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Binding of the Natural Killer Cell Inhibitory Receptor Ly49A to Its Major Histocompatibility Complex Class I Ligand

CRUCIAL CONTACTS INCLUDE BOTH H-2D^d AND β_2 -MICROGLOBULIN*

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Jian Wang[‡], Mary C. Whitman[‡], Kannan Natarajan[‡], José Tormo[§], Roy A. Mariuzza[¶], and David H. Margulies^{‡||}

From the [‡]Molecular Biology Section, Laboratory of Immunology, NIAID, National Institutes of Health, Bethesda, Maryland 20892-1892, [§]Campus de la Universidad Autonoma de Madrid, 28049 Madrid, Spain, and [¶]Center for Advanced Research in Biotechnology, University of Maryland Biotechnology Institute, Rockville, Maryland 20850

Ly49A, an inhibitory C-type lectin-like mouse natural killer cell receptor, functions through interaction with the major histocompatibility complex class I molecule, H-2D^d. The x-ray crystal structure of the Ly49A·H-2D^d complex revealed that homodimeric Ly49A interacts at two distinct sites of H-2D^d: Site 1, spanning one side of the α 1 and α 2 helices, and Site 2, involving the α 1, α 2, α 3, and β_2 m domains. Mutants of Ly49A, H-2D^d, and β_2 -microglobulin at intermolecular contacts and the Ly49A dimer interface were examined for binding affinity and kinetics. Although mutations at Site 1 had little effect, several at Site 2 and at the dimer interface hampered the Ly49A·H-2D^d interaction, with no effect on gross structure or T cell receptor interaction. The region surrounding the most critical residues (in H-2D^d, Asp¹²²; in Ly49A, Asp²²⁹, Ser²³⁶, Thr²³⁸, Arg²³⁹, and Asp²⁴¹; and in β_2 -microglobulin, Gln²⁹ and Lys⁵⁸) of the Ly49A·H-2D^d interface at Site 2 includes a network of water molecules, suggesting a molecular basis for allelic specificity in natural killer cell recognition.

NK¹ cells provide a crucial arm of the innate immune system as they are poised to respond by cytotoxicity and lymphokine production to stimulation by neoplastic or infected cells (1–3). NK cell activation is regulated by a balance of activating and inhibitory signals delivered through receptors of the immunoglobulin and C-type lectin-like superfamilies, that recognize MHC-I or MHC-I-like molecules expressed on target cells (4). In resting NK cells the inhibitory signals dominate, and perturbation of normal expression of MHC-I, by viral infection or oncogenic dysregulation, leads to attenuation of the inhibitory signal and augmentation of the activating one. The best understood inhibitory NK receptor of the C-type lectin family is murine Ly49A, a type II membrane homodimer in which the

structure in complex with the MHC-I ligand, H-2D^d, has been determined crystallographically (5). Direct interaction of Ly49A with H-2D^d has been demonstrated in cell adhesion and protein binding assays (6–9). In addition, biotinylated Ly49A has been employed in staining the naturally expressed H-2D^d ligand on normal cells (10, 11). Labeled tetrameric MHC-I molecules including H-2D^d bind specifically to various Ly49-expressing cells (12, 13). Although Ly49A is a member of the C-type lectin superfamily, Ly49A-mediated recognition is Ca²⁺- and carbohydrate-independent and is MHC-I-restricted but not peptide-specific (9, 14–16).

The crystal structure of the Ly49A·H-2D^d complex (5) indicated that the Ly49A homodimer interacts with two topographically distinct sites on H-2D^d (Fig. 1a). At Site 1, a single Ly49A subunit contacts one end of the MHC-I peptide-binding groove, involving the amino terminus of the α 1 helix and the carboxyl terminus of α 2. Site 2 is a concavity beneath the peptide-binding platform, bounded by amino acid residues of the floor of the β sheet of the α 1 and α 2 domains, as well as by the α 3 domain and the MHC-I light chain, β_2 m. This site overlaps the region where CD8 $\alpha\alpha$ interacts with MHC-I (5). In attempts to understand the details of the interaction of Ly49 receptors with their MHC-I ligands, several groups have analyzed binding and recognition using cells transfected with recombinant Ly49 molecules (17, 18), MHC-I mutants in transfected cells (16, 19–22), and binding of recombinant Ly49A to cells derived from different mouse inbred strains (10, 11). These studies suggest that amino acids in the “neck” region of Ly49A (17) or Ly49C (18), residues of both Site 1 of H-2D^d (10) and Site 2 (22), as well as of its peptide-binding groove (20, 21) may all contribute either directly or indirectly to the Ly49A/MHC-I interaction. In addition, two groups have reported the effects of human β_2 m on the Ly49A/MHC-I interaction (22, 23). Thus, although some evidence supports Site 1 as a primary region of interaction between MHC-I and Ly49A, other experiments are more consistent with Site 2 playing the predominant role, and some studies are inconclusive.

To resolve the issue as to whether Site 1 or Site 2 plays the major role, we have exploited a well defined biochemical system using only recombinant, highly purified molecules. Using a panel of site-directed mutants of Ly49A, H-2D^d, and β_2 m in binding assays, we have determined kinetic and equilibrium parameters quantitatively. These data, viewed in the context of the crystal structure of the Ly49A·H-2D^d complex, define not only the dominant site of interaction of Ly49A with its MHC-I ligand as Site 2, but also provide insight into the importance of homodimeric interaction in maintaining the integrity of Ly49A. Inspection of the region of intermolecular contact at Site 2

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This paper is dedicated to the memory of José Tormo, colleague and friend.

|| To whom correspondence should be addressed: Molecular Biology Section, LI/NIAID, Bldg. 10, Rm. 11N311, National Institutes of Health, Bethesda, MD 20892-1892. Tel.: 301-496-6429; Fax: 301-496-0222; E-mail: dhm@nih.gov.

¹ The abbreviations used are: NK, natural killer; β_2 m, β_2 -microglobulin; mAb, monoclonal antibody; MHC, major histocompatibility complex; TCR, T cell receptor; scTCR, single-chain T cell receptor; HIV, human immunodeficiency virus; MES, 2-(N-morpholino)ethanesulfonic acid.

reveals important contributions from a network of water molecules coordinated by Ly49A, H-2D^d, and β_2m residues and helps to explain the allelic specificity and peptide preference that particular members of the Ly49 family exhibit.

EXPERIMENTAL PROCEDURES

Mutagenesis of Ly49A, H-2D^d, and Mouse β_2m —Mutants were generated by site-directed mutagenesis of the codons of interest to encode alanine substitutions. Using Stratagene's QuickChange Mutagenesis Kit and following the manufacturer's instructions, mutations were introduced into: 1) a cDNA construct encoding the entire extracellular region (residues 67–262) of the C57BL/6 allele of Ly49A in a bacterial expression vector (pET21a; Novagen) (9); 2) a cDNA construct encoding the mature extracellular residues of H-2D^d in pET3a (24); or 3) a murine β_2m -encoding construct based on the C57BL/6 allele of β_2m in pET21d (25). Mutations were confirmed by automated DNA sequencing analysis using an ABI 377 DNA sequencer and accompanying sequencing analysis software (PerkinElmer Life Sciences). *Escherichia coli* strain BL21(DE3) was transformed with these mutant plasmid DNAs for protein expression.

Protein Expression—Bacteria harboring either the parental or mutant Ly49A-, H-2D^d-, or β_2m -encoding plasmid vectors were cultured in LB broth containing 0.25 mg/ml carbenicillin and, following induction of exponentially growing cells for 2 h with isopropyl-1-thio- β -D-galactopyranoside, were lysed with 0.5 mg/ml of hen egg white lysozyme overnight at 4 °C and then with 0.1% deoxycholate for 30 min. The resulting inclusion bodies were washed first with 0.1% deoxycholate in TE buffer (0.1 M Tris pH 8.0, 2 mM EDTA) and then extensively with TE, and they were then denatured in 6 M guanidine hydrochloride and reduced with 0.1 M dithiothreitol. Ly49A was refolded following dilution into an arginine buffer (0.4 M L-arginine-HCl, 0.1 M Tris, pH 8, 2 mM EDTA, 3 mM reduced glutathione, and 0.3 mM oxidized glutathione). Following dialysis against 25 mM MES, pH 5.5, protein was further purified by ion exchange chromatography (Mono-S) followed by gel filtration on a Superdex 75 column. H-2D^d was prepared similarly, with refolding taking place in the presence of mouse β_2m and either a motif (AGPARAAAL) peptide (26) or the HIV IIIB envelope gp160 peptide, P18-I10 (RG-PGRAFVTI) (27, 28). Refolded proteins were further purified by size exclusion chromatography on Superdex-75 (Amersham Biosciences, Inc.).

Monoclonal Antibodies and Recombinant Protein Ligands—Monoclonal antibodies used in BIAcore binding assays were: A1, specific for Ly49A^{C57BL/6} (29, 30), YE1–32, and YE1–48 (derived in rat) (31, 32), and JR9-318 (derived from *Mus spretus*) (33), which binds all allelic forms of Ly49A. The anti-Ly49G2 mAb 4D11 is described by Mason *et al.* (34). Anti-Ly49C/I-reactive SW5E6 (35) was used as control for Ly49A binding. mAbs 34-2-12 (anti-H-2D^d $\alpha 3$ domain) and 34-5-8 (anti-H-2D^d $\alpha 1/\alpha 2$ domain) (36, 37) were from PharMingen (San Diego, CA). KP15, a mAb specific for H-2D^d bound to the HIV envelope glycoprotein-derived peptide Pro¹⁸-Ile¹⁰, has been described elsewhere (38). All antibodies were used as purified proteins. A single-chain TCR (scTCR) with specificity for H-2D^d/Pro¹⁸-Ile¹⁰ was expressed in *E. coli* and purified as described previously (39).

Surface Plasmon Resonance Binding Assays—Assays for binding of Ly49A, H-2D^d, and mAbs were based on kinetic progress curves obtained with the BIAcore 2000 using methods employed previously (9, 40). In general, one ligand (either a mAb, Ly49A, or scTCR) was immobilized on a CM5 biosensor chip using standard coupling procedures, and solution phase ligand (analyte) was offered at a flow rate of 10 μ l/min. All binding experiments were performed at 25 °C. A mock-coupled surface that was activated and blocked but had no protein immobilized was always included as a control for nonspecific binding. When Ly49A mutants were evaluated, a Ly49A wild type-coupled surface was included on the same chip for direct comparison. Kinetic data were collected and analyzed using BIAevaluation 3.1 to calculate kinetic association and dissociation rate constants and equilibrium constants for dissociation. In all cases the potential for sequential deterioration of the integrity of the solid phase ligand was evaluated periodically with appropriate controls introduced throughout the run. Binding parameters obtained by kinetic analysis were compared with equilibrium parameters calculated in parallel and were always internally consistent.

RESULTS

Interface Mutants of the Ly49A Homodimer—To evaluate the contribution of particular residues of Ly49A to recognition by H-2D^d, as well by specific monoclonal antibodies (mAbs), we

made individual alanine substitutions at those positions that, in the crystal structure, appeared crucial to either the homodimeric interaction or the interaction between H-2D^d and Ly49A (Fig. 1). Six Ly49A interface residues, Tyr¹⁴², Trp¹⁴³, Phe¹⁴⁴, Tyr¹⁴⁶, Leu¹⁸⁸, and Val¹⁸⁹ were mutated, and the bacterially expressed mutant proteins were tested for binding to bacterially expressed H-2D^d as well as to a panel of anti-Ly49 mAbs. Although several mutants showed decreased binding to some of the mAbs tested, all preserved the capacity to interact with several at levels at least 50% that of controls (Fig. 2a). Binding to 4D11, which primarily recognizes Ly49G2 and has weak activity on Ly49A, was lower for mutants at positions 142, 143, 146, and 189. Interestingly, 4D11 reactivity was greater for mutants at positions 144 and 188. Significantly, mutation at positions 142, 143, 146, and 189 each revealed decreased binding to either of the H-2D^d preparations (Fig. 2b). These results suggest that the integrity of the homodimer is important for binding to the MHC-I ligand and also influences binding by some mAbs.

Site 2 and Site 1, 2 Mutants of Ly49A—Next, we examined mutants of Ly49A at H-2D^d contact sites. However, no contact residues of Ly49A are unique to Site 1, and therefore any mutation would either include both Sites 1 and 2 or be unique to Site 2. We first studied mutants at Site 2, the interface between Ly49A and H-2D^d that involves Ly49A and all three domains of H-2D^d as well as its light chain, β_2m (Fig. 1a). For H-2D^d binding, point mutations generally either improved binding (N203A and K224A) or impaired binding significantly (S236A, T238A, and R239A). S236A and R239A showed an undetectable level of binding to H-2D^d, and T238A quantitatively reduced binding by about 9-fold (K_D increasing from 1.82 to 15.9 μ M largely as a result of k_d increasing from 0.063 to 0.363 s⁻¹ (Fig. 3b)). The behavior of the triad of Ser²³⁶, Thr²³⁸, and Arg²³⁹ clearly indicates the importance of Site 2 interactions in H-2D^d binding. Binding to a panel of mAbs indicated that the overall integrity of the mutants was preserved and also allowed mapping of epitopes recognized by particular mAbs (Fig. 2). Mutants of N203A, K224A, S236A, T238A, and R239A revealed differences in binding to some of the mAbs. In particular, N203A showed augmented binding to A1, YE1/32, and YE1/48, had no effect on binding to JR9-318, and showed absolutely no binding to 4D11. K224A reduced binding to A1, YE1/48, and JR9-318 and increased binding to YE1/32. Mutants S236A, T238A, and R239A modestly decreased binding to A1, and R239A increased binding to YE1/32, YE1/48, and 4D11.

We then analyzed seven mutants that represented contacts at both Sites 1 and 2: D229A, D241A, N242A, D246A, Q247A, V248A, and F249A (Figs. 1–3). D229A, although it revealed little or no effect on binding of any of the mAbs, eliminated interaction with H-2D^d. D241A, which profoundly reduced binding to A1 but had little effect on any other mAb, also affected binding to H-2D^d, most significantly with an increase in the k_d from 0.054 to 0.363 s⁻¹ (Fig. 3). N242A modestly reduced binding to YE1/32 and 4D11 and also impeded interaction with H-2D^d. D246A, which adversely affects binding of YE1/32 and 4D11, also showed quantitative effects on binding to H-2D^d. Other mutations of residues that structurally contribute at both Sites 1 and 2, Q247A and F249A, did not impede mAb binding and augmented it in some cases. V248A impaired recognition by YE1/32. These three mutants showed quantitative effects on binding to H-2D^d/P18-I10 but less pronounced effects when assayed with H-2D^d-motif complexes. The most consistent of the effects observed was with F249A, which showed a slight increase in k_a , a marked (more than 5-fold) increase in the k_d , and a 5-fold increase in K_D (Fig. 3b).

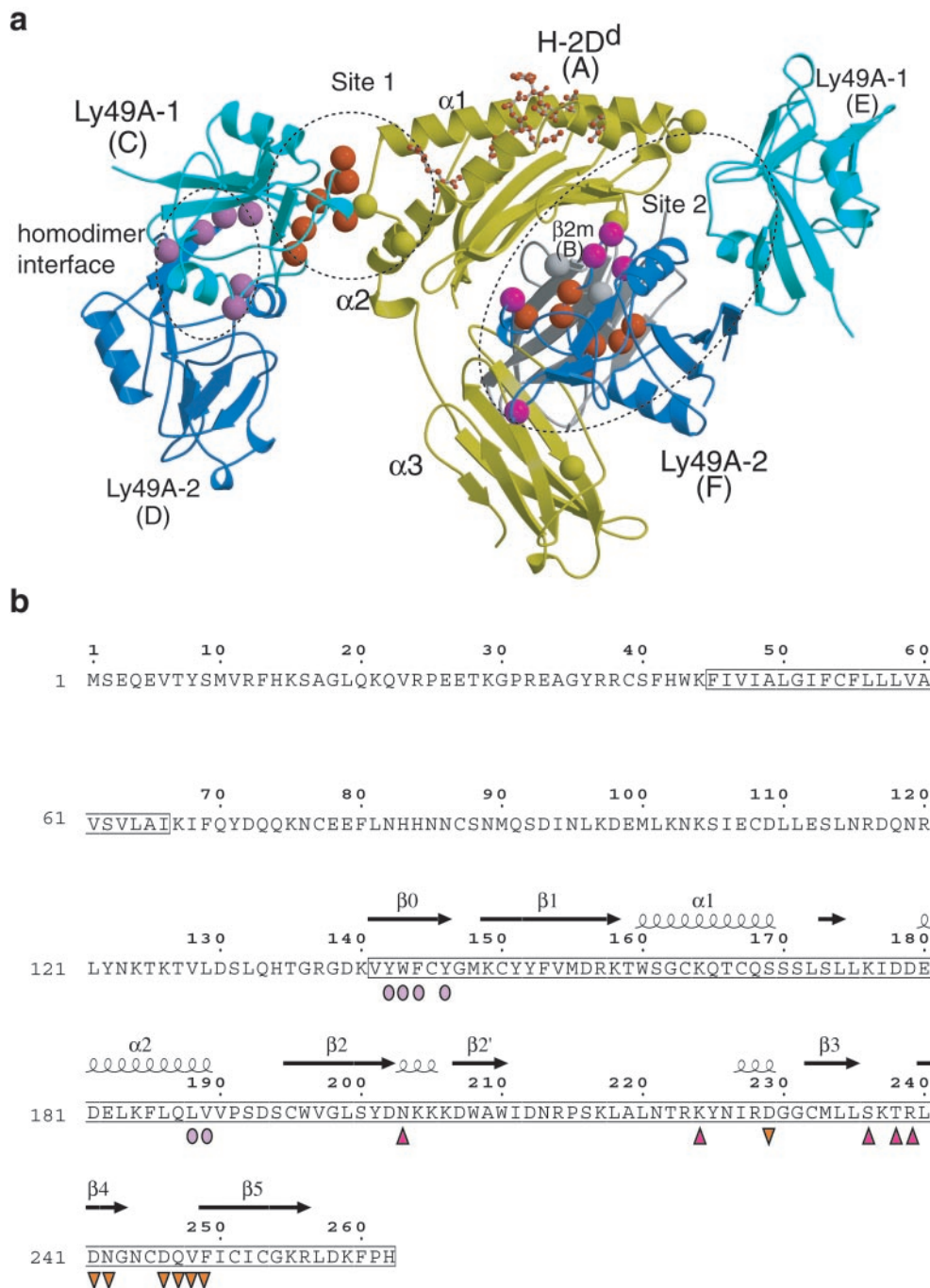


FIG. 1. Location of contacts between Ly49A and H-2D^d. *a*, a ribbon diagram of the structure of the Ly49A-H-2D^d complex (Protein Data Bank code 1qo3) showing sites of interaction. These are referred to as *Site 1*, *Site 2*, and *Ly49A homodimer interface* (labeled only on one dimer). The subunit designations are: A, H-2D^d heavy chain; B, $\beta 2m$; C, Ly49A subunit at Site 1; D, dimeric partner of C; E, symmetry-related Ly49A subunit C; F, major Ly49A subunit interacting at Site 2. The α atoms of residues of the homodimer interface (orchid), Site 2 alone (magenta), and Sites 1 and 2 (orange-red) are indicated as space-filled spheres. *b*, Ly49A amino acid sequence and location of residues selected for mutagenesis. The amino acid sequence of Ly49A^{C57BL/6} is shown with the transmembrane region (amino acids 45–66) and the lectin-like domain (amino acid 141–262) enclosed in boxes. Secondary structure elements are labeled, and the positions of site-directed mutants are indicated: interface contacts of the homodimer (filled circles, orchid), Site 1 and Site 2 (orange-red downward arrows), and Site 2 unique (magenta upward arrows).

Surface Representation of Ly49A Site 2 and Site 1, 2 Mutant Effects—To visualize the location of those Ly49A residues that influence H-2D^d and mAb interaction, we generated a surface representation of their effect on binding (Fig. 4; Ly49A interface mutants are not seen in such a display). Mutations that adversely affected binding are colored in red, orange, yellow, and green, whereas those that improved binding, presumably by eliminating a negative effect, are depicted in light blue and blue. The residues that most affected A1 binding (Lys²²⁴,

Asp²⁴¹, and Arg²³⁹) form a contiguous patch on the surface of the molecule (Fig. 4a). Residues Asp²²⁹, Ser²³⁶, and Thr²³⁸ neighbor this region, and it is straightforward to imagine that the antibody-combining site of A1 could overlay this set of residues on one monomer of the Ly49A homodimer. A1 is an alloantibody raised in BALB/c mice against Ly49A^{C57BL/6} (29). Allelic differences between Ly49A^{BALB/c} and Ly49A^{C57BL/6} in the region where the Ly49A^{C57BL/6} structure is known are at positions 187 and 238 (where Ly49A^{C57BL/6} has Gln and Thr,

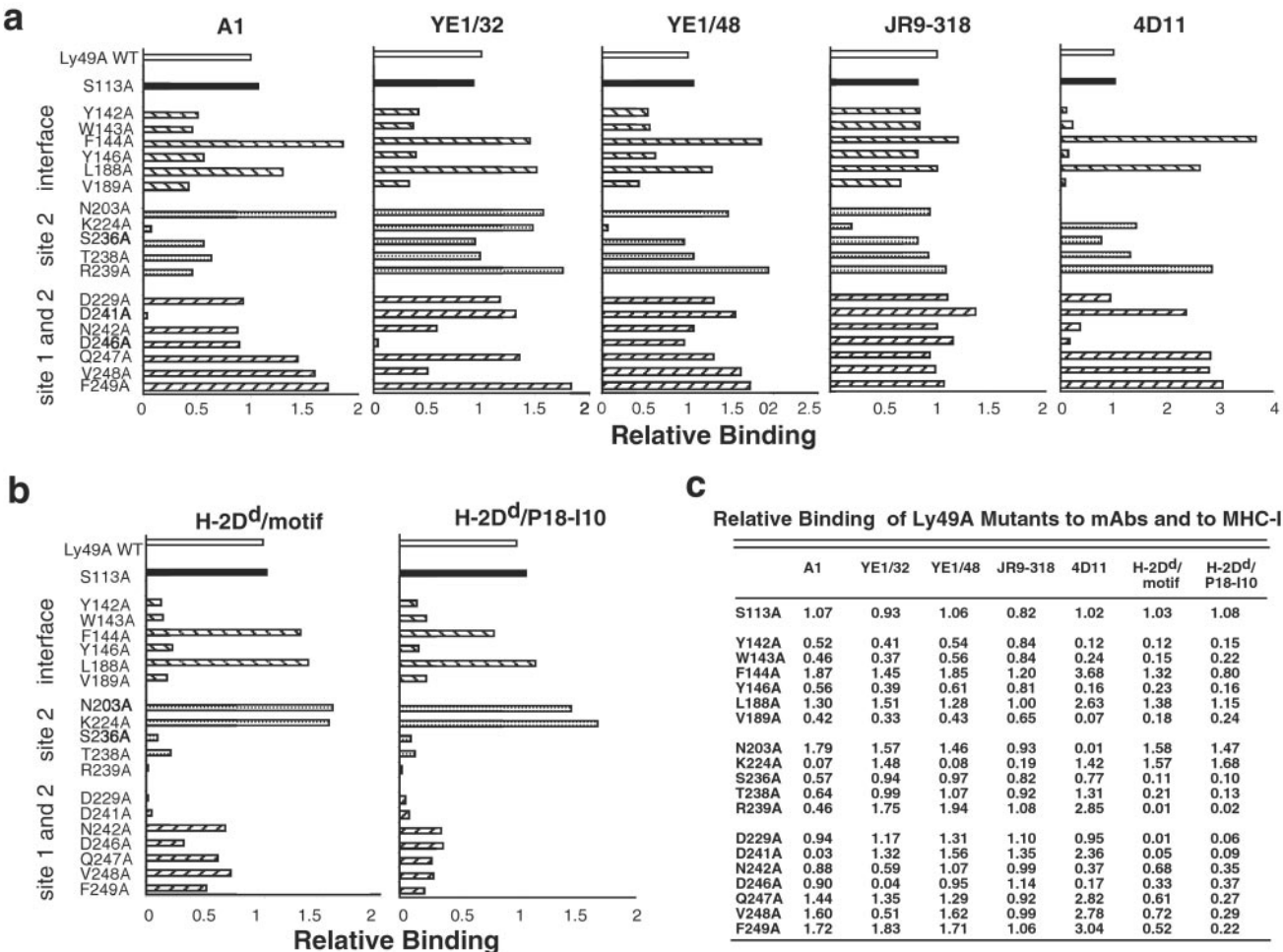


FIG. 2. Binding of mAbs and H-2D^d to Ly49A and its site-directed mutants. *a*, from 4200 to 6000 resonance units of Ly49A and the indicated mutant proteins were coupled to biosensor chips and tested for binding to different specific mAbs. *b*, Ly49A mutants were tested for binding to H-2D^d/motif and H-2D^d/Pro¹⁸-Ile¹⁰ complexes at a concentration of 6.5 μ M. The *Relative Binding*, corrected for background binding to a mock-coupled surface and for level of coupling of the solid phase reagent, was compared with binding to a wild type Ly49A surface and was calculated as follows.

$$RB = \frac{((RU_{\text{bound to Ly49A mutant}} - RU_{\text{bound to mock}})/RU_{\text{Ly49A mutant coupled}})}{((RU_{\text{bound to Ly49A WT}} - RU_{\text{bound to mock}})/RU_{\text{Ly49A WT coupled}})}$$

Resonance unit (*RU*) levels are at steady state. Relative binding (*RB*) of Ly49A wild type therefore = 1. *Open bar*, Ly49A wild type (*WT*); *solid bar*, control mutant S113A; *back-slashed bar*, interface mutants; *gray bar*, Site 2 mutants alone; *forward slashed bar*, Site 1 and Site 2 mutants. *c*, relative binding of Ly49A mutants to mAbs and to H-2D^d was calculated as described above. *JR9-318*, a mAb that binds several Ly49 molecules, was derived from *M. spretus*, and we might expect the Ly49 molecules of this strain to lack Lys²²⁴. We also tested SW5E6, which recognizes Ly49C and Ly49I but not Ly49A (54). In surface plasmon resonance analysis SW5E6 failed to bind Ly49A and also showed no binding to the full panel of Ly49A mutants (data not shown).

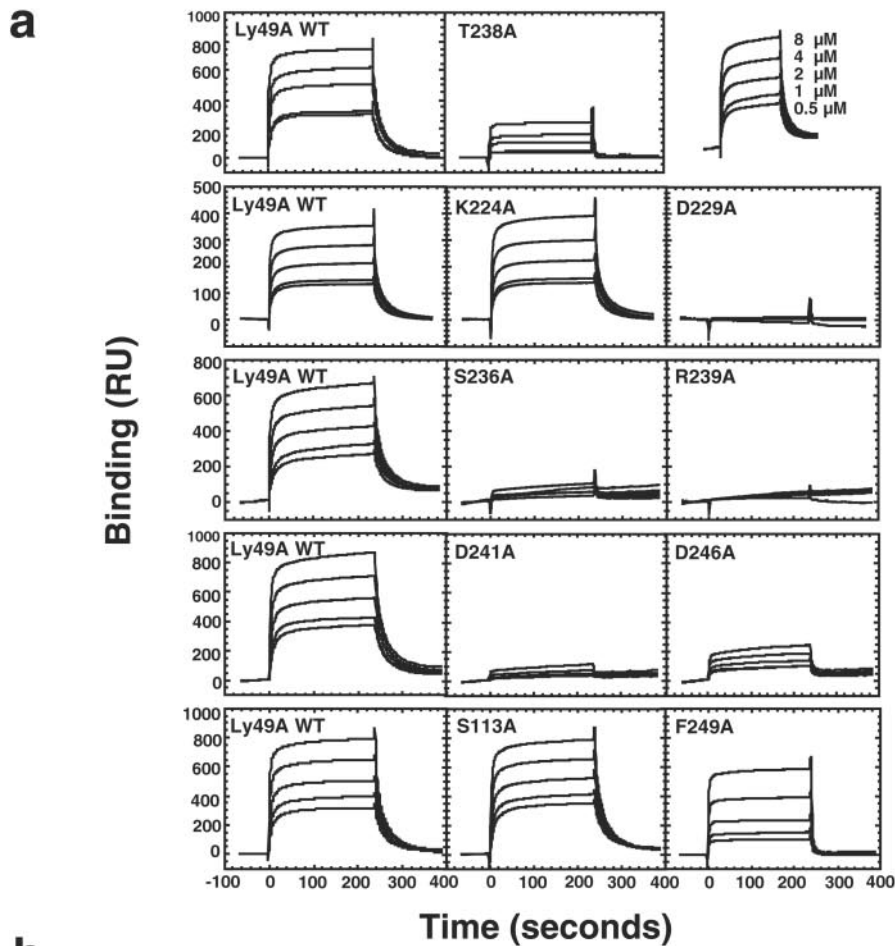
respectively, and Ly49A^{BALB/c} has His and Ile). Residue 187 lies adjacent to residue 238 on the surface of Ly49A. In contrast to those residues in which mutation adversely affected the binding of A1, mutants N203A, Q247A, V248A, and F249A improved binding by A1.

The binding of three other antibodies, YE1/48, YE1/32, and JR9-318, to the panel of mutants reveals different characteristics. The only mutation that adversely affects binding of YE1/48 is K224A (Fig. 4c); YE1/32 is most affected by D246A (Fig. 4d); and JR9-318 is most dependent on K224 (Fig. 4f). These results suggest that YE1/32 interacts more with residues on the “side” of the Ly49A molecule than with those on the “top.”

The surface region that most affects H-2D^d binding is a large area that extends from Asp²⁴⁶ across the top of the molecule that includes Gln²⁴⁷, Val²⁴⁸, and Phe²⁴⁹ as well as a distinct surface consisting of Asp²²⁹, Ser²³⁶, Thr²³⁸, Arg²³⁹, Asp²⁴¹, and Asn²⁴² (Fig. 4, *b* and *e*). In addition to the adverse effects of

mutation of any of the residues in this patch, mutation of Asn²⁰³ and Lys²²⁴ to alanine improves the binding to H-2D^d molecules. Thus, the focus of H-2D^d binding is on Arg²³⁹ and the surrounding residues located along the top of Ly49A.

The Importance of Site 2 Mutants of H-2D^d and β_2m —Although the behavior of Ly49A mutants S236A, T238A, and R239A strongly suggested that Site 2 was the major focus of the Ly49A/H-2D^d interaction, other data, including the analysis of polymorphic MHC-I residues in different mouse strains and the behavior of a single H-2D^d mutation of I52M, had suggested that Site 1 was important (10). To clarify this issue, we tabulated the number of atomic contacts between those residues that seemed to be most involved in binding to H-2D^d (Table I). We looked closely at the contacts made by Ly49A residues Asp²²⁹, Ser²³⁶, Thr²³⁸, Arg²³⁹, Asp²⁴¹, Asn²⁴², Gln²⁴⁷, Val²⁴⁸, and Phe²⁴⁹ at either Site 1 or Site 2. For Site 1 contacts, Gln²⁴⁷, Val²⁴⁸, and Phe²⁴⁹ made 18 atomic contacts with residue Gln⁵⁴ of H-2D^d, and residue Asp²²⁹ made 10 contacts with H-2D^d



	k_a (M ⁻¹ s ⁻¹)	k_d (s ⁻¹)	$t_{1/2}$ (s)	K_D (μM)	χ^2
Group 1:					
Ly49A WT	3.45e ⁴	0.063	11.04	1.82	90.9
T238A	2.29e ⁴	0.363	1.91	15.9	35.5
Group 2:					
Ly49A WT	3.26e ⁴	0.066	10.58	2.01	19.5
K224A	2.71e ⁴	0.058	11.97	2.13	21.3
D229A	ND	ND	ND	ND	ND
Group 3:					
Ly49A WT	2.63e ⁴	0.052	13.28	1.99	58.5
S236A	ND	ND	ND	ND	ND
R239A	ND	ND	ND	ND	ND
Group 4:					
Ly49A WT	3.8e ⁴	0.054	12.83	1.42	116.0
D241A	1.51e ⁴	0.363	1.91	24.1	1.5
D246A	2.13e ⁴	0.135	5.13	6.31	14.7
Group 5:					
Ly49A WT	3.72e ⁴	0.062	11.16	1.67	101.0
S113A	3.12e ⁴	0.059	11.83	1.88	171.0
F249A	4.22e ⁴	0.358	1.94	8.46	20.9

FIG. 3. H-2D^d binding to Ly49A mutants. *a*, H-2D^d-motif and H-2D^d-P18-I10 were injected at five different concentrations ranging from 0.5 to 8 μM in 2-fold increments over biosensor surfaces coupled with different Ly49A mutants. Each row represents a binding assay performed on different flow cells of the same CM5 chip. Because the binding patterns of H-2D^d-motif and H-2D^d-Pro¹⁸-Ile¹⁰ preparations were similar, but the level of H-2D^d-Pro¹⁸-Ile¹⁰ binding was lower, we show only H-2D^d-motif-binding curves. *b*, experimental sensorgram data were obtained from binding curves of experiments similar to *panel a*, and kinetic evaluation was performed using BIAevaluation 3.1. Analysis employed a 1:1 binding model that included a correction for drifting base line. The different groups represent five independent experiments using different biosensor chips. $t_{1/2}$ was calculated according to the relationship $t_{1/2} = 0.693/k_d$. ND, nondetectable; WT, wild type.



To explain the profound effects exerted by mutation of H-2D^d

TABLE I
Atomic contacts between Ly49A and H-2D^d residues

The number of atomic contacts between the indicated amino acids was tabulated from the crystal structure of Ly49A-H-2D^d using QUANTA 2000 (Molecular Simulation, Inc.).

Ly49A residue ^a	H-2D ^d residue	Total contacts	Sites
F:229	A:6	6	2
F:229	A:30	1	2
C:248	A:53	1	1
C:247(6), C:248(8), C:249(4)	A:54	18	1
C:247	A:55	3	1
C:246(2), C:248(1)	A:56	3	1
E:238(7), E:239(1)	A:85	8	2
E:239(8), E:241(2)	A:86	10	2
E:238	A:87	1	2
F:241	A:111	4	2
F:239	A:115	3	2
F:236(6), F:238(7), F:239(10)	A:122	23	2
F:238	A:123	1	2
F:238(3), F:239(1)	A:125	4	2
F:238	A:134	5	2
F:238	A:135	2	2
F:238	A:136	2	2
C:229(10)	A:169	10	1
C:246(3), C:247(4)	A:170	7	1
C:241(4), C:242(3)	A:173	7	1
C:247	A:174	3	1
F:246	A:230	1	2
F:247	A:232	6	2
F:246	A:243	2	2
F:248	B:6	2	2
F:247(9), F:248(9), F:249(3)	B:29	21	2
F:229(7), F:239(3), F:241(8), F:242(2)	B:58	20	2
F:249	B:59	2	2
F:239	B:60	4	2

^a Uppercase letters represent the different domains of the MHC-Ly49A complex: A = H-2D^d; B = mouse β_2m ; C, D, E, and F = Ly49A monomer. C and D form the Ly49A homodimer at Site 1; E and F form Ly49A homodimer at Site 2. Numbers in parentheses represent the number of contacts contributed by the indicated residue. Boldface is used to emphasize those positions that make the greatest number of contacts.

D122A, as well as β_2m Q29A and K58A, we returned to a close inspection of the interface of H-2D^d with Ly49A at Site 2 (Fig. 7). In particular, we have included the water molecules at this interface. The region that surrounds residue Asp¹²² of H-2D^d reveals not only the proximity of the side chains of residues Ser²³⁶, Thr²³⁸, and Arg²³⁹ of Ly49A but also at least seven water molecules that are involved in this interface (Fig. 7b). The β_2m residue Lys⁵⁸, which is highly conserved among species, interacts with Asp²²⁹, Asp²⁴¹, and Asn²⁴² of Ly49A amid a network of water molecules that also coordinate with the side chain of H-2D^d residue Arg⁶ as well as Arg²³⁹ (Fig. 7c). Continuing this general trend, β_2m residue Gln²⁹ (the Gln is unique to mouse β_2m) not only interacts with its contact residues Gln²⁴⁷, Val²⁴⁸, and Phe²⁴⁹ on Ly49A but also interacts with a number of waters in this vicinity (Fig. 7d).

DISCUSSION

Our mutational analysis of the binding of Ly49A, H-2D^d, and β_2m mutants leads to two major conclusions: that the primary binding site of H-2D^d employed by Ly49A is at Site 2, a large surface area that involves contacts from the floor of the peptide-binding groove of the MHC-I and residues from β_2m ; and that integrity of the Ly49A homodimer is critical for preservation of MHC-I binding. The effect of mutation of the homodimeric interface residues Tyr¹⁴², Trp¹⁴³, Tyr¹⁴⁶, and Val¹⁸⁹ in reducing binding to H-2D^d as well as the augmentation of binding by mutations of Phe¹⁴⁴ and Leu¹⁸⁸ suggest that the structure of the surface that contacts H-2D^d is influenced by the nature of the homodimerization. These results are of particular importance when we consider the three-dimensional

structure and mode of dimerization of NK receptors and related molecules. As found previously (41), the dimer interfaces of Ly49A, NKG2D, and CD69 are highly conserved, although in Ly49A it is somewhat asymmetric, whereas NKG2D and CD69 show clear symmetry. The recently determined structure of Ly49I indicates that even within the Ly49 family there is considerable flexibility in the dimerization, because in this molecule the $\alpha 2$ helix is not involved in the dimer interface.² These NK receptor domains dimerize differently from other more distantly related members of the C-type lectin family such as tunicate lectin and coagulation factor IX binding protein. In an evaluation of the binding of Ly49C^{C57BL/6} and Ly49I^{C57BL/6} to various MHC-I molecules in a cell agglutination assay, Lian *et al.* (18) observed that mutation of Tyr¹⁴⁶ (the position equivalent to Tyr¹⁴² of Ly49A) of Ly49C to the histidine found in the closely related Ly49I resulted in reduced binding to H-2^s and H-2^b cell lines as well to an H-2D^d transfectant. These results suggest that variability in the mode of dimerization influences allelic specificity of Ly49 family members.

The importance of Site 2 is indicated by the effect of mutations in Ly49A unique to Site 2 (in particular residues Ser²³⁶, Thr²³⁸, and Arg²³⁹) as well as by mutation of H-2D^d (Asp¹²²) (Fig. 7b) and β_2m (Gln²⁹ and Lys⁵⁸ (Fig. 7, d and c)). Ly49A is unique among NK receptors of either the Ig-like or C-type lectin-like families in its utilization of the β_2m subunit as part of its binding site. A mutational analysis of H-2D^d, based on transfection and agglutination and functional assays, indicated an important role for residues Arg⁶, Asp¹²², and Lys²⁴³ of H-2D^d (22), and the importance of species differences in β_2m has been reported (22, 23). Our results add unequivocal data indicating the critical importance of the two major contact residues of β_2m . This analysis also implicates β_2m as a spatial transducer of subtle structural changes that occur either in the peptide-binding groove or in other parts of the $\alpha 1\alpha 2$ unit of the MHC heavy chain. The disposition of β_2m with respect to the heavy chain varies markedly in different crystal structures (24, 43, 44), and the stability of the MHC/ β_2m interaction is affected by amino acid substitutions in the peptide-binding groove (45). The exquisite sensitivity of Ly49A binding to substitution of either of the two major β_2m contact residues, Gln²⁹ or Lys⁵⁸, explains the observed effects of substitution of bovine or human β_2m in NK recognition (22, 23), because position Gln²⁹ is unique to murine β_2m , whereas human and bovine variants have glycine. The effect of β_2m dislocation at the Site 2 interface explains the behavior of two mutations of H-2D^d, D29N and R35A, that affect Ly49A binding without direct involvement of the Site 2 interface (21). Mutant D29N would be expected to have an indirect effect on β_2m binding because it interacts primarily with H3 of H-2D^d and only indirectly with β_2m residue Arg²²⁸. Residue Arg³⁵ is involved in hydrogen bonds to β_2m M54 backbone oxygen as well as to H-2D^d E32. Thus, mutation of Arg³⁵ to Ala would be expected to change the affinity of the MHC heavy chain for β_2m and distort the disposition of this subunit, thereby altering the interaction with Ly49A. Several other substitutions of H-2D^d that do not make direct contact with either Ly49A or β_2m have been studied, and their behaviors may be explained by similar distant effects. In particular, the double mutant S73W,D156Y, comprising residues that line the peptide-binding groove, has been shown to have functional effects on NK recognition (20), and the double mutants D77S,A99F and N30D,A99F as well as the triple mutant N30D,D77S,A99F showed reduced binding in an Ly49A tet-

² N. Dimasi, M. W. Sawicki, L. N. Reineck, Y. Li, K. Natarajan, D. H. Margulies, and R. A. Mariuzza, manuscript in preparation.

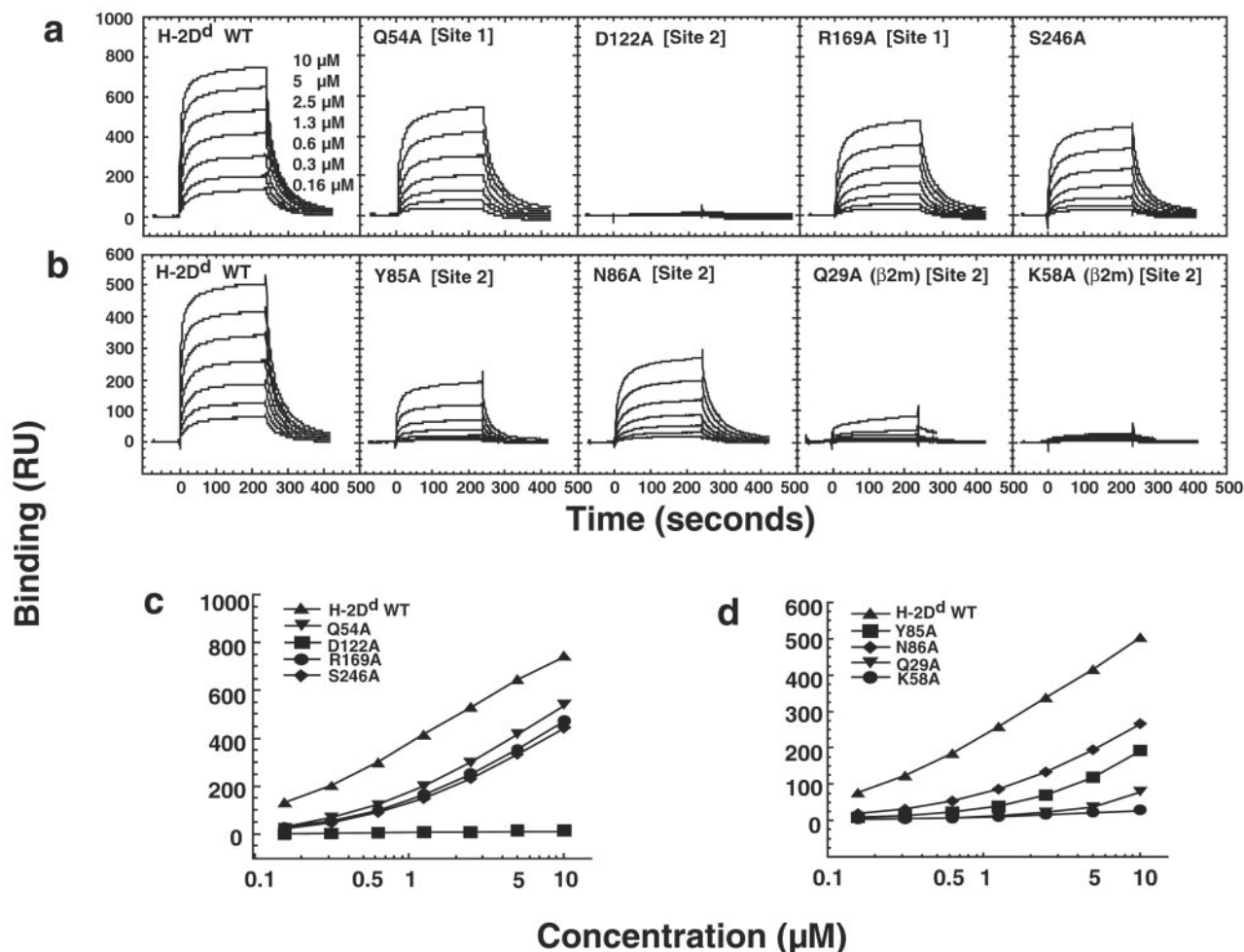


FIG. 5. **H-2D^d and β_2m mutant binding to Ly49A.** The interactions between mutants of H-2D^d, β_2m , and wild type Ly49A were measured in the BIAcore binding assay. Ly49A and mAbs specific for H-2D^d were immobilized on a CM5 chip as described under "Experimental Procedures." Wild type (WT) H-2D^d or its mutants refolded with motif peptide were measured at concentrations from 0.156 to 10 μM in 2-fold increments. Resonance unit (RU) values are corrected for background binding. *a* and *b*, data from two different experiments. *c* and *d*, dose-dependent response comparison of binding of different H-2D^d mutants and β_2m mutants to Ly49A. All preparations were tested in parallel for binding to anti-H-2D^d mAb to confirm the concentration as determined by UV absorbance and the preservation of serological activity (data not shown).

ramer staining assay (21). Residues Ser⁷³, Asp⁷⁷, Ala⁹⁹, and Asp¹⁵⁶ play a role in peptide interaction and thus are likely to contribute to the stability of the interaction with β_2m , thereby affecting the interaction with Ly49A. The nature of the N30D substitution, which seems synergistic with substitution of peptide binding cleft residues, probably is due to the role that N30D plays in the interaction with Ly49A via distant interaction with residue R228 of β_2m . In addition to these experiments with mutant H-2D^d and β_2m , our earlier studies examining the behavior of a single-chain β_2m /H-2D^d molecule in NK cell recognition are also consistent with Ly49A interacting primarily with a site distinct from that of the TCR and subject to inhibition by the peptide linker extending from the carboxyl terminus of β_2m to the amino terminus of H-2D^d (10, 46). Because Ly49G2, a molecule related to Ly49A in both sequence and in its ability to interact with H-2D^d, also is incapable of interacting with the single-chain β_2m /H-2D^d molecule (46), it is likely that Ly49G2 binds H-2D^d at the same site as Ly49A. (Comparison of amino acid sequences of Ly49A^{C57BL/6} and Ly49G2^{C57BL/6} reveals that the residues that contact Asp¹²² of H-2D^d and Lys⁵⁸ of β_2m in the Ly49A complex are conserved, whereas those that interact with the conserved Gln²⁹ of β_2m are polymorphic.)

The analysis of the regions of importance in Site 2 recognition clearly implicates the organization of water molecules in

this large and imperfect interface in mediating the interaction between Ly49A and H-2D^d (Fig. 7). We speculate that the binding specificity of the Ly49 molecules for their MHC/peptide ligands is influenced by a water network exemplified in the Ly49A-H-2D^d structure by residues Asp¹²² of H-2D^d with Ly49A-residues Ser²³⁶, Lys²³⁷, Thr²³⁸, and Arg²³⁹; Lys⁵⁸ of β_2m along with Arg⁶ of H-2D^d and Asp²²⁹, Arg²³⁹, Asp²⁴¹, and Asn²⁴² of Ly49A; and Gln²⁹ of β_2m with Asp⁵⁹ of β_2m , and Gln²⁴⁷, Val²⁴⁸, and Phe²⁴⁹ of Ly49A. Since the description of an important role for water in the carbohydrate specificity of the L-arabinose-binding protein (47), there has been considerable interest in the contribution of water to ligand specificity (48). Recently water has been shown to be important in the specific binding of peptides to MHC molecules (49–53).

The accumulation of the mutational evidence clearly reveals effects of H-2D^d residue Asp¹²² and β_2m residues Gln²⁹ and Lys⁵⁸ on Ly49A binding. These effects seem to be local rather than global, because the TCR binding site of the H-2D^d-Pro¹⁸-Ile¹⁰ complex was preserved both for direct interaction with a cognate TCR and for binding by an MHC-restricted, peptide-specific mAb.

In summary, our mutational analysis of Ly49A has permitted the localization not only of the sites bound by each of a panel of mAbs but has also demonstrated conclusively the importance of the homodimer interface and has localized the

FIG. 6. Binding of different H-2D^d-peptide/β₂m complexes to scTCR. H-2D^d mutant D122A and β₂m mutants Q29A and K58A, which were assembled with parental H-2D^d and with peptide Pro¹⁸-Ile¹⁰, were analyzed in a dose-dependent manner for binding to: *a*, scTCR, 0.6–10 μM; *b*, mAb KP15, 0.015–0.5 μM; *c*, Ly49A, 0.6–20 μM (these were also compared with H-2D^d/motif binding in all cases). All preparations were also tested for binding to mAbs 34-2-12 and 34-5-8 in a similar fashion (data not shown).

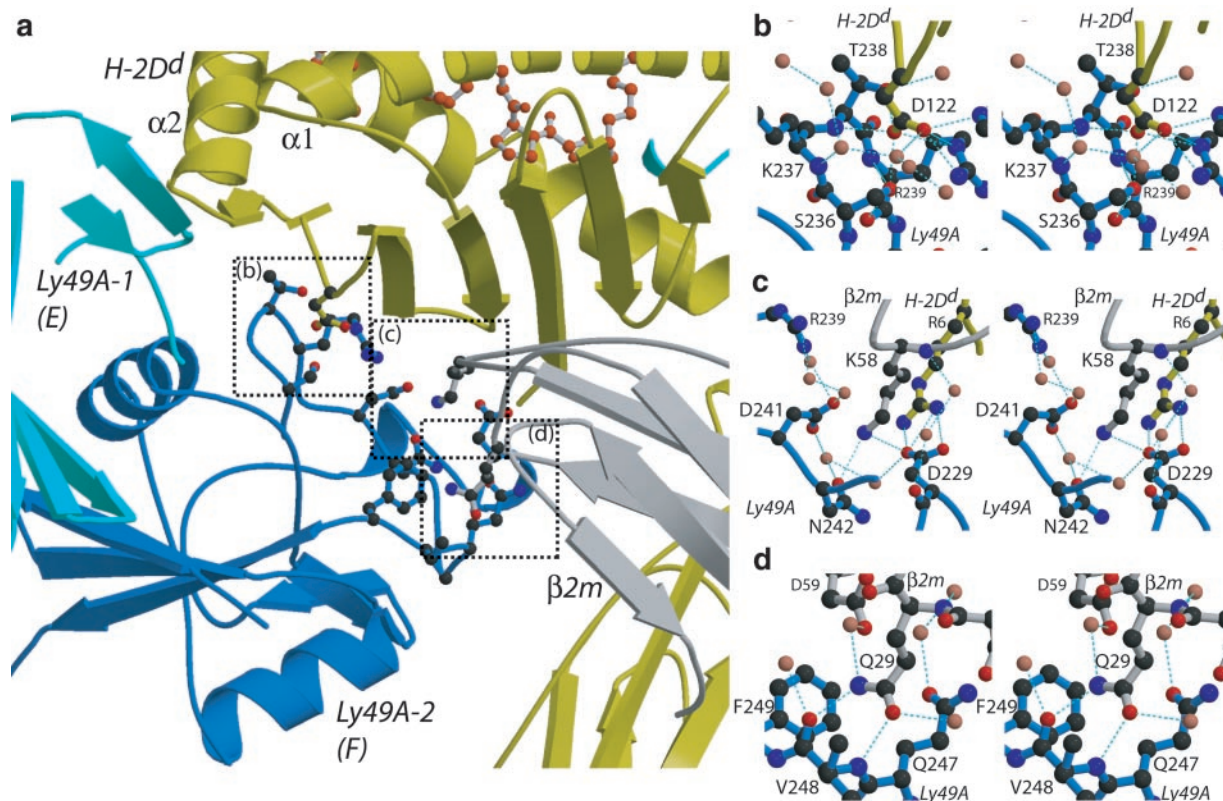
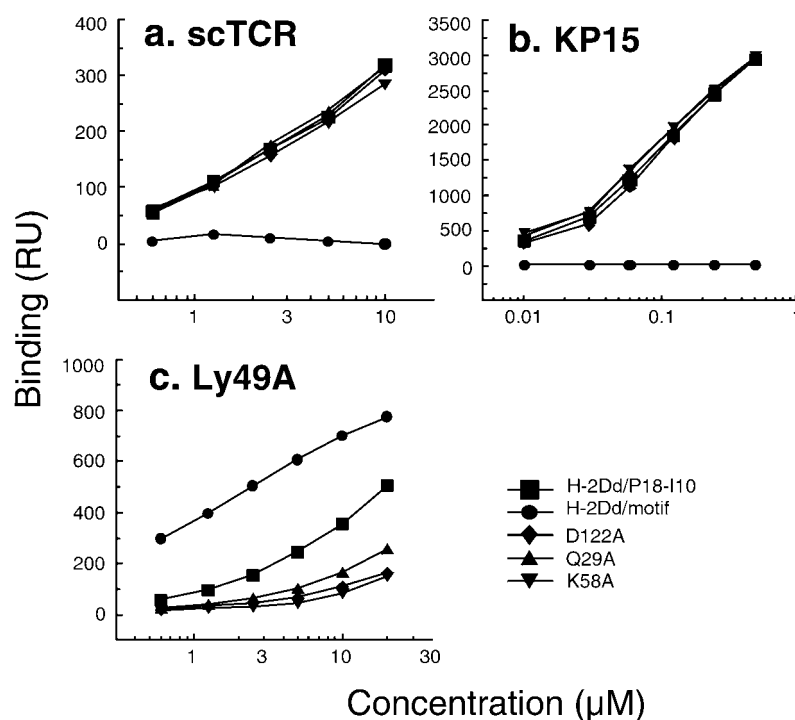


FIG. 7. H-2D^d residue Asp¹²² and β₂m residues Gln²⁹ and Lys⁵⁸ interact with Ly49A at Site 2. Interactions of residues in H-2D^d-Ly49A complex (Protein Data Bank code 1qo3) were illustrated with MOLSCRIPT (56) and rendered with Raster3d (42). *a*, interaction between Ly49A (F domain), H-2D^d, and β₂m mutants at Site 2. *b*, side-by-side stereo view of network of interactions of H-2D^d residue Asp¹²² and Ly49A residues Ser²³⁶, Lys²³⁷, Thr²³⁸, and Arg²³⁹ in the presence of water molecules. Asp¹²² of H-2D^d contacts directly with Ser²³⁶, Thr²³⁸, and Arg²³⁹ of Ly49A through hydrogen bonds. *c*, side-by-side stereo view of network of β₂m residue Lys⁵⁸ with Ly49A residues Asp²²⁹, Asp²⁴¹, and Asn²⁴² in the presence of water molecules. *d*, side-by-side stereo view of network of β₂m residue Gln²⁹ with Ly49A residues Gln²⁴⁷, Val²⁴⁸, and Phe²⁴⁹ in the presence of water molecules. Dashed lines indicate hydrogen bonds. Other atomic contacts are not explicitly shown. Yellow, H-2D^d; blue, Ly49A; lavender, β₂m; orange spheres, water molecules.

critical site of interaction between Ly49A, H-2D^d, and β₂m to Site 2, a region that potentially overlaps with the CD8 binding site. The strength of this interaction and the lack of more than

a 3-fold effect of any of the mutants at Site 1 tested to date suggest that the Site 2 interaction functions in the recognition between the NK cell and the antigen-presenting cell, a *trans*

mode, although it does not eliminate the possibility of *cis* interactions between molecules expressed on the NK cell. Further understanding of the quantitative contributions of the redistribution of water as binding takes place will require careful thermodynamic measurements under conditions that vary the activity of water as solvent.

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**Binding of the Natural Killer Cell Inhibitory Receptor Ly49A to Its Major
Histocompatibility Complex Class I Ligand: CRUCIAL CONTACTS INCLUDE
BOTH H-2Dd AND β 2-MICROGLOBULIN**

Jian Wang, Mary C. Whitman, Kannan Natarajan, José Tormo, Roy A. Mariuzza and
David H. Margulies

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