Mutagenesis of Ly49B Reveals Key Structural Elements Required for Promiscuous Binding to MHC Class I Molecules and New Insights into the Molecular Evolution of Ly49s

Katarzyna M. Mickiewicz, Frances Gays, Richard J. Lewis and Colin G. Brooks

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Ly49B is a potentially important immunoregulator expressed on mouse myeloid cells, and it is thus an unusual member of the wider Ly49 family whose members are ordinarily found on NK cells. Ly49B displays substantial sequence divergence from other Ly49s and in particular shares virtually no amino acid sequence identity with the residues that have been reported to bind to MHC class I (cI) ligands in other Ly49s. Despite this, we show in this study that the BALB/c, but not the C57, isoform of Ly49B displays promiscuous cI binding. Binding was not significantly affected by inactivation of any of the four predicted N-linked glycosylation sites of Ly49B, nor was it affected by removal of the unique 20-aa C-terminal extension found in Ly49B. However, transfer of these C-terminal 20 aa to Ly49A inhibited cI binding, as did the addition of a hemagglutinin tag to the C terminus of Ly49B, demonstrating unexpectedly that the C-terminal region of Ly49s can play a significant role in ligand binding. Systematic exchange of BALB/c and C57 residues revealed that Trp166, Asn167, and Cys251 are of major importance for cI binding in Ly49B. These residues are highly conserved in the Ly49 family. Remarkably, however, Ly49BBALB variants that have C57 residues at positions 166 or 167, and are unable to bind cI multimers, regain substantial cI binding when amino acid changes are made at distal positions, providing an explanation of how highly divergent Ly49s that retain the ability to bind cI molecules might have evolved.

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Ly49 receptors constitute a family of type II transmembrane proteins that belong to subfamily V of the C-type lectin superfamily. They are expressed as disulfide-linked homodimers with each monomer comprising a cytoplasmic domain of ∼45 aa, a transmembrane domain of ∼20 aa, a stalk of ∼75 aa, and a ligand-binding domain, often designated the NK receptor domain (NKD), of ∼120 aa (1, 2).

Sequence alignment reveals that mouse Ly49s fall into four distinct groups, which presumably represent distinct evolutionary lineages (3). The vast majority of mouse Ly49s belong to lineages I and II. The expression of lineage I and II Ly49s is largely confined to NK cells, although they can also be expressed by T cells, especially NKT cells (4) and activated T cells (4, 5). Some of these Ly49s, such as Ly49A, C, and G, bind to the same classical class I (cI) MHC molecules that present peptides in their peptide-binding grooves to T cells, often with considerable selectivity for particular cI isoforms (6–8), and they deliver inhibitory rather than activating signals (9, 10). This selective cI binding and inhibitory signaling, coupled to the unusual stochastic expression of Ly49s on NK cells (10), allows NK cells to kill diseased cells that have lost expression of individual cI molecules, a phenomenon termed “missing self recognition” (11).

Important insights into the molecular basis of Ly49-cI recognition have been obtained from mutagenesis experiments and x-ray crystallography. Crystal structures of the extracellular domains of several Ly49s, including two complexed with cI ligands, have unexpectedly revealed three distinct modes of interaction between Ly49s and cI molecules, two of which were observed in a single Ly49A-D3 crystal. In the first, an Ly49A homodimer was bound, via a single Ly49 monomer, to the edge of the cI peptide-binding groove (site 1) (12). In the second, an Ly49A homodimer was bound to a cavity underneath the cI peptide-binding groove (site 2) via asymmetric contributions from both Ly49 monomers (12). Site 2 lacks polymorphic cI residues, providing no explanation for the cI selectivity of Ly49s, and leading to site 1 being originally favored as the principal binding site (12, 13). However, most (14–19), but not all (13, 20, 21), subsequent mutagenic analyses favored site 2 as the physiological binding site for Ly49s. More recently, the structure of a mutant form of Ly49C in complex with Kb revealed a third binding mode in which the Ly49 homodimer adopts a more open dimerization state and contains an additional α helical region in the middle of loop 3, allowing interactions with a slightly different site underneath the peptide-binding groove (site 3) via a single monomer unit (3).

The remaining two lineages of mouse Ly49s each contain a single member, Ly49Q (lineage III) and Ly49B (lineage IV). These Ly49s display a very different pattern of expression from lineage I and II Ly49s, being generally undetectable in NK cells, and are instead expressed in a nonstochastic manner on various subpopulations of myeloid cells (22, 23). Ly49B in particular is only distantly related to other Ly49s, but despite this it was reported to bind to cI multimers (8). In the present study we demonstrate that the C57 and BALB/c isoforms of Ly49B show major differences in cI binding. Subsequent mutagenic analysis provided a molecular explanation for these differences and revealed novel
insights into the factors underlying the diversification and cl binding capabilities of Ly49 molecules.

Materials and Methods

Constructs and mutagenesis

Ly49B<sup>CSR</sup> and Ly49B<sup>BALB</sup> cDNA constructs with attached 5′ BamHI and 3′ NotI sites were amplified from a plasmid provided by Prof. D. Raulet (University of California Berkeley, Berkeley, CA) and from RAW264 cells, respectively, using KOD polymerase (TuKaRa) and appropriate primers. Other Ly49 cDNAs were amplified in the same way from C57 cells. Variants with C-terminal extensions encoding the hemagglutinin (HA) tag sequence, YPYDVPDYA, and with and without the C-terminal 20 aa of Ly49B were generated by subsequent PCR with appropriate primers. Constructs were cloned into the BamHI and NotI sites of plasmid pMXs-Puro (24) provided by Dr. T. Kitamura (University of Tokyo, Tokyo, Japan), or, where appropriate, a variant in which the internal BglII site had been deleted. Chimeras were generated by excising fragments of Ly49B<sup>CSR</sup> or Ly49B<sup>BALB</sup> DNA using appropriate combinations of BamHI, BglII, EcoRI, and NotI enzymes, and cloning them into Ly49B<sup>CSR</sup> or Ly49B<sup>BALB</sup> plasmids cut with the corresponding enzymes. Mutants were generated by site-directed mutagenesis using the methods of Liu and Naismith (25). In brief, PCRs were set up with plasmids at 2 μg/ml in GC buffer (New England BioLabs), 0.2 mM 2′-deoxyinosine 5′-triphosphates, 1 μM primers, and Velocity polymerase (Bioline) at 20 U/ml. After initial denaturation at 98˚C for 1 min, they were run for 25 cycles at 98˚C for 30 s, 55˚C for 30 s, and at 72˚C for 8 min, followed by 72˚C for 16 min. Products were purified on Quagen MiniElute columns, incubated with DpnI at 300 U/ml for 1 h at 37˚C, and then submitted to SfiI and SalI bacteria (Invitrogen) made competent using a standard CaCl<sub>2</sub> procedure.

Transfection

Plasmids were purified and the fidelity of inserts confirmed by sequencing. Aliquots (0.5 ml) of rat YB20 cells (ATCC CRL-1631) suspended at 1 × 10<sup>6</sup>/ml in AIMV medium (Invitrogen) containing 2.5 μg/ml plasmid DNA were electroporated at room temperature using a Bio-Rad Gene Pulser (4-950 μF, 1,000 V, 960 Ω). Electroporated cells were gently resuspended, added to tubes containing 400 μl cold H2FA, and incubated at 4˚C for 20 min with directly conjugated test mAbs at ice temperature, or with multimers at room temperature, and then washed and analyzed by flow cytometry. Percentage inhibition was calculated as 100 × (1 − MFI<sub>test</sub>/MFI<sub>control</sub>), where MFI<sub>test</sub> indicates MFI with competitor Ab, and MFI<sub>control</sub> indicates MFI with medium.

Conjugate and membrane transfer assay

Cells were washed twice in PBS, resuspended in DMEM buffered with HEPES (Sigma-Aldrich D6171) at 2.5 × 10<sup>6</sup>/ml, and incubated for 10 min at room temperature with the green fluorescent reagent CFSE at 1 μM or with the red fluorescent membrane-binding reagent 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine (DiD) using the Vybrant cell-labeling reagent V-22887 (Life Technologies) at a dilution of 1:400. An equal volume of FBS was then added and cells were incubated for 10 min at 37˚C, after which the cells were washed three times in HBSS containing 5% FBS and resuspended in DMEM/HEPES containing 10% FBS at 1.0 × 10<sup>6</sup>/ml. Aliquots of 50 μl of appropriate CFSE- and DiD-labeled cells were mixed together in triplicates in the wells of V-bottom 96-well plates. In some experiments the labeled cells were incubated for 5 min at room temperature with 1:100 dilutions of hybridoma supernatants containing the following mAbs prior to dispensing into wells: MR3 rat IgG2c anti-nematode Ag A, 2G4 rat IgG2c anti-Ly49B, HB25 16-1-11N mouse IgG2a anti-<sup>2m</sup>, HB87 34-2-125 mouse IgG2a anti-D<sub>β</sub>H<sub>2</sub>, or HB102 34-5-8S mouse IgG2a anti-D<sub>β</sub>H<sub>2</sub> (the last three hybridomas were obtained from the American Type Culture Collection, Manassas, VA). The plates were spun at 100 × g for 2 min and placed in a 37˚C incubator for 30 min. The cells were gently resuspended, added to tubes containing 400 μl cold H2FA, and analyzed on a FACScanto flow cytometer equipped with blue and red lasers. Conjugates were scored as cellular events displaying both green and red fluorescence of similar intensity to control unmixed CFSE- and DiD-labeled cells and forward and side scatter values characteristic of cell aggregates (see Fig. 4 later in the article). Transfer of membrane material from DiD-labeled cells to CFSE-labeled cells was quantified by determining the MFI values of CFSE-labeled cells in the red fluorescence channel.

Results

Relationship of Ly49B to other Ly49s

Ly49B is the only known member of lineage IV of mouse Ly49s, and it shows equally distant amino acid identity (43–46%) to lineage I, II, and III Ly49s. The degree of similarity varies between domains: the cytoplasmic domain shares 66% average sequence identity with that of other mouse Ly49s, the transmembrane domain 73%, the stalk 40%, and the N KD 40%. Remarkably, Ly49B has a similar level of identity (42–48%) with 16 of the 17 putatively functional rat Ly49s in the BN rat strain (28), the exception being Ly49A with which it shares 63% identity. Ly49B is the only mouse Ly49 that has a recognizable ortholog in the rat.

The binding of Ly49s to their cl ligands is determined largely or entirely by their N KDs (29). Fig. 1 shows an alignment of the N KDs of Ly49B proteins with 1) the corresponding regions of representative Ly49s from the other main lineages, namely Ly49A<sup>CSR</sup> (lineage II), Ly49<sup>BC</sup> (lineage I), and Ly49<sup>QC</sup> (lineage III); 2) rat Ly49B; and 3) other selected members of the C-type lectin superfamily. Not only is the extensive sequence difference between Ly49B and other Ly49s readily apparent, but so too is the variation in sequence disparity across the N KD. In particular, in the N-terminal 51 residues of the N KD Ly49B show 61% average identity with other mouse Ly49s, whereas in the next 63 residues there is only 26% average identity with other mouse Ly49s. Thereafter, identity abruptly returns to >60%. Throughout the N KD, Ly49B shows much higher levels of similarity to its rat
counterpart, with 72% identity in the N-terminal region, 52% in the central zone, and 92% toward the C terminus. Additionally, Ly49B and its rat ortholog possess C-terminal extensions of 20 and 26 aa, respectively, compared with lineage I and II Ly49s.

The central region of the NKD, where there is the highest sequence disparity between Ly49B and other mouse Ly49s, contains the main ligand-binding interface in the Ly49A-Dd and Ly49C-Kb cocrystal structures (3, 12) (α, α helix; β, β sheet; L, loop); however, the α3 helix is present only in the latter. Shaded residues are those reported to be in contact with ligand in the Ly49A-Dd, Ly49C-Kb, human CD94/NKG2A-HLA-E, mouse NKG2D-RAE1b, human NKGD-MICA, human NKGD-ULBP, human KLRG1/human E-cadherin, and rat mannose-binding protein A–mannose structures (30–36). The two monomers in the NKGD homodimers are designated a and b. Lowercase residues in the Ly49A-C57 sequence are those that make contact with Dd at site 1 (as well as at site 2). Asterisks in the first line below the Ly49 sequences correspond to positions in contact with the stalk in the Ly49L crystal (38). Arrowheads in the bottom line show the conserved Cys238 and Cys251 residues that form a disulfide bond in virtually all members of the C-type lectin superfamily. Boxed residues are those demonstrated in this study to be principally responsible for the differential ability of Ly49B\textsuperscript{BALB} and Ly49B\textsuperscript{C57} to bind to cl molecules. Circled residues are those that when mutated to Ala compromise the ability of Ly49B\textsuperscript{BALB} to bind to cl molecules. The underlined Asn177 residue is the predicted N-linked glycosylation site in the NKD of Ly49B.

The crystal structures of several other C-type lectins that bind to cl or cl-like molecules have also been solved recently, including the human CD94/NKG2A heterodimer bound to HLA-E (30, 31), the mouse NKG2D-RAE1b, human NKGD-MICA, and human NKGD-ULBP, which share the same ligand-binding protein A–mannose-binding protein A–mannose structures and contain nearly all of the ligand contact residues in Ly49A and C (shaded residues in Fig. 1). Remarkably, although the ligand-contacting residues in these different C-type lectin receptors show no similarity, reflecting the different ligands and/or binding sites with which they interact, they all map to the same regions as the cl contact residues in Ly49s, and they often occupy equivalent positions (Fig. 1). The same is true for the contact residues between KLRG1 and its E-cadherin ligand (35), and even between mannose-binding protein A and its mannose sugar ligand (36) (Fig. 1). It would therefore be expected that the recognition

**FIGURE 1.** Alignment of the NKD of Ly49B with the corresponding regions of selected Ly49 and other C-type lectins. In the Ly49A sequences, dots show identity with the Ly49B\textsuperscript{BALB} sequence. Non-Ly49 sequences are shown in full. The top two lines above the sequence alignment show the secondary structure features found in the Ly49A-Dd and Ly49C-Kb cocrystal structures (3, 12) (α, α helix; β, β sheet; L, loop); however, the α3 helix is present only in the latter. Shaded residues are those reported to be in contact with ligand in the Ly49A-Dd, Ly49C-Kb, human CD94/NKG2A-HLA-E, mouse NKG2D-RAE1b, human NKGD-MICA, human NKGD-ULBP, human KLRG1/human E-cadherin, and rat mannose-binding protein A–mannose structures (30–36). The two monomers in the NKGD homodimers are designated a and b. Lowercase residues in the Ly49A-C57 sequence are those that make contact with Dd at site 1 (as well as at site 2). Asterisks in the first line below the Ly49 sequences correspond to positions in contact with the stalk in the Ly49L crystal (38). Arrowheads in the bottom line show the conserved Cys238 and Cys251 residues that form a disulfide bond in virtually all members of the C-type lectin superfamily. Boxed residues are those demonstrated in this study to be principally responsible for the differential ability of Ly49B\textsuperscript{BALB} and Ly49B\textsuperscript{C57} to bind to cl molecules. Circled residues are those that when mutated to Ala compromise the ability of Ly49B\textsuperscript{BALB} to bind to cl molecules. The underlined Asn177 residue is the predicted N-linked glycosylation site in the NKD of Ly49B.
of cI molecules by Ly49B would involve residues in these same regions.

**MHC cI multimers bind to the BALB/c but not the C57 isofrom of Ly49B**

To analyze in detail the cI binding capabilities of Ly49B, we first transfected rat YB2/0 cells with the C57 and BALB/c isoforms of Ly49B. Both were expressed at high levels as judged by 2G4 mAb staining and, as expected from our previous work (23), transfectants expressing the BALB/c isofrom showed negligible staining with the 1A1 mAb (Fig. 2A). In contrast to an earlier study (8), cells transfected with Ly49B<sup>C57</sup> showed no detectable staining with any of the three cI multimers tested (K<sup>b</sup>-OVA, D<sup>d</sup>-LCMV, and D<sup>d</sup>-HIV). However, all three cI multimers gave clear staining of the same cells transfected with Ly49B<sup>BALB</sup>. Over a series of 32 experiments we found that the MFI for the binding of K<sup>b</sup>, D<sup>d</sup>, and D<sup>d</sup> multimers to YB2-Ly49B<sup>BALB</sup> cells averaged 112, 252, and 150 U, respectively. In contrast, YB2 cells transfected with Ly49A were stained by D<sup>d</sup> but not K<sup>b</sup> or D<sup>d</sup> multimers (Fig. 2B), cells transfected with Ly49G were stained very weakly by D<sup>d</sup> but not K<sup>b</sup> or D<sup>d</sup> multimers, cells transfected with Ly49I were stained by K<sup>b</sup> but not D<sup>d</sup> or D<sup>d</sup> multimers, cells transfected with Ly49Q were stained weakly by K<sup>b</sup> but not D<sup>d</sup> or D<sup>d</sup> multimers, and cells transfected with Ly49E were not stained by any multimers (not shown). These results are consistent with previous studies of the specificities of these receptors (3, 6–8, 13, 39–45), validating the assay methods and reagents. It can be concluded that unlike several other Ly49s, Ly49B<sup>BALB</sup> is a promiscuous cI-binding molecule displaying only a limited selectivity (D<sup>d</sup> > D<sup>d</sup> > K<sup>b</sup>) for the cI isoforms in the present study.

A possible explanation for the failure of Ly49B<sup>C57</sup> molecules transfected into rat YB2 cells to bind to cI multimers could be that Ly49B<sup>C57</sup> molecules are sequestered and masked by rat cI molecules of RTI-u haplotype expressed on YB2 cells. Such cis interactions with cI molecules expressed on the same cells have been reported for several Ly49s, and they can strongly inhibit the binding of these Ly49s to soluble cI multimers (8, 46, 47). Although in one of these studies it was reported that Ly49B does not engage in cis interactions with mouse cI molecules (8), the possibility that Ly49B engages in cis interactions with rat cI molecules on YB2 cells was directly addressed by exposing Ly49B-transfected cells to acid under conditions previously demonstrated to selectively disrupt cI molecules and liberate Ly49s from sequestration by endogenous cIs (46). As expected, such treatment reduced the expression of rat cI molecules on YB2-Ly49B transfecants to almost undetectable levels as judged by staining with the RT1A<sup>a</sup>-specific mAb 68-1-D2 (and also with the rat β<sub>2</sub>m-specific mAb 4C9; data not shown), but it had no significant effect on the staining of the same cells with the anti-Ly49B mAb 2G4 or with D<sup>d</sup> or D<sup>d</sup> multimers (Fig. 3). In particular, YB2 cells transfected with Ly49B<sup>C57</sup> molecules did not acquire any detectable ability to bind D<sup>d</sup> or D<sup>d</sup> multimers following acid treatment.

**FIGURE 2.** Binding of cI multimers and mAbs to cells transfected with Ly49B. (A) YB2 cells transfected with Ly49B<sup>C57</sup> (B-C57), Ly49B<sup>BALB</sup> (B-BALB), or Ly49B<sup>BALB</sup> bearing an HA tag at the C terminus (BHA-BALB) were stained with cI multimers and mAbs 1A1 and 2G4. (B) YB2 cells transfected with Ly49A (A) or Ly49A bearing a C-terminal HA tag (AHA) were stained with cI multimers, and YE1/48 anti-Ly49A. Numbers in each panel show the MFI. Data in (A) are representative of 11 experiments, and in (B) of 5 experiments.
To establish whether the interaction between Ly49BBALB and cI molecules could also be demonstrated in functional assays where receptor and ligand were expressed on opposing cell surfaces, YB2-Ly49BBALB transfectants labeled with CFSE were incubated with YB2-Dd transfectants labeled with DiD. A much higher proportion of cells formed conjugates than when CFSE-labeled YB2-Ly49BBALB cells were incubated with DiD-labeled untransfected YB2 cells as judged by either the frequency of double-positive cellular events displaying high levels of both CFSE and DiD fluorescence (Fig. 4A) or scatter parameters (Fig. 4B). Additionally, the CFSE-labeled YB2-Ly49BBALB cells that at the 30 min assay point were not conjugated to YB2-Dd cells were observed to have captured substantial amounts of DiD-labeled membrane material compared with CFSE-labeled YB2-Ly49BBALB cells that had been incubated with DiD-labeled untransfected YB2 cells, as shown by the DiD MFI values of unconjugated YB2-Ly49BBALB cells in the top left quadrant of the dot plots in Fig. 4A. Importantly, conjugate formation and membrane transfer were specific events that occurred at above-background levels with cells transfected with Ly49BBALB but not Ly49BC57 (Fig. 4C). The direct involvement of Ly49BBALB and Dd cI molecules in conjugate formation and membrane transfer was confirmed by the finding that these events were blocked by the anti-Ly49B mAb 2G4 but not by the isotype control mAb MR3, and by the anti-Dd mAbs 34-2-12 and 34-5-8 but not by the isotype control mAb 16-1-11 specific for the cI molecule Kk (Fig. 4D). The finding that mAb 34-5-8 (HB102), which binds to an epitope dependent on residues in the α2 domain of Dd (15, 48–51), inhibits the binding of Dd-transfected cells to Ly49BBALB-transfected cells is consistent with previous reports that this mAb inhibits the interaction of Dd with Ly-49A and Ly49D as measured in cytotoxicity assays (6, 52–54) or physical binding assays (13, 55). However, the inhibition of binding of Ly49BBALB-transfected cells to Dd-transfected cells by mAb 34-2-12 (HB87), which binds to an epitope entirely dependent on residues in the α3 domain of Dd (49–51, 56), contrasts with the failure of this mAb to interfere with the recognition of Dd by Ly49A in either cytotoxicity (6, 52) or physical binding assays (13, 55), indicating that Ly49B may interact with cI molecules differently to Ly49A.

The binding of cI multimers and mAbs to acid-treated cells

To potentially facilitate subsequent mutational analysis of cI binding to Ly49B, we created transfectants expressing Ly49B molecules with a C-terminal HA tag. Such transfectants showed similar levels of staining with 2G4 to those expressing untagged Ly49B, but they showed markedly reduced binding of all three cI multimers and mAbs to acid-treated cells. YB2 cells transfected with Ly49BC57 (B-C57) or Ly49BBALB (B-BALB) were pretreated with either PBS (open bars) or acid buffer (filled bars) then stained with an anti-RT1Au mAb (Au), the 2G4 anti-Ly49B mAb, or Dd or Dk cI multimers. The values plotted are the mean MFI values from four independent experiments.
multimers (Fig. 2A). Over a series of 11 experiments, the MFIs for the binding of K^b, D^p, and D^d multimers to cells transfected with HA-tagged Ly49B were 23, 44, and 21%, respectively, of those for binding to cells transfected with untagged Ly49B, whereas the corresponding figure for mAb 2G4 binding was 101%. The reduction in binding of each of the multimers was highly significant (p < 0.001). The relatively greater reduction in both K^b and D^d binding compared with D^p binding was also highly significant (p < 0.001). The addition of the 8-aa HA tag to the C terminus of Ly49A also had significant effects, in this case slightly increasing the binding of D^d multimers and causing the dramatic appearance of a low but significant ability to bind K^b and D^d multimers (Fig. 2B).

In line with these findings, the binding of cI multimers to cells transfected with HA-tagged Ly49B^BALB was profoundly inhibited by anti-HA mAbs (Fig. 5B). The binding of multimers to cells transfected with both HA-tagged and untagged Ly49B^BALB was also inhibited by 2G4 (Fig. 5C). These data provide direct confirmation that cI multimers bind to Ly49B molecules on the Ly49B-transfected cells, and they indicate that the sites at which cI molecules bind to Ly49B are sufficiently close to both the C-terminal HA tag and to the 2G4 binding site (which probably resides in the C-terminal 20 aa; see below) to be affected by Abs bound to these sites. Mutual inhibition between 2G4, 1A1, and anti-HA mAbs was also frequently observed on the various transfectants, but the degree of inhibition was often low and in some cases undetectable (Fig. 5B–D), indicating that the binding sites for the three mAbs are in close proximity but nonetheless mAbs can sometimes bind independently to these sites.

To directly examine whether the additional 20 aa found at the C terminus of Ly49B influence ligand binding, we created a YB2 transfectant expressing HA-tagged Ly49B^BALB molecules in which the C-terminal 20 aa of Ly49B had been replaced by a single histidine residue (as found at the C terminus of Ly49A and several other Ly49s). This transfectant (BHA-BALB-Cde120) showed no significant alteration in cI multimer binding but, surprisingly, was completely unable to bind 2G4 (Fig. 6A). The same result was obtained with similar transfectants lacking the HA tag (not shown). In contrast, addition of the C-terminal 20 aa of Ly49B to the C terminus of Ly49A had no discernible effect on the binding of Ly49A-specific mAbs but markedly reduced the binding of D^d multimers (Fig. 6B). In a series of six experiments, the MFIs for the binding of 1A1, JR9, and YE1/48 mAbs to cells expressing Ly49A-Bterm molecules were 101, 100, and 87%, respectively, of those of cells expressing wild-type Ly49A, whereas the corresponding figure for D^d multimers was 32% (p < 0.001). Remarkably, Ly49A-Bterm transfectants could efficiently bind mAb 2G4. The addition of the same sequence to Ly49G markedly lowered surface expression, affected the relative binding activity of three anti-Ly49G mAbs, but again created a 2G4 binding site (not shown).

Taken together, the data described in this section demonstrate that changes at the C termini of Ly49 molecules can have significant effects on the binding of mAbs and cI ligands, and in some cases on surface expression. They also demonstrate that the epitope recognized by the 2G4 mAb is highly dependent on the C-terminal 20 aa, and may be entirely contained therein, a conclusion supported by the finding that the addition of the C-terminal 20 aa of Ly49B to two distantly related C-type lectin molecules, NKRP1D and Dectin-1, conferred reactivity to 2G4 (F. Gays and C.G. Brooks, unpublished observations).

**Mapping of residues required for the binding of cI multimers**

The all-or-nothing difference in cI binding between the C57 and BALB/c isoforms of Ly49B must arise from differences in their amino acid sequences. Of the 12 differences, 1 is a conservative change in the transmembrane region, and 11 are mostly nonconservative changes in the NKD (see Fig. 1). Five of the differences correspond to contact residues in Ly49C-Kb and/or Ly49D-Dd. To determine whether changes at these residues or elsewhere were responsible for the loss of cI binding in Ly49B^C57, we took advantage of conserved EcoRI and BglII sites in the coding sequence to make a series of chimeric constructs between the BALB/c isoform and the C57 isoform. These chimeras were subsequently mutated by site-directed mutagenesis. The entire set of chimeras and mutants was transfected into YB2/0 cells to create stable transfectants that were stained with mAbs and cI multimers. As shown in Fig. 7, the level of staining with 2G4 was remarkably constant, indicating that the mutant Ly49B molecules were generally expressed at similar levels to wild-type.

Neither a chimera in which the first eight variable residues were BALB/c (chimera 1) nor one in which the last four variable residues were BALB/c (chimera 2) showed detectable cI multimer binding (Fig. 7A), demonstrating that BALB/c residues from both these regions are required. Similarly, chimeras in which either the first three (chimera 3) or the last nine (chimera 4) variable residues...
were BALB/c failed to bind cI multimers (Fig. 7A). Each of these four chimeras also failed to form cI conjugates or engage in membrane transfer with YB2-Dd transfectants (data not shown). Collectively, these results indicate that cI binding requires at least one of the first three and at least one of the last four variable residues to be of BALB/c origin. This was confirmed by the finding that chimera 5, in which only the first three and last four variable residues were of BALB/c origin, bound cI multimers as efficiently as did the wild-type BALB/c isoform.

One of the last four variable residues, Cys251, is highly conserved and forms a disulfide bridge with Cys238 in virtually all members of the C-type lectin superfamily whose structure has been determined. However, in the C57 Ly49B isoform this residue is replaced by an Arg. Remarkably, not only did mutation of Arg251 in chimera 3 to Cys (mutants 3.1 and 3.2) restore efficient multimer binding in general (Fig. 7B), but it also created a molecule that bound Dd (Fig. 7B) and Kb (not shown) cI multimers, and mAb 2G4 anti-Ly49B. (B) YB2 cells transfected with Ly49A (A) or a mutant bearing the C-terminal 20 aa from Ly49B (A-Bterm) were stained with the anti-Ly49A mAbs A1, JR9, and YE1/48, Dd multimers, and mAb 2G4. Numbers in each panel show the MFI. Data in (A) are representative of three experiments, and in (B) of six experiments.

![Diagram](image)

**FIGURE 6.** Changes at the C terminus of Ly49 molecules affect the binding of cI multimers and mAbs. (A) YB2 cells transfected with wild-type Ly49B(BALB) (BHA-BALB) or a mutant in which the C-terminal 20 aa had been replaced with a single His residue (BHA-BALB-Cdel20), both of which bore a C-terminal HA tag, were stained with mAb 16.43 anti-HA, cI multimers, and mAb 2G4 anti-Ly49B. (B) YB2 cells transfected with Ly49A (A) or a mutant bearing the C-terminal 20 aa from Ly49B (A-Bterm) were stained with the anti-Ly49A mAbs A1, JR9, and YE1/48, Dd multimers, and mAb 2G4. Numbers in each panel show the MFI. Data in (A) are representative of three experiments, and in (B) of six experiments.

To determine which of these three variable residues is required for cI binding, additional mutations were introduced into chimeras 2 and 3. Mutation of the first variable residue, residue 59 (located in the transmembrane region) from the BALB/c form (Leu) to the C57 form (Val), had no adverse effect on cI binding (mutant 3.2 versus 3.1; Fig. 7B), a result that is perhaps not surprising given the conservative nature of this substitution. In contrast, mutating the second variable residue (position 166) from the BALB/c form (Trp) to the C57 form (Leu) (mutant 3.3) virtually eliminated multimer binding. Mutating the third variable residue (position 167) from the BALB/c form (Asn) to the C57 form (Lys) (mutant 3.4) also reduced multimer binding but to a much lesser extent. Similar results were obtained when the same mutations were introduced into an HA-tagged version of mutant 3.1 (not shown). The retention of substantial cI multimer binding in mutant 3.4 demonstrates that the BALB/c residues Trp166 and Cys251 are largely sufficient by themselves to create a cI binding site in a C57 Ly49B molecule.

Interestingly, the loss of multimer binding caused by mutating residue 166 in mutant 3.3 could be significantly reversed (p = 0.001) by reintroducing three BALB/c-specific residues near the C terminus, Glu236, Ser242, and Thr254 (mutant 2.1) (Fig. 7B). Paradoxically, when these same residues were introduced into mutant 3.4, cI multimer binding was abrogated (mutant 2.2). Looking at this same situation from the opposite viewpoint, the loss of multimer binding caused by mutation of residue 167 from the BALB/c form (Asn) in chimera 5 to the C57 form (Lys) in mutant 2.2 could be significantly reversed (p < 0.001) by the subsequent introduction of additional C57 residues at positions 236, 242, and 254 (mutant 3.4). As noted above, all three of these residues correspond to ligand contact sites in Ly49A-Dd and Ly49C-Kb.

These findings were confirmed and extended by introducing Trp166 or Asn167 into chimera 4 that contains BALB/c residues at all variable positions except the first three and is unable to bind cI multimers. Asn167 (mutant 4.1) restored significant but low cI binding, at least of Db (Fig. 7C). In contrast, Trp166 (mutants 4.2 and 4.3) restored negligible cI binding. Thus, rather surprisingly, although Trp166 can support substantial cI binding in an Ly49B molecule that is entirely of C57 origin apart from Cys251 (mutant 3.4), it is unable to do so in a molecule that is entirely of BALB/c origin apart from Lys167 (mutant 4.3). Direct comparison of mutant 4.2 or 4.3 with mutant 3.4 confirms the conclusion above that in certain contexts BALB/c-specific residues actually inhibit cI
binding, and that the loss of cI binding in Ly49BBALB caused by the mutation of residue 167 from Asn to Lys (mutant 4.3) can be partially reversed by introducing C57 residues at other positions (mutant 3.4).

To determine whether residues in Ly49B that correspond to ligand contact residues in the Ly49A-Dd and Ly49C-Kb structures would be important for ligand binding by Ly49BBALB, we introduced Ala mutations at five such positions. At four of these positions, Arg198, Asn230, Ser242, and His255 (shown by ovals in Fig. 1), this caused significant (p, 0.001) and often severe loss of cI binding (Fig. 7D). At the fifth, Lys209, there was no significant reduction in cI binding, but interestingly mutation of the corresponding residue in Ly49A in a previous study (18) also caused no reduction in cI binding. Thus, although Trp166, Asn167, and Cys251 are clearly critical for the binding of Ly49BBALB to cI ligands, so too are at least four of the residues that correspond to ligand contact residues in Ly49A, Ly49C, and other C-type lectins (see Fig. 1).

General importance of residues 166 and 167 for the binding of cI multimers and mAbs to Ly49 molecules

Trp166 is highly conserved, being found in all known members of the Ly49 family in all species examined, with the single exception of the C57 isoform of Ly49B. Position 167 is also conserved, being Ser in all C57 Ly49s except Ly49B and Ly49Q. To determine whether a Ser at position 167 is compatible with cI binding to Ly49B, we transfected cells with a mutant Ly49BBALB molecule in which Asn167 was changed to Ser. This mutant (4.4; Fig. 7C) could indeed bind cI multimers, but at a much reduced level compared with the wild-type. To determine whether Trp and Ser at these positions are important for ligand binding to other members of the Ly49 family, we mutated the corresponding residues, Trp160 and Ser161, in the HA-tagged Ly49A construct. As shown in Fig. 7E, mutation of Trp160 in Ly49A to Leu (as found in Ly49B(C57)) abolished the binding of Dd multimers. Unexpectedly, it also abolished the binding of all three anti-Ly49A

FIGURE 7. The binding of cI multimers to mutant Ly49 molecules. (A–D, F) YB2 cells transfected with wild-type Ly49B(C57) (B-C57), wild-type Ly49BBALB (B-BALB), and various chimeric or mutant Ly49B constructs were stained with Dd and Dd multimers, mAb 1A1, and mAb 2G4. (E) YB2 cells transfected with wild-type or mutant Ly49A constructs, all bearing C-terminal HA tags, were stained with Dd and Dd multimers, mAb A1 anti-Ly49A, and mAb 16.43 anti-HA. In all cases the box at the left side of each section shows the amino acids present at each of the 12 positions corresponding to those at which the C57 and BALB/c isoforms of Ly49B differ. In panels (A)–(D), BALB/c residues are shown in uppercase, C57 residues in lowercase, and non-Ly49B residues in underlined lowercase type. Data for cells transfected with wild-type Ly49BBALB are shown in all panels, and some other results appear in more than one panel to facilitate comparisons. In (E), mutated residues are underlined. Note that the data in (F) were obtained with different preparations of multimers from those used for the data in the other panels. Bars and numbers in the histogram panels show the average MFI values from all experiments performed, with the number of experiments being shown on the right side of the descriptive left-hand box.
mAbs that were tested, A1, JR9, and YE1/48 (Fig. 7E and data not shown). Coupled with the reduced expression of this mutant, as judged by staining with the anti-HA mAb, the data indicate that this mutation may have had adverse structural effects. In contrast, mutation of Ser166 to Lys (as found in Ly49B Bal) did not inhibit the binding of Dα multimers or mAbs to Ly49A. Thus, the conserved Ser residue found at alignment position 167 is clearly not essential for cl binding and can be replaced by either Lys (in the Ly49A mutant S161K or Ly49B mutant 3.4) or Asn (in wild-type Ly49B Bal).

The panel of chimeric and mutant Ly49B molecules also allowed us to determine which of the variable amino acids were responsible for the selective binding of mAb 1A1 to the C57 isoform. The results for chimeras 1, 2, and 3 demonstrate that 1A1 binding requires at least one residue of C57 origin in the first three variable positions, but in contrast to cl multimer interactions, the binding is affected minimally by whether the last four variable residues, including the critical Cys251, are of C57 or of BALB/c origin (Fig. 7A). Switching the transmembrane residue 59 between its C57 and BALB/c equivalents had no effect on the interactions of 1A1 with various Ly49B molecules (e.g., mutant 3.1 versus 3.2 in Fig. 7B). In contrast, mutation of either of the C57 residues Leu166 or Lys167 in chimera 4 to their BALB/c equivalents of Trp166 and Asn167, respectively, abolished 1A1 binding (mutants 4.1 and 4.2; Fig. 7C), suggesting that both are required for the formation of the 1A1 epitope. However, when the reverse changes were introduced into chimera 3.1, mutation of Trp166 to Leu (mutant 3.3) was sufficient by itself to restore maximal 1A1 binding (Fig. 7B). Essentially identical results were found when the same mutations were introduced into chimera 3, which has the C57 Arg residue at position 251 (not shown). Taken together, the results demonstrate that, in contrast to cl binding, Cys251 is not required for 1A1 binding, and that in an Ly49B molecule that is composed mostly of C57 residues (chimera 3 or mutant 3.1), Leu166 but not Lys167 is by itself sufficient to create a 1A1 binding site, whereas in an Ly49B molecule composed mostly of BALB/c residues (chimera 4), Leu166 and Lys167 are both required to create the 1A1 epitope. Consistent with the importance of residues 166 and 167 for 1A1 but not 2G4 binding, removal of the C-terminal 20 aa, which was shown above to eliminate 2G4 binding, had no effect on 1A1 binding (not shown).

Effect of glycosylation on ligand binding to Ly49B

Ly49B contains four potential Asn-linked glycosylation sites. Three of these, at positions 94, 105, and 114, are in the stalk region, but only one, at position 105, is conserved in other Ly49s. The fourth, at position 177, is located in the NKD (underlined in Fig. 1) but away from the main ligand binding regions of C-type lectin receptors and is not conserved in other Ly49s. To determine whether glycosylation at these four sites influenced ligand binding, a series of mutants was created in which the Asn residues were mutated to Gln. SDS-PAGE analysis showed that each of the mutant molecules, when immunoprecipitated from transfected cells, migrated faster than the wild-type Ly49B Bal molecule, indicating that all four predicted glycosylation sites normally bear sizeable glycans (data not shown). The three stalk mutants bound Dα and Dδ cl multimers with a similar efficiency to wild-type molecules (Fig. 7F), demonstrating that glycosylation at these sites is not required for, and does not interfere with, cl binding. Similarly, as shown by the N177Q mutant, glycosylation of the NKD was also not required for cl binding. The noticeably lower cl binding to this mutant suggests that glycosylation at this site might enhance ligand binding, but the differences were not statistically significant.

Discussion

In this study, we have demonstrated that the BALB/c isoform of Ly49B is able to efficiently bind at least three cl molecules, Kα, Dα, and Dδ, whereas the C57 isoform displays no measurable binding to any of these molecules. The promiscuous cl binding of Ly49B Bal is similar to that reported for Ly49C (7, 8), but it is quite distinct from the highly restricted cl binding of Ly49A, D, G, I, and Q (3, 6–8, 13, 39–45). By constructing a library of chimeric and mutant Ly49B molecules containing various permutations of BALB/c and C57 residues at the 12 positions at which these isoforms differ, we have established that the inherent inability of Ly49B C57 to bind cl molecules is largely due to changes at three of these positions, Trp166, Asn167, and Cys251. The location of these residues is shown by boxes in the primary sequences in Fig. 1, and the location of the corresponding residues in the Ly49A-Dα and Ly49C-Kα crystal structures is illustrated in Fig. 8. None of these positions corresponds to ligand contact residues common to the Ly49A-Dα and Ly49C-Kα structures (3, 12), or to residues at the Ly49 dimer interface (12), or to residues that were observed to interact with the stalk in the Ly49L structure (38) (see Fig. 1).

Cys251 (shown in light blue in Fig. 8) is the second residue of loop 6 in the Ly49 crystal structures and forms disulfide bonds with the preceding cysteine (Cys238, dark blue in Fig. 8). These two cysteines, and presumably the disulfide bonds between them, are highly conserved throughout the animal C-type lectin superfamily, and they are present in all known members of the Ly49 family in all species examined with the single exception of Ly49B C57. The equivalent residue to Cys251 is not a ligand contact residue in Ly49A-Dα and Ly49C-Kα. However, it lies in the middle of a sequence of 14 residues (242–255), every one of which, except the conserved Cys, makes contact with the cl ligand in either or both the Ly49A-Dα and Ly49C-Kα structures (Fig. 1). Similarly, all seven residues (229–237) immediately preceding Cys251 are in contact with ligands in either or both the Ly49A-Dα and Ly49C-Kα structures.

Images were generated using PyMOL.
structures. The nonconservative replacement of Cys\textsuperscript{251} with Arg in the Ly49B\textsuperscript{C57} molecule, and the consequential loss of the Cys\textsuperscript{251}–Cys\textsuperscript{238} disulfide bond, would therefore cause major disruption of the main ligand-binding interface. The strategic importance of the disulfide in each of the three described binding sites is clearly shown in Fig. 8A–C.

Trp\textsuperscript{166} is found near the N-terminal end of the α1 helix in each of the Ly49 structures solved to date. Trp\textsuperscript{166} does not make direct contact with ligands, its side chain pointing toward the hydrophobic core of the protein (Fig. 8). The Ly49 NKD fold places Trp\textsuperscript{166} directly below the ligand-binding interface, interestingly in close proximity to Cys\textsuperscript{251}. Similar to Cys\textsuperscript{251}, Trp\textsuperscript{166} is found in all known members of the Ly49 family except Ly49B\textsuperscript{C57}, implying that it has a critical structural or functional role. Consistent with this, a Ly49A variant in which the equivalent Trp residue was changed to Leu (as in Ly49BC\textsuperscript{57}) not only lost its ability to bind cI ligands, but also showed low expression at the cell surface and failed to react with any of three Ly49A-specific mAbs.

Asn\textsuperscript{167} is also well conserved, being Ser in all C57 Ly49s except Ly49B and Ly49Q (Fig. 1). However, Ly49 proteins in other mouse strains and in other species sometimes have nonconservative substitutions at this position, including several that have Lys, indicating that the presence of Lys at position 167 of Ly49B\textsuperscript{C57} would not have major structural effects and would not necessarily be incompatible with cI binding. Direct confirmation was provided by the finding that a Ser → Lys substitution at the homologous position (161) in Ly49A had no adverse effect on cI binding. In contrast, an Asn → Lys substitution at position 167 in Ly49B\textsuperscript{BALB} caused an almost complete loss of binding to all three cI ligands, whereas an Asn → Ser substitution had a markedly reduced effect. In the Ly49A-D\textsuperscript{3} and Ly49C-K\textsuperscript{b} crystal structures, the Ser\textsuperscript{161} side chain points toward the ligand, and in Ly49C-K\textsuperscript{b} but not Ly49A-D\textsuperscript{3}, Ser\textsuperscript{161} is in van der Waal contact with a cI residue (Ile\textsuperscript{225} of K\textsuperscript{b}) (Fig. 8C). Taken together, these observations suggest that residue 167 in Ly49B is not of major structural importance, but that it is positioned sufficiently close to ligand that the particular side chain present could make a decisive positive (Asn) or negative (Lys) contribution to overall binding energy.

The dominant importance of the residues at positions 166, 167, and 251 in the binding of Ly49B\textsuperscript{BALB} to cI ligands was most clearly shown by the efficient cI binding displayed by mutants 3.1 and 3.2 that contained C57 residues at the other eight variable positions in the NKD. Importantly, however, the corollary that these eight positions have no influence on cI binding was shown to be incorrect. Thus, even in mutants 3.1 and 3.2 there was a noticeable shift in specificity; that is, K\textsuperscript{b} and D\textsuperscript{3} ligands bound with increased efficiency compared with their binding to the wild-type BALB/c molecule, indicating that C57 residues at sites other than 166, 167, and 251, in the appropriate context, be more conducive to cI binding than their BALB/c counterparts. For example, mutation of residue 167 of Ly49B\textsuperscript{BALB} from Asn to Lys (mutants 2.2 and 4.3) abrogated cI binding, a loss of function that could be largely reversed by introducing C57 residues at other positions in the NKD (mutant 3.4). Similarly, the loss of cI binding caused by mutating residue 166 of mutant 3.1 from Trp to Leu (mutant 3.3) could be partially reversed by introducing additional mutations in the C-terminal region of the molecule (mutant 2.1). Thus, mutations that reduce or eliminate cI binding in Ly49 molecules, even when they occur in highly conserved and probably structurally critical residues such as Trp\textsuperscript{166}, can be compensated for by mutations elsewhere.

Residues at positions corresponding to ligand contact residues in Ly49A and Ly49C were also important for cI binding by Ly49B\textsuperscript{BALB}, in particular Arg\textsuperscript{198}, Asn\textsuperscript{236}, Ser\textsuperscript{242}, and His\textsuperscript{255}. However, although mutation of any one of these residues to Ala reduced or eliminated cI binding, Ly49A and Ly49C have natural nonconservative substitutions at three of these positions that must presumably compensate for each other (and changes elsewhere) to maintain cI binding.

Positions 166 and 167 also played a critical role in the formation of the binding site for the C57 isoform-specific mAb 1A1 but, as in the case of cI ligands, changes at these positions could sometimes be compensated for by changes elsewhere. Thus, both Leu\textsuperscript{166} and Lys\textsuperscript{167} were required for 1A1 binding to molecules that contained BALB/c residues at all other positions, but Lys\textsuperscript{167} was not required when C57 residues were present at most of the other variable positions. Remarkably, neither the 1A1 epitope nor the 2G4 epitope was affected by the presence or absence of the conserved and presumably structurally important Cys\textsuperscript{238}/Cys\textsuperscript{251} disulfide bond. The 2G4 but not 1A1 binding site was in fact dependent on, and most likely contained within, the 20-aa C-terminal extension found in Ly49B molecules. The finding that 2G4 can bind to Ly49B molecules denatured by the reduction of disulfide bonds, boiling in SDS, and transfer to nitrocellulose membranes (K.M. Mickiewicz and C.G. Brooks, unpublished observations) suggests that this extension sequence is unstructured or can readily refold independently of the rest of the molecule. In the crystal structures of Ly49A-D\textsuperscript{3} and Ly49C-K\textsuperscript{b}, the C termini of Ly49A and Ly49C are on the opposite side of the molecule to the ligand-binding interface, extending out into the solvent as apparently unstructured polypeptides (Fig. 8D). They would, therefore, not be expected to influence cI binding. However, our findings that 1) a C-terminal HA tag reduced cI binding to Ly49B and enhanced cI binding to Ly49A, 2) an Ab (mAb 16.43) bound to the HA tag on Ly49BHA completely eliminated cI binding, 3) 2G4 (which probably binds an epitope contained within the C-terminal extension of Ly49B) also eliminated cI binding, and 4) transfer of the Ly49B C-terminal extension to Ly49A inhibited cI binding collectively demonstrate that amino acids at the C terminus of Ly49 molecules can significantly affect cI binding. This could occur via direct interaction with ligands, interactions with other transmembrane proteins, or alterations to the Ly49 structure.

The results of this study lead to a substantive paradox. Although Ly49B\textsuperscript{BALB} is able to bind to the same cI isoforms as Ly49A and Ly49C, it shows little sequence similarity to Ly49A or Ly49C in the ligand-binding regions. Notably, most of the charged contact residues in Ly49C that contribute to the exquisite electrostatic complementarity between Ly49C and K\textsuperscript{b} (57) are lost in Ly49B, and of the five residues in Ly49A that contribute most of the binding energy between Ly49A and Dd, and that when individually mutated to Ala abolish binding to D\textsuperscript{3} (18), four have undergone nonconservative substitutions in Ly49B\textsuperscript{BALB} with no apparent adverse effect on D\textsuperscript{3} binding. The paradox extends to Ly49A and Ly49C: in the crystal structures, both Ly49s bind to a largely invariant region in cI molecules, but they show amino acid identity at only 4 of 30 contact positions. From the functional perspective, this paradox is resolved by our finding that nonconservative alterations in critical contact residues that inactivate cI binding can be complemented by additional mutations elsewhere that restore cI binding. However, this leaves unexplained why Ly49s should have undergone such extensive diversification if they bind to a non-polymorphic site in cI molecules.

One possibility would be that this diversity is driven by the interaction of Ly49s with other molecules, the most likely being pathogen-encoded molecules. Inhibitory receptors, such as Ly49A and Ly49C, are presumably highly susceptible to exploitation by pathogens that could evolve ligands that bind to these molecules and thereby inactivate immune cells. Pressure to counteract this would favor the survival of individuals whose inhibitory Ly49
molecules possessed mutations that converted them into activatory receptors, or which prevented interaction with pathogen-modified or encoded molecules (58–60). Two likely examples of this evolutionary struggle have been documented: interaction of the cl-like MCMV-encoded protein m17 with the inhibitory Ly49C and Ly49l molecules and the activatory Ly49H molecule (61–63), and the interaction of the activating Ly49P molecules with an endogenous cl molecule modified in some unknown manner by MCMV infection (64). Pathogen-driven diversification of individual Ly49 molecules would be facilitated by the redundancy provided by multigenism, allowing Ly49s that had acquired inactivating mutations to regain functionality via counteracting mutations. Under these circumstances, pathogen-imposed selection pressures would generate extreme diversification, consistent with the fact that mouse lineage I and lineage II Ly49s have no obvious orthologs in the rat and, therefore, must have evolved independently since the separation of the species. In contrast, Ly49B possesses a clear ortholog in the rat, with which it still shares identity at 11 of the 30 contact positions in the Ly49A-D and Ly49C-K structures. Clearly, Ly49B has undergone much less diversification than other Ly49s, with apparently no duplication in either species, implying that, although it recognizes the less diversification than other Ly49s, with apparently no duplica- tion in either species, implying that, although it recognizes the

## References


