (hereafter Ly49I) receptors and the inhibitory Ly49I<sub>129</sub>, Ly49C<sup>57BL/J6</sup> (hereafter Ly49C), and Ly49I<sub>129</sub> receptors (hereafter Ly49<sub>129</sub>) receptors. These receptors are distinguished by their binding properties to m157 and MHC class I and therefore have different effects on the activation of MCMV-specific T cells. The ly49<sub>129</sub> mice lack activating Ly49H and Ly49<sub>129</sub> receptors and have reduced MCMV-specific T-cell responses. Therefore, comparison of the three-dimensional structures of the Ly49H and Ly49I<sub>129</sub> receptors illuminated the critical amino acid residues that define these functional differences.
whereas Ly49C and Ly49IB6 show no m157 recognition but have many MHC ligands. Ly49H\textsuperscript{229} also binds multiple MHC class I molecules (27). Structure-based amino acid sequence alignment of these five receptors indicated 14 variable positions of the 127 residues in the CTLD (Fig. 1A). However, only amino acids at positions 146, 151, 179, 180, and 226 are conserved in the receptors binding to m157 but are different in receptors that do not bind to m157 (Fig. 1A). These residues map to three well-defined regions of the CTLD, which might be significant for m157 recognition (Fig. 1, A and D): the linker region between the β0-β1 sheets (sites 146 and 151), the linker region between the α1-α2 helices (sites 179 and 180), and binding “site 2” for MHC class I (residue 226) (Fig. 1A, rectangles).

Three-dimensional structures for Ly49A (21), Ly49I (22), and Ly49C (23) have been resolved and reveal that Ly49 receptors share a well-conserved CTLD core consisting of two α-helices (α1 and α2) and two anti-parallel β-sheets. However, these proteins vary in their mode of homodimerization. Ly49C and Ly49I monomers are linked together with four hydrogen bonds contributed by their respective first β-strand, β0. In contrast to Ly49C and Ly49I, Ly49A monomers are also linked by β-strand β0 but the dimer interface at the hydrophobic core differs, as the β0-strands are shifted by four residues compared with Ly49C and Ly49I. More importantly, the α2 helices are side-by-side in the Ly49A interface, closing up the Ly49A dimer when compared with Ly49C and Ly49I (29). Taking into account these structural constraints, we build a three-dimensional model for Ly49H (Fig. 1B) by using the coordinates of Ly49I (22). As seen in Fig. 1, B–D, the residues Tyr\textsuperscript{146} and Gly\textsuperscript{151}, which were mutated in this study, map primarily within the Ly49H (or Ly49I) homodimer interface and are important for homodimer formation. The other residues targeted for mutations in this study map distantly from the Ly49 homodimer interface. Specifically, Val\textsuperscript{179} and Lys\textsuperscript{180} are located within the core of the molecule; Ile\textsuperscript{226} maps to the Ly49 L6 loop (Fig. 1, B and D). This loop region corresponds to the MHC class I “site 2” binding site and is highly distorted in both the Ly49I and the Ly49C crystal structure (29).

Construction and functional assessment of the Ly49 mutant panel

To investigate the functional significance of the five naturally occurring amino acid substitutions, individual target residues were replaced in a panel of 18 chimeric receptors carrying wild-type or mutant Ly49C-branch CTLDs on an Ly49H backbone (Figs. 2–4). Ly49H, Ly49U, and Ly49IB6 recombinant receptors were expressed and studied in 2B4 cells stably transfected with DAP12 adapter protein cDNA carrying an N-terminal FLAG epitope and with a GFP reporter gene under the control of an NFAT promoter (11). Expression of the chimeric receptor and the FLAG-DAP12 complex was confirmed by flow cytometric analysis by using a polyclonal Ab against the intracellular region of Ly49H, the 1F8 mAb against the Ly49C/I/HU extracellular domain, and anti-FLAG mAb (Figs. 2 and 3). Physical binding to m157 was monitored by staining of the reporter cells with the m157-Ig fusion protein (11). Functional activity of these reporter cells signifying Ly49 receptor activation was detected by flow cytometric analysis (Fig. 2 and 3) of GFP expression following their coculture with Ba/F3 cells expressing m157.

Inhibitory receptors Ly49C and Ly49I\textsuperscript{229}, which recognize the H2\textsuperscript{D}-encoded MHC class I molecules endogenously expressed on the 2B4 reporter cells, were studied in transiently transfected human 293T cells expressing FLAG-DAP12 by staining with anti-Ly49H Abs and m157-Ig fusion protein (Fig. 4).

![Figure 2](https://example.com/figure2.png)

**FIGURE 2.** Different physical and functional requirements for m157 recognition by Ly49H are revealed by systematic replacement of five CTLD discordant amino acids. A, Binding of m157-Ig to mutant Ly49H receptors expressed on 2B4 reporter cells. Each of the stable 2B4 reporter lines was stained with the m157-Ig fusion protein; m157 binding is expressed as a relative value to Ly49-FLAG-DAP12 expression on the cell surface (see Materials and Methods for details). The wild-type Ly49H construct is shown in black, β0-β1 loop mutants are represented by diagonal lines, α1-α2 loop mutants are represented by squiggles, and the “site 2” mutant is represented by vertical lines. B, Ly49 receptor expression on 2B4 reporter cells was assayed by anti-FLAG and anti-Ly49 staining and flow cytometric analysis. Ly49-transfected 2B4 reporters were also cultured on plates coated with an anti-FLAG Ab to verify DAP12 expression and correct activation of the NFAT-GFP pathway (data not shown). Subsequently, reporter cells were cultured with Ba/F3 cells expressing the m157 protein in 1:1 ratio. GFP expression was detected by using flow cytometric analysis.

**Single amino acid substitutions of Ly49H CTLD alter differentially physical binding to m157 and functional activation of Ly49H reporter cells**

Physical binding to m157 and functional activation of the reporter cells correlated with the swapping of the CTLD between the Ly49H and Ly49I\textsuperscript{229} receptors. The m157-Ig fusion protein stained reporter cells expressing a chimeric receptor carrying the Ly49H CTLD on the Ly49U backbone, but not the Ly49U CTLD on the Ly49H backbone. Reporter cells expressing the Ly49H CTLD also produced GFP after coculture with m157-transfected Ba/F3 cells, indicating that binding activity is conferred by the CTLD region of Ly49H (results not shown). Systematic replacement of the five discordant amino acid positions in the Ly49H CTLD demonstrated that each of the individual mutations had
some effect on m157 binding, but none abolished the staining with the m157-Ig fusion protein completely as measured by relative binding of the anti-FLAG mAb (Fig. 2A). The “site 2” mutant receptor Ly49H (I226T) showed ~50% of relative binding to m157-Ig as compared with wild-type Ly49H. Remarkably, the two continuous mutations at the α1-α2 loop behaved very differently, with the nonconservative K180N substitution retaining significant binding to m157-Ig in Ly49H (K180N), and the conservative V179L mutation showing the most severe effect by reducing binding to 10% in the mutant Ly49H (V179L). In contrast, Ly49H receptors with mutations at the α0-α1 loop (Y146H and G151S) all retained significant binding to m157-Ig.

An analysis of 2B4 reporter cells for GFP synthesis following overnight coculture with m157-expressing Ba/F3 showed a more severe effect of the mutations. Ly49H (I226T) and Ly49H (K180N) reporters produced slightly reduced GFP levels as compared with the wild-type Ly49H reporters, and Ly49H (V179L) reporter cells produced no GFP. Surprisingly, reporter cells carrying the receptors Ly49H (G151S) and Ly49H (Y146H) had dramatically reduced GFP production despite their ability to physically bind m157-Ig as described above. These two mutations, which map to the β0-β1 loop, affected physical binding and receptor activity differently following an encounter with m157. These results suggested that there are different structural constraints for the physical binding of m157 and functional activation by Ly49H. However, it should be noted that m157-Ig is an artificial construct and its function may not be identical with that of the native m157 protein expressed on the surface of the target Ba/F3 cell.

Single amino acid substitutions at the β0-β1 loop confer functional m157 recognition

To further characterize the functional significance of these five naturally occurring amino acid substitutions, the reciprocal mutations were introduced into the CTLDs of Ly49U and Ly49IB6, which do not recognize m157 or H2k molecules. The Ly49H and Ly49U CTLDs differ at eight amino acid positions, but only positions 151, at the α0-α1 loop, and 180, at the α1-α2 loop, are conserved in the m157 binding receptors, but not Ly49U. The staining of reporter cells with m157-Ig was negative for wild-type Ly49U but positive for mutant Ly49U (N180K)- and Ly49U (S151G)-expressing cells (Fig. 3A), indicating that both positions are important for physical binding to m157. In contrast, whereas neither wild-type Ly49U nor Ly49U (N180K) reporter cells synthesized GFP following coculture with m157-Ba/F3 targets, the Ly49U (G151S) reporters showed similar activation levels to those of wild-type Ly49H reporter cells (Fig. 3B). These results indicated that a single conservative S151G substitution at the β0-β1
loop is necessary and sufficient for functional recognition of m157 by the activating Ly49U receptor.

There are seven amino acid differences between the CTLDs of Ly49B86 and Ly49H, but only residue 146, localized at the C terminus of the β0 sheet, and residue 226, mapping on the “site 2” of MHC class I binding region, are conserved in m157 binding receptors. Wild-type and mutant Ly49B86 (T226I) and Ly49I (H146Y) were expressed in 2B4 reporter cells. Of these, only Ly49I (H146Y) reporter cells stained with the m157-Ig fusion protein (Fig. 3A). Similarly, only Ly49I (H146Y) reporter cells expressed GFP following overnight coculture with m157-Ba/F3 cells (Fig. 3B). The effect of the substitutions H146Y and T226I on Ly49B86 binding to m157 was confirmed by using the transient transfection system in 293T cells (Fig. 4), where staining with m157-Ig was solely observed in cells expressing the Ly49B86 (H146Y) construct. Altogether, these results suggested that the H146Y substitution at the β0-β1 loop is critical for physical binding to m157 by Ly49B86 and its functional activation. These gain-of-function mutations were confirmed in a biologically relevant situation by coculturing chimeric Ly49 bearing reporter cells with MCMV-infected mouse embryonic fibroblast cells (data not shown).

Transient expression experiments were also used to determine binding to m157-Ig by the Ly49C (which does not bind m157) and Ly49I129 (which binds m157) receptors carrying key mutations. Ly49C differs from m157 binding receptors at the conserved positions 151 and 179, which map to the β0-β1 loop and α1-α2 loop, respectively. Wild-type or mutant Ly49C (L179V) had no or poor binding to m157. In contrast, cells expressing the β0-β1 loop mutant Ly49C (S151G) were stained by m157-Ig at levels comparable to those for Ly49B86 (Fig. 4). Reciprocally, the introduction of a G151S substitution in Ly49I129 completely abolished binding to m157, confirming the important role of this position in the recognition of m157. An Ly49I129 construct was also used to assess the role of the “site 2” MHC class I-binding residue 226, which had been previously shown to be crucial to MHC class I binding by Ly49 receptors (40). Remarkably, staining of Ly49I129 (T226I) with m157-Fc was comparable to that for the wild type, demonstrating that this position does not affect the recognition of m157 by Ly49I129. These results also indicated that residues mapping on the β0-β1 sheet, but not substitutions at position 226, appear critical to m157 recognition by Ly49 receptors.

Discussion

NK cell-mediated killing of healthy “self” cells is prevented by the interaction of classical MHC class I molecules expressed on their surface with inhibitory NK cell receptors (41). Intriguingly from the perspectives of NK cell function and evolution, very different gene families in humans and mice encode receptors recognizing classical MHC class I molecules (17, 42). The mouse Ly49s and the human KIRs fulfill an identical and essential function in these two species, which emphasizes their importance to the host. Another remarkable feature of MHC class I receptors is the emergence of activating isoforms that may specialize in the recognition of infected cells. New activating Ly49 receptors have been formed principally through gene recombination that replaces exons encoding the signaling domain of an inhibitory receptor with an exon encoding the signaling domain of an activating receptor (17). Of 21 Ly49 receptors (Ly49A to Ly49W) identified to date, eight encode activating isoforms (30, 43). As clearly demonstrated by Ly49H-mediated resistance to MCMV (7, 8, 10, 33), the presence of activating receptors may provide an important mechanism of host resistance against infection. Similarly, in the case of a human HIV infection, individuals who possess a gene encoding the activating KIR3DS1 and certain HLA-B allotypes demonstrate a delayed progression to AIDS (44, 45). However, the precise molecular determinants between activating NK cell receptors and infected cells in mice and humans remain to be clarified.

We have taken advantage of natural variation in Ly49 receptors closely related to Ly49H and the availability of crystal structures to understand the molecular details of Ly49H recognition of m157. Amino acid sequence alignments identified positions 146, 151, 179, 180, and 226 as conserved in the Ly49H and Ly49I129 m157 binding receptors and different in Ly49U, Ly49C, and Ly49B86, which do not bind m157. Analysis of a three-dimensional model of the Ly49H homodimer threaded on the atomic structure of Ly49I129 indicated that the variable sites between Ly49H-binding and nonbinding receptors resided within three well-defined regions of the Ly49 monomer: 1) “site 2” or the functional binding site for MHC class I; 2) the α1-α2 loop; and 3) the β0-β1 sheet.

The “Site 2” mutation, at position 226, did not change the phenotype of wild-type and mutant cell lines studied. In particular, Ly49H (I226T) and Ly49I129 (I226T) reporter cells behaved similarly to reporters expressing corresponding wild-type receptors, indicating that this residue is not important for binding to m157. This position, however, is important for the recognition of MHC class I by Ly49C because the Ly49C mutant carrying a T226 mutation lost binding to H2-Kb in a cell-cell adhesion assay (40), pointing to a significant difference in the mechanism of self and viral MHC class I recognition. Inspection of the crystal structure of the Ly49C and H2-Kb complex indicated that the side chain of I226 forms hydrogen bonds and >10 van der Waals contacts with R111 and E128 on H2-Kb (23), emphasizing its importance for this receptor-ligand complex. However, loop 3 containing I226 (23) has a high degree of conformational flexibility (29) and is the most variable region of the CTLD among Ly49 receptors. Notably, most of the differences between Ly49H and Ly49I129 are clustered there, further suggesting that this region is not involved in m157 recognition.

The two mutations of the α1-α2 loop at positions 179 and 180 have very different effects despite their physical proximity in the receptor molecule. Whereas mutant Ly49H (K180N) retained both binding and signaling, the V179L mutation on the Ly49H backbone significantly decreased binding and function as compared with wild-type Ly49H. Ly49U carries N180 and Ly49C carries V179. Detailed analysis of the V179 position in our Ly49H model (Fig. 5A) indicated that V179 is likely buried in the monomer exactly at the center of the α1-α2 loops and that the side chain of V179 is probably strictly constrained by the side chains of residues of the α2 helix with an average distance of 4 Å (Fig. 5A). Alteration of these constraints by the introduction of L179 might displace the α2 helix with deleterious consequences for the binding of Ly49H to m157. Remarkably, a Ly49C (V179L) carrying the reciprocal mutation lost both binding to the H2-Kb tetramer and function in a cell-cell adhesion assay (40), suggesting that position 179 is equally important for the recognition of MHC class I.

Mutations of the β0-β1 loop at positions 146 and 151 had the most remarkable effect in all of the receptors tested. Mutant Ly49H (G151S) or Ly49H (Y146H) had severely decreased binding to m157, whereas the single-residue mutants Ly49U (S151G), Ly49C (S151G), and Ly49B86 (H146Y) acquired recognition of m157. The possible role of these positions in Ly49H function might be extracted from the Ly49I129 and Ly49C crystal structures. In these molecules, the two homodimer subunits interact mainly through their β0 strands, which, together with the β1 strand, create an extended anti-parallel β-sheet (Fig. 5B). The presence of the Y146H substitution would introduce a positive charge at the center of this β-sheet. This might alter the geometry of the anti-parallel
β-sheet with possible structural changes for both the Ly49H monomer and the mode of dimerization, thus explaining the loss of m157 binding. The change introduced by G151S is seemingly more subtle, considering that position 151 resides at a β-hairpin linkinng the C-terminal half of h01 to the end of h01 (29). This position, however, would be buried between the subunits of Ly49H, which may affect the homodimerization mode. Notably, the Ly49I129 (G151) and Ly49C (S151) amino acid sequences are identical at the dimer interface except for position 151, but the homodimers present clear structural differences, suggesting that this might be the case. First, as shown in Fig. 5C, the homodimers present clear structural differences, suggesting that this might be the case. First, as shown in Fig. 5C, the homodimers have a different arrangement of the side chain orientation of Y146 and F148 (Fig. 5C). Also, the buried surface between Ly49H and Ly49I variation in the Ly49 receptors could account for side chain reorientation of residues Tyr146 and Phe148 at the homodimerization interface. These slight changes could also account for the homodimerization variability seen in the Ly49 receptors. D. Surface representations (top view) of the Ly49C (left) and Ly49I (right) homodimer. In these surface representations, the amino acid corresponding to the MHC class I binding site 2, as revealed by the Ly49C and H2-Kb cocrystal structure, are outlined in green. Asterisks show the location of residues Ile226 and Arg233 in Ly49I. These residues were not seen in the Ly49I crystal structure and, therefore, were omitted from the three-dimensional model. The location of the helices α2 in both Ly49C and Ly49I are schematically shown seated in the surface representation. The distances in angstroms (Å) between the helix α2 in Ly49C and Ly49I are shown. In addition, the homodimer length is also shown for both Ly49C and Ly49I. The small distance for Ly49C and Ly49I shows that the two homodimers have similar three-dimensional shape.
(23). The presence of two independent binding sites for class I in the homodimer allows for relatively relaxed structural constraints in the dimerization geometry in Ly49C (23). This finding is in contrast to the more stringent constraints on the architecture of other C-type lectin-like receptors, where each subunit binds the ligand at different sites. A good example of this is NKG2D, whose single-ligand binding site recognizes a single MHC class I-like molecule, and NKG2D has to be precisely aligned with the ligand for efficient binding (46–48). In a model where a single M157 molecule is accessed by either Ly49H, Ly49C(S151G), or Ly49U(S151G), the change of dimerization mode introduced by a variation at position 151 would be crucial to confer or abolish recognition to m157. Mutation studies (40, 49) and the three-dimensional crystal structures available for Ly49 proteins (21–23) have revealed a remarkable variability in the manner by which Ly49 inhibitory receptors bind MHC class I. Much of this variation resides in a degree of plasticity at the dimer interface, which imparts notable changes in subunit orientation. Our combined functional and three-dimensional modeling approach indicates that the architecture of the Ly49H dimer is crucial for accessing m157. Further mutational analysis of Ly49 receptors and of m157, together with studies of the atomic structure of the Ly49H and m157 complex, will be necessary to test this model. Understanding the mechanism of Ly49H recognition of m157 will provide insight into how NK cells recognize pathogen “tags” on infected cells. The presence of multiple activating Ly49 receptors and several phenotypically defined loci linked to the Ly49 gene family suggest that there are likely other viral products specifically recognized by activating NK cell receptors.

Acknowledgments

We thank Fumio Takei for providing the Ly9c cDNA and Andrew Makrigiannis for the Ly9i cDNA construct.

Disclosures

The authors have no financial conflict of interest.

References