The Functional Binding Site for the C-type Lectin–like Natural Killer Cell Receptor Ly49A Spans Three Domains of Its Major Histocompatibility Complex Class I Ligand

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Abstract

Natural killer (NK) cells express receptors that recognize major histocompatibility complex (MHC) class I molecules and regulate cytotoxicity of target cells. In this study, we demonstrate that Ly49A, a prototypical C-type lectin–like receptor expressed on mouse NK cells, requires species-specific determinants on β2-microglobulin (β2m) to recognize its mouse MHC class I ligand, H-2D^d. The involvement of β2m in the interaction between Ly49A and H-2D^d is also demonstrated by the functional effects of a β2m-specific antibody. We also define three residues in α1/α2 and α3 domains of H-2D^d that are critical for the recognition of H-2D^d on target cells by Ly49A. In the crystal structure of the Ly49A/H-2D^d complex, these residues are involved in hydrogen bonding to Ly49A in one of the two potential Ly49A binding sites on H-2D^d. These data unambiguously indicate that the functional effect of Ly49A as an MHC class I–specific NK cell receptor is mediated by binding to a concave region formed by three structural domains of H-2D^d, which partially overlaps the CD8 binding site.

Key words: β2-microglobulin • inhibitory receptor • cytotoxicity • mutation • H-2 antigens

Introduction

NK cells are a population of lymphocytes with an ability to spontaneously kill tumor cells and infected cells (1). Target recognition by NK cells involves MHC class I molecules on target cells (2). NK cells express C-type lectin–like or Ig-like receptors for MHC class I molecules (3, 4). Engagement of these MHC class I receptors by its ligands inhibits or activates NK cells, depending on a motif found in the cytoplasmic region or a positively charged amino acid residue in the transmembrane segment of the receptors (3, 5, 6).

Mouse NK cells express receptors of the Ly49 family, comprised of >10 members. These molecules are homodimers of type II transmembrane proteins with C-type lectin–like domains in the extracellular region (7–12). Ly49A, the prototype member of this family, is an inhibitory receptor specific for the mouse MHC class I molecules H-2D^d and H-2D^k (13). MHC class I is a ternary complex of a heavy chain, which consists of α1/α2 and α3 domains, β2-microglobulin (β2m),[1] and a peptide bound to a groove in the α1/α2 domain (14). Several lines of evidence suggested the involvement of the α1/α2 domain in the recognition of H-2D^d by Ly49A. The 34-5-8S antibody, which recognizes the α1/α2 domain of H-2D^d, but not an antibody against the α3 domain (34-2-12S) inhibits functional and physical interaction between Ly49A and H-2D^d (13, 15). Ly49A recognizes the natural mutant MHC class I molecule dm-1, which has the α1 and NH_2-terminal half of the α2 domain of H-2D^d with the rest of the molecule derived from H-2L^d, which is not a ligand for Ly49A (16). Ly49A recognizes only the peptide-bound form of H-2D^d molecules, but there is no apparent specificity for peptides as long as they have the anchoring residues required to bind H-2D^d (17, 18). Despite the homology of Ly49A to C-type lectins, the ability of Ly49A to bind certain carbohydrates like fucoidan or dextran sulfate (19), and the presence of two Asn-linked carbohydrates in H-2D^d, binding of Ly49A

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[1] *Abbreviations used in this paper:* ADCC, antibody-dependent cellular cytotoxicity; β2m, β2-microglobulin; CHO, Chinese hamster ovary; MFI, mean fluorescence intensity; sLy49A, soluble Ly49A.

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to H-2D^d does not depend on carbohydrates on H-2D^d (20). Our previous study using D^d/K^d chimeric molecules has shown that the polymorphic determinant of H-2D^d that restricts Ly49A reactivity lies in the NH_2-terminal halves of α1 and α2 regions of H-2D^d that form the bottom of the α1/α2 domain (20). Recently, Tormo et al. (21) resolved the crystal structure of the Ly49A/H-2D^d complex, providing two possible Ly49A binding sites on H-2D^d, and suggested that site 1, which includes the NH_2 terminus of α1 α-helix and COOH terminus of α2 α-helix, is the functional binding site for Ly49A rather than site 2, a concave region formed by α1/α2 and α3 domains, and β2m. However, experimental data explaining which represents the functional Ly49A binding site on H-2D^d that leads to inhibition of NK cell cytotoxicity had been missing.

In this study, we first focused on β2m, which had not been thought to be involved in the recognition of H-2D^d by Ly49A, and demonstrate the essential role of β2m in recognition. This result led us to explore a panel of individually Ala-substituted mutants of H-2D^d, in which mutations were introduced into α1/α2 or α3 domains, to functionally interact with Ly49A. We found specific residues in the α1/α2 and α3 domains of H-2D^d that are critically important for Ly49A interaction, as determined by soluble Ly49A (sLy49A) binding and inhibition of cytotoxicity by Ly49A$^+$ NK cells. Surprisingly, our results indicate that site 2 rather than site 1 is the functional binding site of Ly49A on H-2D^d that results in the inhibition of NK cell cytotoxicity.

Materials and Methods

Mice. C57BL/6J mice were obtained from Clea. This study was approved by the Animal Experiment Review Board of the Faculty of Pharmaceutical Sciences at The University of Tokyo, Tokyo, Japan.

Cells and Antibodies. C1498 cells, which have C57BL/6 origin with H-2^b and β2m^b, and Daudi cells were obtained from American Type Culture Collection. Ly49A-transfected Chinese hamster ovary (CHO) cells and H-2D^d-, K^d-, or L^d-transfected C1498 cells were established as described (13, 19, 20). Ly49A$^+$ IL-2–activated NK cells were prepared from C57BL/6 mouse splenocytes as described (13). S19.8 (anti–mouse β2m^b; reference 22), BBM1 (anti–human β2m; reference 23), 34-5-8S (anti–H-2D^d α1/α2; reference 24), 34-2-12S (anti–H-2D^d α3; reference 24), and A1 (anti-Ly49A; reference 25) were purified from culture supernatants. Fab and F(ab′)$_2$ fragment of antibodies were prepared with standard methods. Because both of the anti-β2m antibodies are mouse IgG2b isotypes, it is difficult to make F(ab′)$_2$ fragments; Fab fragments of these antibodies were instead used in cell-mediated cytotoxicity assays to avoid antibody-dependent cellular cytotoxicity (ADCC). Daudi cells were transfected by electroporation with mouse β2m^b or human β2m cDNA (a gift from Dr. R.K. Ribaudo, Molecular Applications Group, Silver Spring, MD) that was cloned into pApuro vector (26) together with wild-type H-2D^d cDNA cloned into pHβAPr-1neo vector, and stable transfectants were established as described (20).

Cell-mediated Cytotoxicity Assay and Cell–Cell Adhesion Assay. Cell-mediated cytotoxicity of Ly49A$^+$ NK cells against H-2D^d–transfected C1498 cells was tested by a 4-h ^{51}Cr-release assay as described (20). All cytotoxicity assays were done in triplicate. When indicated, target or Ly49A$^+$ NK cells were preincubated for 15 min with anti–MHC class I or anti-Ly49A antibodies, respectively. To prevent ADCC, anti-β2m and anti–H-2D^d antibodies were used as Fab and F(ab′)$_2$ fragments, respectively. Intact antibodies and F(ab′)$_2$ fragments were used at 10 μg/ml and Fab fragments were used at 40 μg/ml. Binding of H-2D^d–transfected cells to Ly49A-transfected CHO cells was examined as described previously (15, 20). When indicated, target cells or Ly49A$^+$ NK cells were preincubated for 15 min with anti–MHC class I antibodies or anti-Ly49A antibody, respectively.

Preparation of sLy49A Tetramer. sLy49A was prepared as described elsewhere (Matsumoto, N., K. Tajima, M. Mitsuki, and K. Yamamoto, manuscript submitted for publication). In brief, the extracellular domain of Ly49A with NH_2-terminal biotinylation sequence tag (27) was expressed in *Escherichia coli* using an efficient T7 RNA polymerase-based system (28). The recombinant protein was in vitro refolded by dilution (29) and purified by cation exchange and gel filtration column chromatography. The sLy49A was biotinylated by biotin ligase BirA (Avidity). sLy49A tetramer was formed by incubating the biotinylated sLy49A with R-PE–conjugated streptavdin (BD PharMingen) at a molar ratio of 4:1.

β2m Replacement Studies. H-2D^d–transfected C1498 cells were cultured for 16 h in the presence or absence of 4 μM human β2m (purified from plasma; Calbiochem) in RPMI 1640 free from FCS at 37°C. Then the cells were used for flow cytometry or cell-mediated cytotoxicity assay. After 16 h of culture under FCS-free condition, >99% of the cells were viable.

Site-directed Mutagenesis and Stable Transfection of Cells. Point mutations were introduced by primer extension with T4 DNA polymerase using the Altered Sites® II system (Promega) or by sequential PCR steps as described by Cormack (30). To introduce point mutation by the PCR-based technique, we introduced individual mutations into the 5′ fragment of H-2D^d cDNA, which encodes a signal sequence and α1/α2 domain, or 3′ fragment, which encodes the rest of H-2D^d, by PCR using the following terminal primers: 5′-C*CTGCAG*GTCGACTCTAGAG-3′ and 5′-GTT*CTTAAG*AGCGTAGCATTCCCGTTC-3′ for the 5′ fragment and 5′-CT*CTTAAG*AACAGATCCCCCAAAGGC-3′ and 5′-*GGATCC*ACACCAGGCAGCTG-3′ for the 3′ fragment. These primers contain SalI, AflII, or BamHI site (indicated in italics). The sequence of primers used in the first PCR step will be provided on request. All the PCR reactions were performed using KOD-Plus-DNA polymerase (Toyobo). Each of the 5′ or 3′ fragments was subcloned into the SmaI site of pBluescript® II SK$^+$ (Stratagene), and the sequence was confirmed by reading both strands using an LS-2000 sequencer (LI-COR). Then, each of the mutant 5′ fragments and wild-type 3′ fragment or wild-type 5′ fragment and each of the mutant 3′ fragments was directionally cloned into pHβApr-1neo vector and the constructs were used for the transfection of C1498 cells by electroporation as described (20).

Flow Cytometry. Cells were stained with 10 μg/ml of indicated primary antibodies and then with FITC-goat anti–mouse IgG F(ab′)$_2$. For sLy49A staining, cells were stained with PE-labeled sLy49A tetramer and then fixed with 0.5% paraformaldehyde in PBS. In both cases, the stained cells were analyzed using a FACScalibur™ with CELLQuest™ software. Binding of the sLy49A tetramer to each mutant H-2D^d was calibrated, with the expression of H-2D^d detected by 34-2-12S or 34-5-8S antibodies in the following formula: sLy49A tetramer binding index = (mean fluorescence intensity [MFI] of sLy49A tetramer stained cells − MFI of streptavidin-PE stained cells)/(MFI of 34-2-12S

or 34-5-8S stained cells − MFI of control antibody stained cells). sLy49A tetramer binding of each H-2D^d mutant is expressed as the relative value of the binding index when wild-type H-2D^d is adjusted to 100. Because introduction of E227A mutation into H-2D^d abrogated the 34-2-12S epitope, we evaluated the expression of E227A mutant by reactivity with the 34-5-8S antibody.

Results

Anti–Mouse β2m^b Antibody Inhibits Recognition of H-2D^d by Ly49A. To investigate the possible involvement of β2m in the recognition of H-2D^d by Ly49A, we examined the effect of the S19.8 antibody, which reacts with the b allele of mouse β2m, on the interaction of Ly49A with H-2D^d in target cell killing assays. In these experiments, antibody fragments lacking the Fc region were used to avoid the potential effect of target lysis by ADCC mediated by NK cells through their Fc receptors. F(ab′)$_2$, or Fab for IgG2b isotypes, fragments were used. As reported previously (13), Ly49A$^+$ LAK cells were unable to kill H-2D^d–transfected C1498 (H-2^b) lymphoma cells efficiently (Fig. 1 A). Importantly, the addition of S19.8 Fab fragments to the killing assay, as well as the positive control anti–H-2D^d α1/α2 antibody (34-5-8S) F(ab′)$_2$ fragments or intact anti-Ly49A antibody (A1), reversed the H-2D^d–mediated inhibition of the target cell killing by Ly49A$^+$ LAK cells (Fig. 1 A). By contrast, negative control anti–H-2D^d α3 antibody (34-2-12S) F(ab′)$_2$ fragments had no effect. We also investigated the effect of S19.8 antibody on the physical binding of H-2D^d–transfected C1498 cells to Ly49A-transfected CHO cells (Fig. 1 B). Anti–mouse β2m antibody, as well as anti–H-2D^d α1/α2 or anti-Ly49A antibody, completely abrogated the binding of H-2D^d–transfected C1498 cells to Ly49A-transfected CHO cells (Fig. 1 B). These results clearly demonstrate inhibition of the functional and physical interaction between H-2D^d and Ly49A by the anti-β2m^b antibody S19.8 and suggest the possible involvement of β2m in Ly49A binding to H-2D^d.

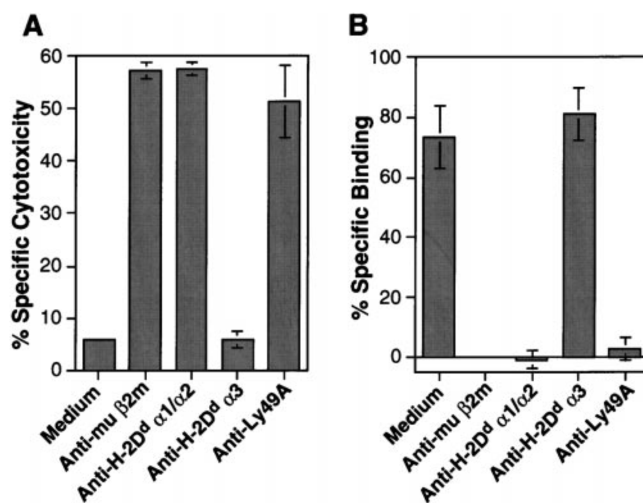

Figure 1. Anti-β2m antibody inhibits Ly49A interaction with its MHC class I ligand H-2D^d. (A) Reversal of H-2D^d/Ly49A–mediated inhibition of target cell killing by anti-β2m antibody. Killing of H-2D^d–transfected mouse lymphoma C1498 cells by Ly49A$^+$ NK cells in the presence of various antibodies was examined by standard ^{51}Cr-release assay at an E/T ratio of 20:1. Anti–mouse β2m or anti–H-2D^d antibodies were used as Fab or F(ab′)$_2$ fragments to prevent ADCC. Means ± SD of triplicate studies are shown. (B) Inhibition of H-2D^d–transfected cells binding to Ly49A-transfected cells by anti-β2m antibody. Binding of H-2D^d–transfected C1498 cells to Ly49A-transfected CHO cells was examined in the presence of various antibodies. The antibodies were used at 10 μg/ml. Means ± SD of triplicate studies are shown.

Failure of Ly49A To Recognize H-2D^d Complexed with Human β2m. β2m bound to MHC class I molecules on the cells in culture can be replaced by exogenously added β2m (31, 32). It is conceivable that some MHC class I molecules on C1498 cells and their transfectants were associated with bovine β2m, because those cells were maintained in the presence of FCS. The observation that anti–mouse β2m antibody completely abrogated the recognition of H-2D^d by Ly49A (Fig. 1) suggested that H-2D^d complexed with β2m from bovine or other species might not be recognized by Ly49A. To investigate the species-specific involvement of β2m in Ly49A recognition of H-2D^d, we used human β2m to which a serological reagent was available. Incubation of H-2D^d–transfected mouse C1498 cells with human β2m induced expression of human β2m epitope detected by BBM1 antibody (Fig. 2 A) and decreased expression of mouse β2m epitope by 34% in MFI compared with H-2D^d–transfected C1498 cells cultured in the absence of human β2m (Fig. 2, A and B). Incubation of H-2D^d–transfected C1498 cells with human β2m also increased expression of H-2D^d by 12.4%, consistent with the reported ability of human β2m to stabilize surface expression of mouse MHC class I molecules (33). These data indicate that a substantial number of H-2D^d molecules were complexed with human β2m. These cells were then tested for killing by Ly49A$^+$ NK cells in the presence or absence of various an-

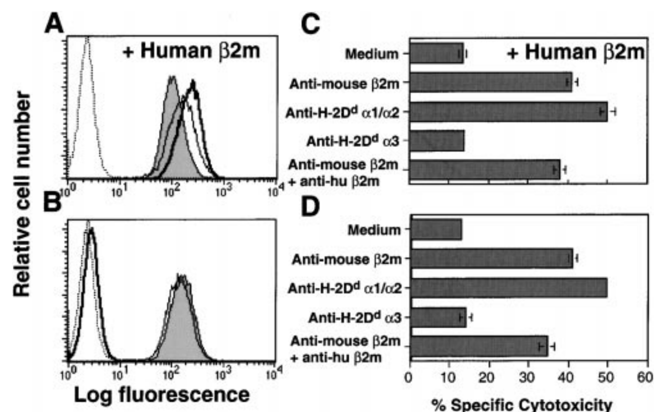

Figure 2. H-2D^d complexed with human β2m cannot protect target cell killing by Ly49A$^+$ NK cells. H-2D^d–transfected mouse lymphoma C1498 cells were cultured in the presence (A and C) or absence of human β2m (B and D). Then the cells were tested for binding of anti–mouse β2m antibody S19.8 (shaded area), anti–H-2D^d α3 antibody 34-2-12S (thin line), anti–human β2m antibody BBM1 (bold line), or control antibody (dotted line) by flow cytometry (A and B). The cells were tested for killing by Ly49A$^+$ NK cells in the presence of various antibodies (C and D) at an E/T ratio of 20:1. Anti-β2m or anti–H-2D^d antibodies were used as Fab or F(ab′)$_2$ fragments, respectively.

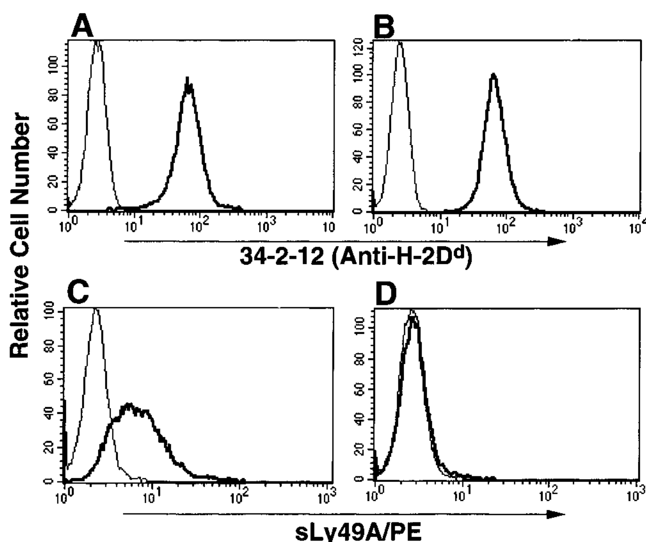

Figure 3. sLy49A tetramer binds H-2D^d complexed with mouse β2m but not with human β2m. β2m-defective human Daudi cells were stably transfected with mouse β2m (A and C) or human β2m (B and D) together with H-2D^d heavy chain. The cells were assayed for binding of anti–H-2D^d antibody 34-2-12S (A and B, bold line), control antibody (A and B, thin line), PE-labeled sLy49A tetramer (C and D, bold line), or streptavidin-PE (C and D, thin line) by flow cytometry. Staining of these cells with 34-5-8S gave similar results to 34-2-12S (data not shown).

tibodies (Fig. 2 C). To evaluate the interaction of Ly49A with H-2D^d complexed with human β2m, H-2D^d complexed with mouse β2m was masked by anti–mouse β2m antibody. Addition of anti–mouse β2m antibody Fab fragments as well as the anti–H-2D^d α1/α2 antibody F(ab′)$_2$ fragments reversed the inhibition of Ly49$^+$ NK cell–mediated killing of H-2D^d–transfected C1498 cells cultured in the presence of human β2m (Fig. 2 C). Further addition of anti–human β2m antibody Fab fragments did not show any effect. These data imply that H-2D^d complexed with human β2m is unable to inhibit killing by Ly49A$^+$ LAK cells; however, a firm conclusion could not be obtained due to the incomplete exchange of human β2m for mouse β2m.

To further address this issue, we transfected β2m defective human cell line Daudi with human or mouse β2m together with H-2D^d heavy chain (Fig. 3, A and B). We recently prepared sLy49A tetramer, which specifically binds H-2D^d (Matsumoto, N., K. Tajima, M. Mitsuki, and K. Yamamoto, manuscript submitted for publication). sLy49A tetramer bound Daudi cells transfected with mouse β2m and H-2D^d (Fig. 3 C) but not those transfected with human β2m and H-2D^d (Fig. 3 D), despite the equivalent level of H-2D^d expression on those cells (Fig. 3, A and B). These results clearly demonstrate the species-specific ability of mouse β2m to support Ly49A binding to H-2D^d and suggest the direct involvement of β2m in the recognition of H-2D^d by Ly49A.

Disruption of Ly49A Recognition of H-2D^d by the Introduction of Three Individual Mutations in α1/α2 or α3 Domains of H-2D^d. Previous observations indicate the importance of the α1/α2 domain, especially the NH_2-terminal halves of the α1 and α2 regions of H-2D^d that form the bottom of the α1/α2 domain, in Ly49A recognition of H-2D^d (16, 34). Moreover, our analysis indicates that β2m may be directly involved in the recognition. These observations prompted us to prepare a panel of Ala substituted mutants of H-2D^d and to explore their interaction with Ly49A. Residues substituted with Ala were chosen from solvent-exposed residues in α1/α2 or α3 domain based on the crystal structure of H-2D^d (35, 36). These sites include the residues involved in hydrogen bonding between H-2D^d

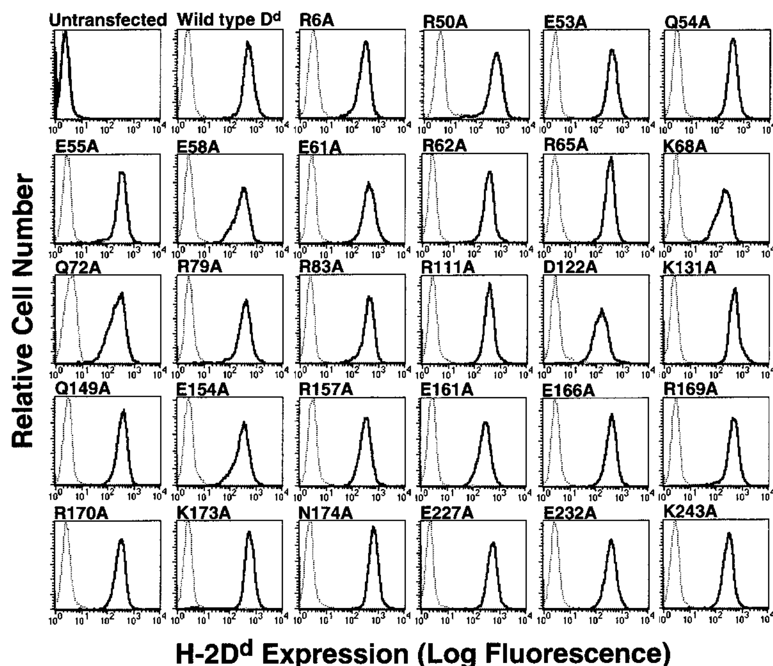

Figure 4. Expression of H-2D^d mutants on transfected C1498 cells. Individual Ala substitution of each residue was introduced into the indicated residues of H-2D^d. Each mutant is described as original amino acid residue in one letter code followed by residue number and by A, the one letter code for Ala. C1498 cells that were untransfected or transfected with wild-type H-2D^d or individual H-2D^d mutants other than E227A mutant were stained with 34-2-12S antibody (bold lines) or control antibody (broken lines). C1498 cells transfected with H-2D^d E227A mutant were stained with 34-5-8S (bold lines) or control antibody (broken lines), because E227A mutation disrupted the 34-2-12S epitope.

and Ly49A at the two putative interaction sites in the crystal structure of the Ly49A/H-2D^d complex reported by Tormo et al. during the course of this study (21).

C1498 mouse lymphoma cells were stably transfected with the mutant H-2D^d cDNA constructs. The transfectants with a similar level of H-2D^d expression, as assayed by staining with 34-2-12S or 34-5-8S antibodies, were selected for further analysis (Fig. 4). Most of the H-2D^d mutants tested here were equally reactive with both 34-2-12S and 34-5-8S antibodies (data not shown). However, substitution of Glu227 in the α3 domain of H-2D^d with Ala (E227A) disrupted the epitope recognized by 34-2-12S but not of 34-5-8S as reported by Connolly et al. (37). The panel of H-2D^d mutant transfectants was then assayed for binding of the sLy49A tetramer (Fig. 5). Individual Ala substitution of the residues Arg6, Asp122 in the α1/α2 domain of H-2D^d, or Lys243 in the α3 domain completely abrogated the ability of H-2D^d to bind sLy49A tetramer, whereas substitution of Arg111 partially inhibited the binding. The rest of the mutants had similar capacities to bind the sLy49A tetramer as wild-type H-2D^d. The same panel of H-2D^d mutants was also tested for the ability to protect tumor cells from killing by Ly49A$^+$ LAK cells (Fig. 6). Introduction of R6A, D122A, or K243A mutation into H-2D^d completely abrogated the protective activity of H-2D^d against killing by Ly49A$^+$ LAK cells. None of the other mutations tested here significantly impaired the ability of H-2D^d to protect C1498 cells from killing by Ly49A$^+$ LAK cells (Fig. 6). Neither one of the H-2D^d mutants tested here nor wild-type H-2D^d protected C1498 cells from killing by Ly49A$^-$ LAK cells (data not shown). These results indicate that Arg6, Asp122, and Lys243 are essential for the physical binding of Ly49A and also for the functional binding of Ly49A that leads to inhibition of NK cell cytotoxicity. Importantly, these residues are involved in hydrogen bonding to Ly49A in one out of two binding sites in the crystal structure of the Ly49A/H-2D^d complex (Fig. 7; reference 21).

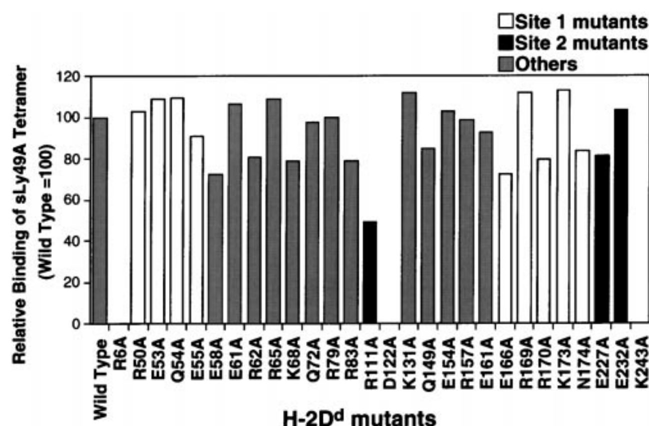

Figure 5. Binding of sLy49A tetramer to H-2D^d mutant transfectants. Each of the H-2D^d mutant transfectants was stained with sLy49A tetramer or streptavidin-PE alone. Binding of sLy49A tetramer to each H-2D^d mutant transfectant is expressed as relative value to wild-type H-2D^d transfectants as described in Materials and Methods. The mutants in site 1 and site 2 are represented by white and black bars, respectively. The other mutants and wild-type are represented by gray bars; R6A, D122A, and K243A mutations, which virtually disrupted sLy49A tetramer binding, are located in site 2. Definition for site 1 and site 2 followed that of Tormo et al. (21) and is shown in Fig. 7.

Discussion

Our data clearly indicate the functional Ly49A binding site on H-2D^d that is associated with inhibition of NK cell cytotoxicity. The crystal structure of the Ly49A/H-2D^d complex defined two possible binding sites for Ly49A on a single H-2D^d molecule (site 1 and site 2 in Fig. 7; reference 21). Site 2 spans the three structural domains that constitute the MHC class I molecule, α1/α2, α3, and β2m. Several new lines of evidence indicate that site 2, rather than site 1, is the functional binding site for Ly49A. (a) 34-5-8S antibody, which recognizes the α1/α2 domain, inhibits the interaction between Ly49A and H-2D^d (Figs. 1 and 2; reference 13). We recently mapped the epitope of 34-5-8S to a region containing Glu104 and Gly107 of the H-2D^d heavy chain (Matsumoto, N., W. Yokoyama, S. Kojima, and K. Yamamoto, manuscript submitted for publication), which is located in the neighborhood of site 2 (Fig. 7). (b) The anti-β2m^b antibody S19.8 completely inhibited the functional and physical interaction between Ly49A and H-2D^d (Fig. 1). S19.8 recognizes an epitope containing Ala85 and His34 of mouse β2m of b allele (38, 39), which is also juxtaposed to site 2 (Fig. 7). (c) To bind H-2D^d, Ly49A required a complex of H-2D^d with mouse β2m, but not with

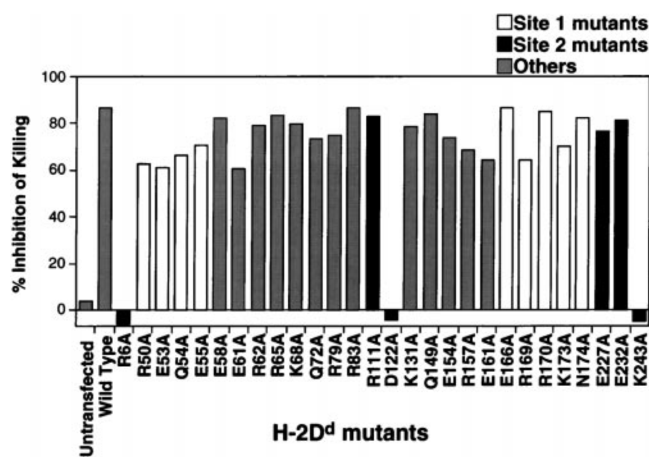

Figure 6. Inhibition of Ly49A$^+$ LAK cell–mediated killing of tumor cells by H-2D^d mutants. Each H-2D^d mutant transfectant was assayed for killing by Ly49A$^+$ LAK cells in the absence or presence of anti-Ly49A antibody or control antibody at an E/T ratio of 20:1. Inhibitory activity of each H-2D^d mutant is expressed as percent inhibition of target cell killing mediated by Ly49A in the formula: % inhibition of killing = (% specific cytotoxicity in the presence of the anti-Ly49A antibody A1 − % specific cytotoxicity in the absence of the antibody)/(% specific cytotoxicity in the presence of antibody 100×). Killing assay at an E/T ratio of 4:1 gave similar results (data not shown). Control antibody did not show any significant effect on killing by Ly49A$^+$ LAK cells (data not shown). The mutants in site 1 and site 2 are represented by white and black bars, respectively. The other mutants and wild-type are represented by gray bars.

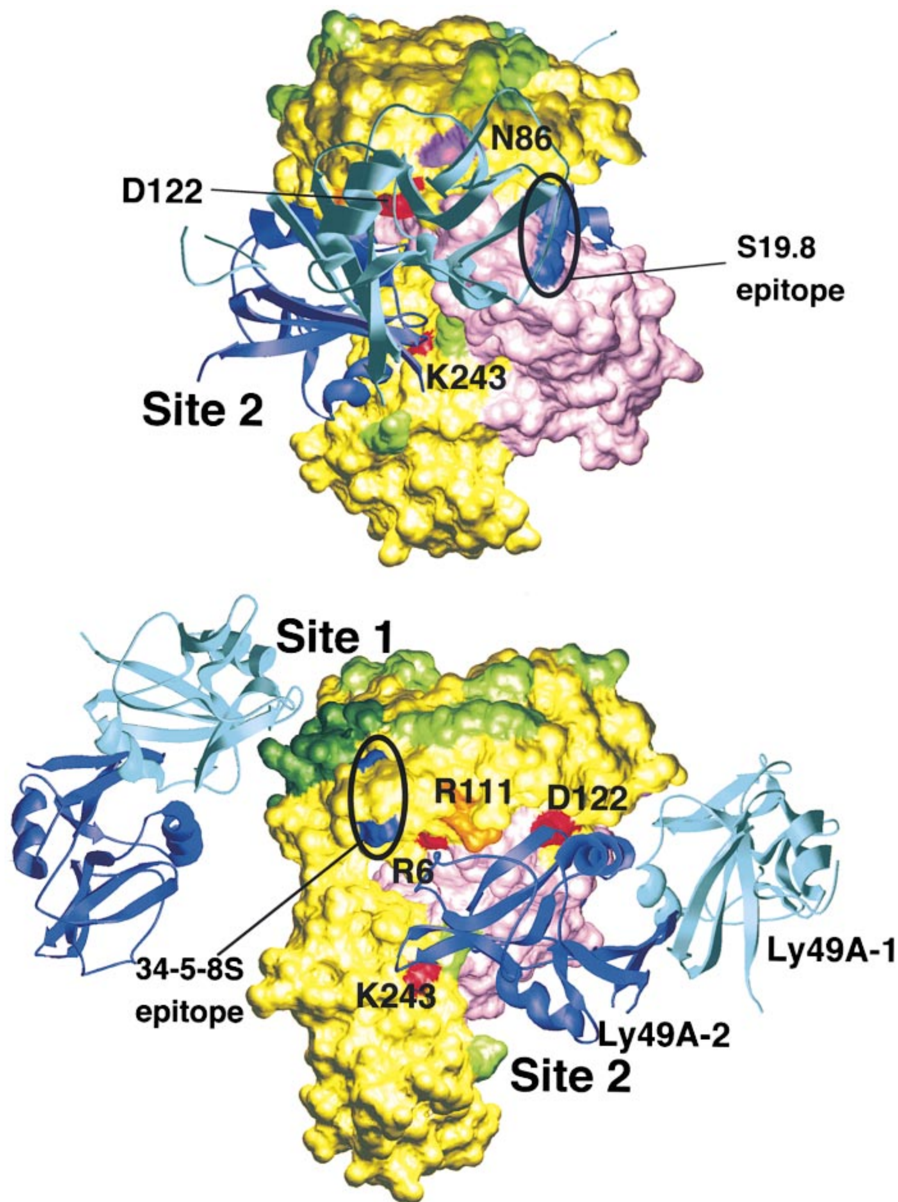

Figure 7. H-2D[d] mutations and antibody epitopes in the Ly49A/H-2D[d] complex. An MHC class I molecule and Ly49A molecules are depicted as molecular surface and ribbon diagrams, respectively (the coordinates were provided by Dr. D.H. Margulies, National Institutes of Health, Bethesda, MD). The bottom view is orthogonal to the top view. The molecular surface of H-2D[d] heavy chain and β2m are yellow and pink, respectively. The residues of H-2D[d] that affected Ly49A binding upon Ala substitution are red or orange and are labeled, whereas those that did not affect Ly49A binding upon Ala substitution are dark green (those in site 1) or light green (others). The residues involved in antigenic epitopes recognized by the anti–mouse β2m antibody S19.8 and the anti–H-2D[d] α1/α2 antibody 34-5-8S are blue. The glycosylation site Asn86 is magenta. The graphics image was prepared with Swiss-PdbViewer (reference 59).

human or bovine β2m (Figs. 1–3). On the other hand, H-2D[d] complexed with rat β2m can bind Ly49A as shown by Sundback et al. (40). β2m contributes 25% of the binding surface in site 2 of the Ly49A/H-2D[d] complex structure (21). Importantly, the surface of β2m has the species-specific residues found in mouse and rat β2m but not in human or bovine β2m (data not shown). In particular, in the crystal structure of the Ly49A/H-2D[d] complex, site 2 encompasses the side chains of Lys3, Gln29, and Lys58 of mouse β2m. Of these, Lys3 and Gln29 are replaced with Arg and Gly, respectively, in human and bovine β2m but are conserved in rat β2m, suggesting significance of Lys3 and Gln29 in species-specific contribution of β2m to the interaction between Ly49A and H-2D[d]. However, we cannot exclude the possibility that the replacement in other residues in human or bovine β2m forces H-2D[d] heavy chain to have a different conformation from H-2D[d] complexed with mouse or rat β2m. Mutation analysis is in progress to identify β2m residues that account for the species specificity. (d) Ala substituted mutation into any one of the residues of H-2D[d] heavy chain, Arg6 and Asp122 in α1/α2 and Lys243 in α3, completely abrogated the physical and the functional interaction between H-2D[d] and Ly49A (Figs. 5 and 6). Introduction of R111A mutation into α1/α2 of H-2D[d] partially inhibited the ability of H-2D[d] to bind Ly49A to such an extent that the effect was not evident in the functional protection assay (Figs. 5 and 6). Importantly, these four residues are involved in hydrogen bonding to Ly49A in site 2 in the crystal structure of the Ly49A/H-2D[d] complex (Fig. 7; reference 21). Interestingly, not all of the Ala substitution of the residues that putatively disrupt hydrogen bonds between Ly49A and H-2D[d] in site 2 resulted in loss in binding and function: E227A and E232A mutants of H-2D[d] were fully functional in

binding and protection from killing (Figs. 5 and 6). However, detailed examination of the crystal structure of the Ly49A/H-2D^d complex revealed that multiple hydrogen bonds are provided by each side chain of the residues of which Ala substitution completely (R6, D122, K243) or partially (R111) abolished the interaction between Ly49A and H-2D^d. By contrast, each side chain of the residues E227 and E232 forms only single hydrogen bond (data not shown). These differences could account for the observed absence in binding and functional effects of the E227A and E232A mutants. (e) Another possible Ly49A binding site on H-2D^d (site 1) was also revealed by the crystal structure of the Ly49A/H-2D^d complex (21). None of the Ala substitutions that were expected to disrupt hydrogen bonds between Ly49A and site 1 in the crystal structure showed any effect on sLy49A tetramer binding, or on the functional interaction of H-2D^d with Ly49A (Figs. 5–7). However, our data do not exclude the possibility that Ly49A interacts with H-2D^d through site 1 so weakly that the interaction was not detectable by sLy49A tetramer staining. The weak interaction through site 1 might be associated with cis interaction between Ly49A and H-2D^d on NK cells that leads to modulation of the Ly49A receptor as observed by Kåse et al. (43).

Collectively, our current results combined with the crystal structure of the Ly49A/H-2D^d complex (21) unambiguously indicate that the functional binding site of Ly49A lies in a concave region formed by the bottom of the $\alpha 1/\alpha 2$ and $\alpha 3$ domains, and $\beta 2$m. These results also explain the previous observation that a single chain H-2D^d molecule, in which $\beta 2$m is covalently linked to H-2D^d heavy chain through a peptide spacer, fails to interact with Ly49A (41). The peptide spacer is expected to cross in the middle of site 2, thereby interfering with the binding of Ly49A to site 2. Also, the current data provide a basis for the observation that the Ly49A binding site on MHC class I is distinct from the binding site for T cell receptor, which binds the top of the $\alpha 1/\alpha 2$ domain (42). Similarly, the identification of the binding site for Ly49A provides an understanding of other observations.

Functional binding of Ly49A to site 2 of H-2D^d well explains the observation that binding of Ly49A to H-2D^d requires the presence of peptide in the groove of H-2D^d $\alpha 1/\alpha 2$ domain (17, 18). In the crystal structure of the Ly49A/H-2D^d complex, the side chains of Thr238 and Arg239 of Ly49A-1, which has a smaller contact area with H-2D^d than the other Ly49A subunit Ly49A-2 (Fig. 7), are hydrogen bonded to main chain carbonyl oxygens of Tyr85 and Asn86, respectively, both of which are located in the COOH-terminal end of $\alpha 1$ α-helix (21). The side chain of Ser192 of Ly49A-1 is hydrogen bonded to the amide groups of Met138 and Ala139, both of which are in the NH_2-terminal end of $\alpha 2$ α-helix. Binding of a peptide to the peptide-binding groove of H-2D^d would bring two α-helices of H-2D^d to a position where Tyr85, Asn86, Met138, and Ala139 are available for hydrogen bonding to the residues in Ly49A-1. The notion that mutations in the peptide-binding groove of H-2D^d deteriorate the interaction between H-2D^d and Ly49A is remarkable in this context (Matsumoto, N., W. Yokoyama, S. Kojima, and K. Yamamoto, manuscript submitted for publication; references 45, 46). It is also noteworthy that the binding of Ly49C and Ly49I to H-2K^b and K^d, respectively, is restricted by the peptide bound to MHC class I (44, 47). Interestingly, position 7, which is proximal to the end of the groove, of H-2K^b–bound peptide is critically involved in the peptide specificity of Ly49C binding to H-2K^b (47). Provided that Ly49C binds to a similar site on H-2K^b as Ly49A functionally binds H-2D^d (site 2), the peptide bound to H-2K^b might affect the conformation of the end regions of the α-helices and thereby influence the binding of Ly49C.

The functional Ly49A binding site on H-2D^d is located beneath the $\alpha 1/\alpha 2$ domain (Fig. 7), and it partially overlaps the CD8 binding site (48, 49). CD8 has a stalk region of 30–50 residues, which is highly *O*-glycosylated (50), and its extended structure enables the unique Ig-like domain to reach MHC class I on the opposing target cell (49). Similarly, Ly49A has a stalk region of 68 residues, which connects the C-type lectin–like domain to the transmembrane domain and has three potential *N*-glycosylation sites (7, 8). *N*-glycosylation on these sites might keep the stalk region of polypeptide in an extended conformation to enable the C-type lectin–like domain of Ly49A to reach the recognition surface beneath the $\alpha 1/\alpha 2$ domain of H-2D^d. It is of note that two of the three *N*-glycosylation sites were highly conserved among other Ly49 members (data not shown).

H-2D^d has two *N*-glycosylation sites at Asn86 and Asn176. Results from investigations on the role of carbohydrate moieties in recognition of H-2D^d by Ly49A were controversial (20, 51, 52). However, it was established that Ly49A does not require carbohydrates on H-2D^d to interact with H-2D^d (20, 42). Because site 2 is located in the neighborhood of the *N*-glycosylation site at Asn86, the carbohydrate attached to this site might influence the Ly49A binding. We modeled H-2D^d with a high mannose-type glycochain on Asn86 by transplanting that from human CD2, of which dynamic structure including the carbohydrate moiety was determined by nuclear magnetic resonance (53) (Matsumoto, N., H. Iijima, and K. Yamamoto, unpublished data). The carbohydrate in any conformation found in CD2 is well accommodated in the interface of Ly49A at site 2. Moreover, the model raises the possibility that the carbohydrate might interact with the surface of Ly49A that corresponds to the carbohydrate-recognition surface found in typical carbohydrate-binding C-type lectins (54). This could account for the finding that optimal binding of H-2D^d–expressing cells to immobilized Ly49A is compromised by a sulfation inhibitor (51). The modeling of the H-2D^d with carbohydrate moieties on Asn86 also provides an insight into the stoichiometry of Ly49A binding to H-2D^d. From the model provided by the crystal structure of the Ly49A/H-2D^d complex, one Ly49A dimer could associate with two H-2D^d molecules. However, the carbohydrate modeled on Asn86 of H-2D^d

occupied the space where the H-2D^d molecule that interacts through site 1 is supposed to fill. Therefore, the stoichiometry of binding of the Ly49A dimer to the *N*-glycosylated H-2D^d is postulated to be one to one; when a single Ly49A dimer on NK cells binds H-2D^d on target cells via site 2, the same Ly49A molecule would not be able to interact with MHC class I on NK cells via site 1.

Ly49A distinguishes polymorphic MHC class I molecules (Matsumoto, N., K. Tajima, M. Mitsuki, and K. Yamamoto, manuscript submitted for publication; reference 44). However, the critical residues identified in this study are conserved among mouse MHC class I molecules, including H-2D^d and D^k, which are ligands for Ly49A, and H-2D^b, K^b, and K^d, which are not ligands for Ly49A (data not shown). Some of the polymorphic residues exposed on the surface of non-Ly49A ligand MHC class I that correspond to site 2 (data not shown) might determine the reactivity with Ly49A. In this context, we previously reported that NH_2-terminal halves of α1 and α2 regions of H-2D^d are critically important for the recognition of H-2D^d by Ly49A by analyzing H-2D^d/K^d chimeric molecules (20). Sundback et al. (40) also reported the inability of the H-2D^b α2 domain to support recognition of H-2D^d by Ly49A by exon shuffling between H-2D^d and D^b. Mutational studies on H-2D^d revealed that polymorphic residues inside and outside of the peptide-binding groove affect the recognition of H-2D^d by Ly49A (45, 46) (Matsumoto, N., W. Yokoyama, S. Kojima, and K. Yamamoto, manuscript submitted for publication). Of particular importance, we recently found that the substitution of polymorphic Asn30 of H-2D^d with Asp, which is found in non-Ly49A ligands K^b and K^d, partially abolished the functional as well as physical recognition of H-2D^d by Ly49A (Matsumoto, N., W. Yokoyama, S. Kojima, and K. Yamamoto, manuscript submitted for publication). The effect of the mutations in these sites may be conformational, since these residues identified by mutational studies are not found in the interface between Ly49A and H-2D^d that was functionally identified in this study (site 2). It should also be noted that a comparison of the crystal structure of MHC class I molecules revealed variation of the relative orientation of α3 domain or β2m to α1/α2 domain among mouse and human MHC class I molecules (36). Configuration of these domains might be critical for the interaction with Ly49A because Ly49A interacts with the surface of H-2D^d that spans the α1/α2 and α3 domains, and β2m in site 2 (Fig. 7). Therefore, the structural basis for MHC class I allele specificity of Ly49A remains to be examined in detail by site-directed mutagenesis.

The structure of the human Ig-like NK cell receptor KIR2DL2 in complex with its MHC class I ligand, HLA-Cw3, has been determined (55). Our analysis clearly demonstrated that Ly49A recognizes the surface of MHC class I that is distinct from the surface recognized by KIR2DL2. The interaction of KIR2DL2 with MHC class I is abrogated by individual amino acid substitutions of the residues in KIR2DL2 that disrupt hydrogen bonds between KIR2DLD and MHC class I (55). Similarly, the presence of hydrogen bonds critical for Ly49A/H-2D^d association was shown in our assays (Figs. 5 and 6). Thus, despite the structural difference and the difference in the binding sites on MHC class I, binding of the functionally similar receptors Ly49A and KIR2DL2 to MHC class I is critically mediated by hydrogen bonds and is very sensitive to individual disruption of hydrogen bonds.

The identification of the functional Ly49A binding site on H-2D^d provides a molecular basis for understanding the recognition of the MHC class I or related molecules not only by other members of the Ly49 family but also by other C-type lectin–like NK cell receptors, including HLA-E or Qa-1 recognition by CD94/NKG2A (56) and MIC-A, B, and RAE recognition by human and mouse NKG2D, respectively (57, 58). While our studies predict that other C-type lectins, such as CD94/NKG2A, may interact with MHC class I–related molecules in the same way, several findings suggest that there may be differences. CD94/NKG2A is a heterodimer of two related chains with unique C-type lectin–like domains, whereas Ly49A is a homodimer. CD94/NKG2A recognizes the nonclassical MHC class I molecule HLA-E (Qa-1 in mouse), whereas Ly49A recognizes the classical MHC class I molecules like H-2D^d and D^k. Recognition of the MHC class I ligand by CD94/NKG2A is dependent on the sequence of the MHC class I–bound peptide (59, 60), whereas Ly49A has no apparent specificity for MHC class I–bound peptide (17, 18). CD94 and NKG2A have relatively short stalk regions, 28 residues in human CD94 and 24 residues in human NKG2A, compared with Ly49A, which has a stalk region of 68 residues. One might argue that the short stalk region is not compatible with the idea that CD94/NKG2A interacts with the similar site on HLA-E as Ly49A functionally interacts with H-2D^d. However, the stalk regions with 24–28 residues are able to stretch for at least 8 nm in an extended conformation and would be capable of placing C-type lectin–like domains for CD94/NKG2A to bind the similar site on HLA-E. Biochemical as well as structural studies on the interaction between other members of the C-type lectin–like NK cell receptors and their ligands are needed to show whether the similar sites on MHC class I or its related molecules are used as receptor binding interface.

Structurally based studies such as this work together with the recently resolved crystal structures of the Ly49A/H-2D^d and the KIR2DLD/HLA-Cw3 complexes (21, 55) have unveiled the mode of recognition of MHC class I molecules by MHC class I–specific NK cell receptors of the two structurally different families. These studies are also important with respect to NK cell biology in general. The missing-self hypothesis predicts that NK cells monitor the expression of MHC class I molecules and kill the cells with aberrant expression of MHC class I associated with such events as tumorigenesis or infection (2). The structural studies suggest how NK cell receptors can sense the aberrant expression of MHC class I molecules, in addition to global loss of expression.

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