Epitope mapping of Ly-49G and G-like receptors: CK-1 antibody defines a polymorphic site of functional interaction with class I ligand

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Abstract: Ly-49 receptors regulate mouse natural killer cell functions. Members of the polymorphic Ly-49 multigene family recognize specific alleles of major histocompatibility complex class I (MHC I) or MHC I-like proteins. Previous studies have provided insight into the nature of Ly-49A and -C interaction with their high-affinity MHC I ligands, H-2D^d and K^b, respectively. Unlike Ly-49C, recognition of MHC I by Ly-49A is regulated in part by residues within the β4–β5 loop of its ectodomain. Ly-49A and -G are within the same Ly-49 subfamily, and both receptors recognize D^d. However, there have been no studies that define specific sites on Ly-49G that mediate class I MHC recognition. The Ly-49G receptors of different inbred mouse strains can differ as a result of amino acid polymorphisms within their ectodomains. In this report, we have generated a novel antibody, CK-1, which recognizes Ly-49G^{B6} and a Ly-49G^{B6}-like receptor, Ly-49M$^{nonobese\ diabetic}$, but not Ly-49G$^{BALB/c}$. By exploiting the differences within ectodomains of C57BL/6 and BALB/c *Ly-49G* allele products, we identified epitopes recognized by the Ly-49G-specific antibodies CK-1 and Cwy-3, whose epitopes mapped within the β4–β5 loop and the β1 strand, respectively, and were nonoverlapping. Although both antibodies specifically recognized the Ly-49G^{B6} ectodomain, Cwy-3 was unable to block its interaction with MHC I, and CK-1 significantly inhibited it. The importance of residues within the β4–β5 loop in Ly-49G recognition demonstrates that its interaction with MHC I is similar to that of Ly-49A but not Ly-49C. *J. Leukoc. Biol.* 77: 644–651; 2005.

Key Words: *Ly-49 receptors · motif · ligand interaction*

INTRODUCTION

Natural killer (NK) cells are large, granular lymphocytes that constitute a major component of innate resistance to tumors and viruses [1, 2]. NK cells mediate their protective functions by direct cytolysis or release of cytokines and chemokines [3–5]. Activities of NK cells are controlled through a balance between signals generated by inhibitory and activating receptors and response results from reduction of inhibitory signals or enhancement of activating signals [6, 7].

NK cells express inhibitory receptors that recognize class I major histocompatibility complex (MHC) proteins, preventing NK cell aggression toward cells expressing normal levels of self class I MHC molecules [8, 9]. Virally infected cells and transformed cells typically express reduced levels of class I MHC products, rendering them susceptible to NK cell effector functions as a result of reduction or absence of class I-dependent NK cell inhibitory signals. NK cells express a variety of activating receptors that trigger NK cell-mediated cytotoxicity or cytokine release [7]. Such receptors recognize ligands expressed on virally infected, transformed, or otherwise "stressed" cells [10].

In mice, NK cell receptors that directly recognize classical MHC I molecules are members of the *Ly-49* multigene family of lectin-like receptors [11]. The *Ly-49* gene family is encoded within the NK gene complex on mouse chromosome 6 [12, 13]. The number of *Ly-49* genes can vary in different inbred mouse strains, and *Ly-49* genes display extensive allelic variation [14]. Ly-49 receptors are disulfide-linked homodimers expressed on NK cells, NKT cells, and some CD8$^+$ memory T cells [15–17]. The Ly-49 family contains two receptor types: inhibitory Ly-49 receptors are able to disrupt NK cell activation; activating Ly-49 receptors stimulate NK cell activation [11]. Each subunit of an inhibitory Ly-49 receptor contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) in its cytoplasmic tail. Following Ly-49 engagement with a class I MHC ligand, the ITIMs become phosphorylated, leading to Src homology-containing tyrosine phosphatase 1 recruitment and dephosphorylation of molecules in the NK cell activation cascade [18, 19]. Ly-49-activating receptors generally share a high degree of amino acid identity in their ectodomains with inhibitory Ly-49 receptors but lack an ITIM. Instead, activating Ly-49 receptors contain an arginine residue in the transmembrane segment that facilitates association with the signaling adaptor protein DAP12 [20]. Engagement of Ly-49-activating

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receptors stimulates NK cell signaling cascades associated with NK cell activation [21, 22].

In this report, we identified the amino acids controlling the specificity of two Ly-49G-reactive antibodies, CK-1 and Cwy-3. We defined a critical region of functional interaction between the C57BL/6 allele product of the inhibitory Ly-49G receptor with a class I MHC ligand: the loop connecting the predicted β4 and β5 strands of Ly-49G. Our functional analyses used native Ly-49 receptor ectodomains and ligands, and serological reagents to define receptor epitopes. Thus, it was different from strategies that result in changes in primary amino acid sequences of the ectodomain, which could influence receptor conformation. In addition to distinguishing between Ly-49G allele products, we found that CK-1-recognized Ly-49M but not the closely related Ly-49W receptor, possibly allowing the discrimination and characterization of NK cell subsets that express Ly-49M but not one or more related activating Ly-49 receptors.

MATERIALS AND METHODS

Antibodies

Hybridomas producing antibodies 4D11 [rat immunoglobulin G (IgG)2a], anti-Ly-49G [23]; M1/42 (rat IgG2a), anti-mouse class I MHC [24]; Y13–238 (rat IgG2a), anti $p21^{ras}$ [25]; and BB7.1 (IgG1), anti-human leukocyte antigen-B7 [26] were obtained from the American Type Culture Collection (Manassas, VA). The Cwy-3 and CK-1 hybridomas were generated in this laboratory. The CK-1 (anti-Ly-49G, IgG1) hybridoma was generated following immunization of BALB/c mice with interleukin (IL)-2-activated C57BL/6 spleen cells, as described for the Cwy-3 (anti-Ly-49G, IgG1) hybridoma [27]. Antibodies were prepared by ammonium sulfate precipitation and phosphate-buffered saline (PBS) dialysis of tissue-culture supernatants obtained from hybridomas grown in protein-free hybridoma medium. Purified OX-8 (IgG1) anti-rat CD8α were purchased from BD PharMingen (San Diego, CA) [25]. Fluorescein isothiocyanate (FITC)-coupled mouse anti-rat and rat anti-mouse IgG secondary antibodies were purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Purified rat IgG was purchased from Sigma-Aldrich (Oakville, ON).

Cell lines

COS-7 Simian virus 40-transformed African green monkey kidney cells were grown in Opti-MEM I medium (Life Technologies, Burlington, ON, Canada), containing 10% heat-inactivated fetal calf serum (FCS; Invitrogen Canada, Burlington, ON) and 5×10^{-5} M 2-mercaptoethanol (2-ME). RNK-16, a spontaneous F344 rat strain NK cell leukemia [28], was maintained in RPMI 1640 supplemented with 10% FCS, L-glutamine, penicillin, streptomycin, and 5×10^{-5} M 2-ME (RNK medium).

Cloning of Ly-49G cDNA and site-directed mutagenesis

The cDNAs encoding the B6 and BALB/c alleles of Ly-49G or Ly-49G-related receptors (Ly-49W1$^{\text{nonobese diabetic (NOD)}}$, Ly-49L$^{BALB/c}$, Ly-49M^{NOD}) were prepared by reverse transcriptase-polymerase chain reaction from total RNA isolated from IL-2-activated NK cells of corresponding mouse strains, as described [29], and were then inserted into the *Xba*I/*Eco*RI sites of the mammalian expression vector pCI-neo (Promega, Madison, WI). Ly-49G mutants were generated using the Stratagene QuikChange kit (Stratagene, La Jolla, CA).

Cell transfection

The cDNA encoding inhibitory and activating Ly-49 receptors was transiently expressed in COS-7 cells using Lipofectamine (Life Technologies) as described [29]. Vectors encoding activating receptors were co-transfected with a cDNA encoding mouse DAP12 in the pFLAG-CMV-1 expression vector (Sigma-Aldrich, St. Louis, MO). The generation of RNK-16 cells, stably expressing Ly-49G2^{B6} (clone 1B6) or a chimeric receptor consisting of the ectodomain of Ly-49G2^{B6} fused to the transmembrane and intracellular domains of Ly-49W^{NOD} (Ly-49W/G^{B6}, clone 2G2), has been described previously [25].

Flow cytometric detection of Ly-49G and related receptors

Approximately 48 h after transfection to express Ly-49 receptors, COS-7 cells were incubated with 4D11, Cwy-3, CK-1, or isotype control antibodies M1/42 or BB7.1. The appropriate FITC-coupled secondary antibodies, mouse anti-rat or rat anti-mouse, were subsequently added for an additional incubation, whereupon samples were analyzed on a flow cytometer. To examine the expression of Ly-49G epitopes expressed on the surface of RNK-16 transfectants, Cwy-3, CK-1, or their isotype control BB7.1 was labeled directly with Alexa-Fluor® 488 or 647 fluorochromes (Molecular Probes, Eugene, OR) using the manufacturer's instructions. The RNK-Ly-49G^{B6} transfectant, 1B6, was resuspended at a density of 1×10^6 cells/ml and incubated with rat IgG at 40 μg/ml for 15 min to block rat Fc receptors (FcRs). Saturating levels of Alexa-488-labeled Cwy-3 or BB7.1 antibodies were added to FcR-blocked cells and incubated for 45 min, and then saturating amounts of Alexa-647-labeled CK-1 or BB7.1 were added to the cells stained with the Alexa-488-labeled antibodies and incubated for 30 min. All incubations were conducted at 4°C. Cells incubated with labeled antibodies were fixed with 4% *p*-formaldehyde in PBS and then analyzed on a FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA).

Cytotoxicity assays

Concanavalin A (Con A)-activated blasts were prepared from DBA/2 and C57B46 strain spleen cells, as described [30]. The Con A blasts were labeled at 37°C with 100–150 μCi $Na^{51}CrO_4$ (Mandel Scientific, Guelph, ON) for 1.5 h. Rat FcRs on RNK-16 Ly-49W/G^{B6} 2G2 effector cells were first blocked by incubation with rat IgG at 40 μg/ml, and then the effector cells were incubated for 30 min with medium, Ly-49G-specific antibodies, or isotype control antibodies prior to addition of labeled target cells, which were incubated with 2G2 effector cells in the presence or absence of antibody for 4 h at 37°C in V-bottom microtiter plates at various effector:target (E:T) ratios in triplicate. After incubation, supernatant samples were counted in a MicroBeta TriLux liquid scintillation counter (Perkin Elmer, Wellesley, MA). Percent specific lysis was determined as (experimental release–spontaneous release)/(maximum release–spontaneous release) × 100. Cytotoxicity experiments were performed four separate times.

RESULTS

Ly-49 receptor specificities of alloantibodies

Individual mouse NK cells can express several Ly-49 receptors simultaneously, and this is determined by a stochastic process [31]. Most described serological reagents that bind Ly-49 receptors are cross-reactive and recognize multiple Ly-49 receptors, making it difficult to attribute a ligand specificity or functional property to a particular Ly-49 receptor. Ly-49G and a number of Ly-49G-related activating receptors, Ly-49W, Ly-49M, and Ly-49L, are reactive with the 4D11 antibody. These different receptors share a high degree of amino acid identity within the carbohydrate recognition domain (CRD). Two allele products of *Ly-49G*, Ly-49G2$^{BALB/c}$ and Ly-49G2^{B6}, share over 96% amino acid identity in their CRD. Nevertheless, strain-specific differences in Ly-49 gene content and allelic variation offer opportunities to generate serological reagents with highly restricted Ly-49 specificity. Such antibodies would be useful not only to detect receptor expression but also

to map receptor site(s) that are important for ligand interaction. We generated the antibody-producing CK-1 hybridoma after injecting BALB/c mice with C57BL/6 IL-2-activated NK cells, similar to the generation of the Cwy-3 hybridoma [27]. We compared the reactivity of CK-1, Cwy-3, and 4D11 with Ly-49G and Ly-49G-related receptors. COS-7 cells were transiently transfected with cDNAs encoding Ly-49G receptors from the C57BL/6 and BALB/c mouse strains or Ly-49G-related activating receptors (Ly-49L$^{BALB/c}$, M^{NOD}, W^{NOD}) co-transfected with mouse DAP12. After 48 h, the transfectants were stained with 4D11, Cwy-3, CK-1, or a corresponding isotype-matched control. All Ly-49G and Ly-49G-related receptors were strongly expressed on the COS-7 cells, as detected by substantial rat 4D11 antibody staining (**Fig. 1**, left panels). As the Cwy-3 and CK-1 hybridomas were produced from the BALB/c mouse, it was expected that they would recognize the BALB/c allele product of Ly-49G poorly, if at all, as is the case in Figure 1, middle and right panels. However, the Cwy-3 and CK-1 antibodies recognized the Ly-49G^{B6} receptor (Fig. 1, middle and right panels), and CK-1 stained cell-surface Ly-49G2^{NOD} (data not shown). The Cwy-3 antibody also recognized the Ly-49W activating receptor from the NOD strain, as we previously reported [29], as well as another activator from the NOD strain, Ly-49M, but the BALB/c activating receptor Ly-49L only weakly, similar to the Ly-49G$^{BALB/c}$ control (Fig. 1, middle panels). CK-1, like Cwy-3, recognized Ly-49M^{NOD} but unlike Cwy-3, did not recognize Ly-49W^{NOD} or Ly-49L$^{BALB/c}$ (Fig. 1, right panels). Thus, unlike 4D11 and Cwy-3, CK-1 has the ability to detect the expression of Ly-49M, as opposed to Ly-49W.

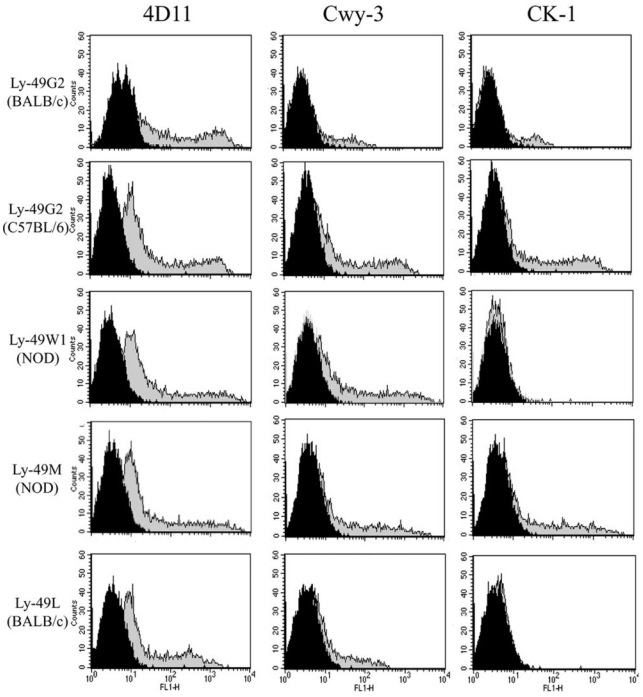

Fig. 1. The CK-1 and Cwy-3 antibodies recognize partially overlapping sets of Ly-49 receptors. COS-7 cells were transiently transfected to express the inhibitory Ly-49G2 receptor from C57BL/6 or BALB/c mice or Ly-49W1, Ly-49M, or Ly-49L receptors along with murine DAP12 and were then analyzed by flow cytometry using 4D11, Cwy-3, or CK-1 antibodies (gray) or isotype controls, M1/42 and BB7.1 (black).

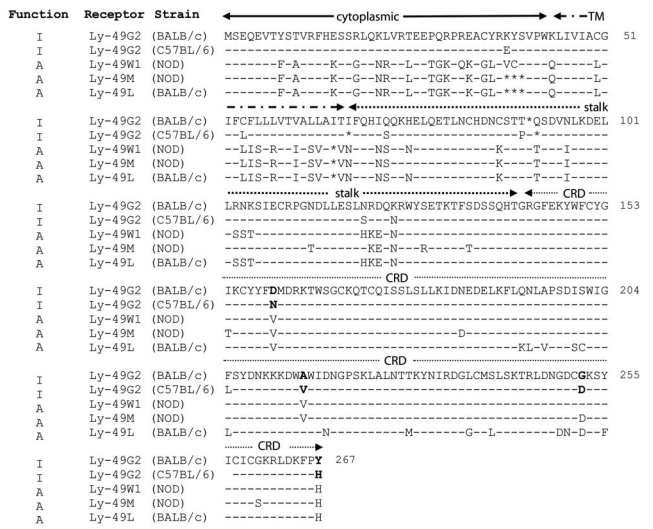

Fig. 2. Amino acid alignment of Ly-49G and Ly-49G-related receptors from different mouse strains. Regions corresponding to the cytoplasmic, transmembrane (TM), stalk, and CRD are indicated with arrows. Amino acids in bold identify residues that differ between BALB/c and B6 allele products of Ly-49G2 and are consistent with the staining patterns of Ly-49G and related receptors. Determined or expected receptor function is designated by inhibitory (I) or activating (A). GenBank accession numbers include AF307946 (Ly-49G2$^{BALB/c}$), NM_014194 (Ly-49G2$^{C57BL/6}$), AF283250 (Ly-49W1^{NOD}), AF283252 (Ly-49M^{NOD}), and AF307947 (Ly-49L$^{BALB/c}$).

CK-1 and Cwy-3 epitope mapping

The differential reactivity of Cwy-3 and CK-1 for Ly-49G-related receptors suggested that they may bind distinct epitopes on these receptors. Residues contributing to strong Cwy-3 staining are not present in Ly-49G2$^{BALB/c}$ or Ly-49L$^{BALB/c}$ but are present in Ly-49G2^{B6}, -W, and -M. Similarly, the CK-1 epitope may involve residues in common to Ly-49G2^{B6} and Ly-49M^{NOD} but not the other examined Ly-49G-related receptors. To identify candidate residues that could contribute to or define the Cwy-3 or CK-1 epitopes, we examined stalk and CRD domains for amino acid residues that differed between the BALB/c and C57BL/6 alleles of Ly-49G. To further refine potential residues that contributed to the Cwy-3 and CK-1 epitopes, residues were considered that differed between the BALB/c and C57B46 alleles of Ly-49G and were also consistent with the patterns of Cwy-3 and CK-1 reactivity with Ly-49G-related receptors. There were four residues in the CRD at positions 159, 214, 251, and 267, which could contribute to the Cwy-3 or CK-1 epitopes (**Fig. 2**). Polymorphisms in the stalk regions did not fulfill the criteria for residues that may define Cwy-3 or CK-1 epitopes, and the four identified residues were not in positions that could affect predicted receptor glycosylation sites.

We mutated the BALB/c allele of Ly-49G to contain each of the identified candidate residues in the C57B46 allele that may confer Cwy-3 or CK-1 antibody reactivities. The four single amino acid mutants were examined for reactivity by 4D11, Cwy-3, and CK-1. The 4D11 antibody recognized the wild-type

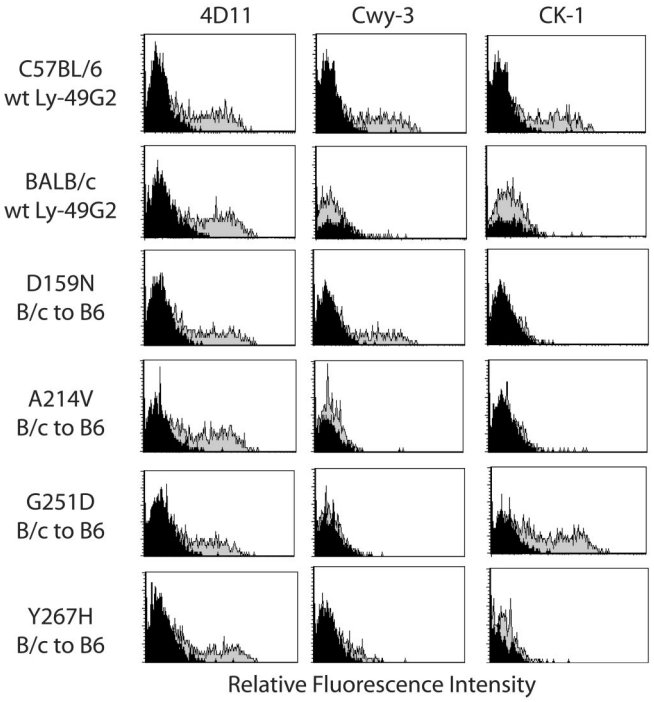

Fig. 3. Recognition by Cwy-3 and CK-1 antibodies requires distinct Ly-49G^{B6} amino acid residues. COS-7 cells were transfected with Ly-49G2 from BALB/c or C57BL/6 mice or mutants that substitute individual Ly-49G^{B6} residues for Ly-49G$^{BALB/c}$ residues and were then analyzed by flow cytometry using 4D11 to monitor Ly-49G surface expression (gray) or the M1/42 isotype control (black). Alternatively, transfected cells were analyzed using Cwy-3 or CK-1 (gray) or an isotype control (BB7.1, black) to identify mutants that conferred the epitope recognized by either Ly-49G^{B6}-binding antibody. wt, Wild-type.

BALB/c and C57B46 allele products of Ly-49G as well as all of the mutant BALB/c Ly-49G receptors in transfected COS-7 cells (**Fig. 3**, left panels). This indicated that the mutant receptors folded in a manner similar to the wild-type, and expression of the mutant receptors was clearly detectable. It is more important that a single amino acid substitution at position 159 (D to N) of the BALB/c Ly-49G conferred recognition by the Cwy-3 antibody but not by the CK-1 antibody (Fig. 3, middle and right panels). In contrast, a single amino substitution at position 251 (G to D) on a BALB/c template, resulted in CK-1 reactivity similar to that toward Ly-49G^{B6} but not Cwy-3 reactivity (Fig. 3, middle and right panels). Substitutions of residues 214 and 267 did not result in Cwy-3 or CK-1 recognition (Fig. 3, middle and right panels), suggesting that residues at these positions do not contribute to the epitopes detected by Cwy-3 or CK-1. Thus, epitopes recognized by Cwy-3 and CK-1 can be conferred with single amino acid substitutions at positions 159 and 251, respectively. The epitopes recognized by Cwy-3 and CK-1 appear to be independent, as a change at position 159 only affected Cwy-3 recognition, and a change at position 251 only affected CK-1 reactivity.

To confirm the importance of aspartic acid 251 for the CK-1 epitope, the C57BL/6 allele of the Ly-49G receptor was mutated at position 251 to glycine found at this position in the BALB/c allele of Ly-49G (D251G). This mutant receptor was expressed on the cell surface and folded properly, as demonstrated by 4D11 staining comparable with that of the C57B46 wild-type receptor (**Fig. 4**, left panels). Substituting aspartic acid with glycine at this position resulted in a complete loss of CK-1 staining compared with the wild-type Ly-49G2^{B6} receptor (Fig. 4, right panels) but not Cwy-3 (Fig. 4, middle panels), demonstrating the critical role aspartic acid plays at this position for CK-1 recognition.

Ly-49G and Ly-49G-related receptors that are CK-1-positive, such as Ly-49G^{B6} and Ly-49M, contain the amino acid sequence DCD at positions 249–251 (Figs. 1 and 2). In addition, the mutant Ly-49G2$^{BALB/c}$ receptor (G251D) recognized by CK-1 also contains the 249–251 sequence DCD. We directly tested a possible requirement for simultaneous expression of aspartic acids at positions 249 and 251 to observe CK-1 reactivity. Thus, a second mutant was constructed of the Ly-49G2^{B6} allele, substituting an alanine for the aspartic acid at position 249. This mutant receptor was expressed on the cell surface at similar levels to wild-type Ly-49G2^{B6}, as detected by 4D11 staining (Fig. 4, bottom left panel), and Cwy-3 binding was not affected (Fig. 4, bottom middle panel). However, the D249A mutant was not recognized by CK-1 (Fig. 4, bottom right panel). We conclude that recognition by CK-1 requires the presence of both aspartic acids at positions 249 and 251 in Ly-49G^{B6} and likely also, Ly-49G-related receptors.

The preceding experiments indicate that Cwy-3 and CK-1 antibodies have distinct requirements for the presence of specific amino acids in Ly-49 receptors for recognition. This raised the possibility that these antibodies recognize nonoverlapping epitopes on Ly-49G^{B6}. To test this possibility directly, we determined whether these two antibodies could bind to Ly-49G^{B6} simultaneously. Ly-49G^{B6}-transfected RNK-16 cells were stained with saturating concentrations of Cwy-3, CK-1, or both antibodies. The antibodies were labeled with distinct fluorochromes, Alexa 488 and Alexa 647. As expected, each Ly-49G^{B6}-reactive antibody was able to bind the Ly-49G^{B6} transfectants (**Fig. 5**, lower left, upper right panels); in addi-

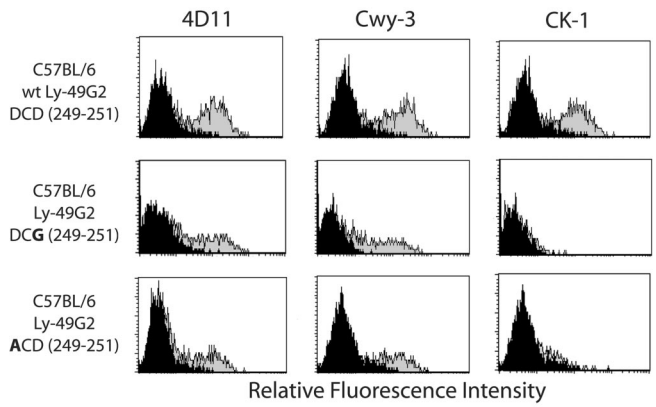

Fig. 4. CK-1 recognition requires two aspartic acid residues, 249 and 251, of Ly-49G2^{B6}. COS-7 cells were transfected with wild-type (wt) Ly-49G2^{B6} or amino acid substitution mutants of Ly-49G2^{B6}, as indicated, and were then analyzed by flow cytometry using 4D11 (gray) or an M1/42 isotype control (black) to monitor Ly-49G^{B6} expression. Cwy-3 or CK-1 (gray) or an isotype control (BB7.1, black) staining was performed to identify Ly-49G^{B6} mutants that affected the CK-1-recognized epitope. Differences in β_4–β_5 loop amino acid sequences of mutant receptors are in bold.

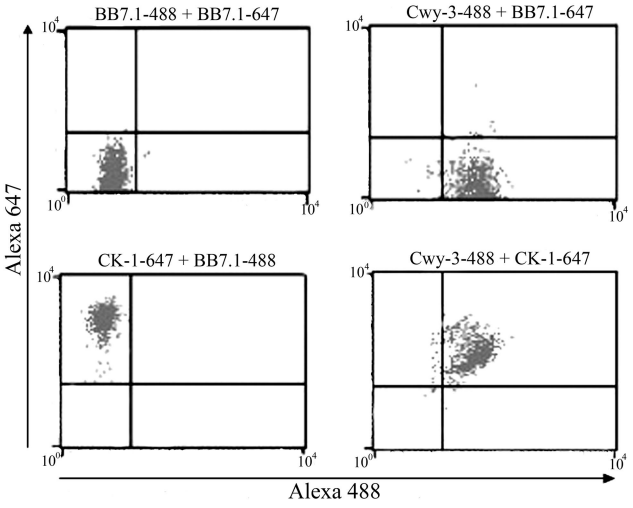

Fig. 5. CK-1 and Cwy-3 recognize distinct, nonoverlapping epitopes. RNK-16 cells stably transfected with Ly-49G2^{B6}, clone 1B6, were preincubated with rat IgG to block antibody binding to cell FcRs. 1B6 cells were treated with Alexa-488-labeled antibodies (Cwy-3 or BB7.1) followed by treatment with Alexa-647-labeled antibodies (CK-1 or BB7.1), as indicated. Alexa-488 staining is indicated on the horizontal axis, and Alexa-647 staining is on the vertical axis. All antibodies were added at saturating concentrations.

tion, both antibodies were able to stain the transfectants simultaneously (Fig. 5, lower right panel). No staining was observed with isotype controls (Fig. 5, upper left panel). Similar antibody staining was obtained using 2G2 cells, RNK transfectants expressing the Ly-49W/G^{B6} chimeric receptor (data not shown). Thus, Cwy-3 and CK-1 bind nonoverlapping epitopes, consistent with the results of the mutagenesis experiments.

Differential inhibition of Ly-49G^{B6} ectodomain functional interaction with its ligand

We expressed a chimeric receptor that has the cytoplasmic and transmembrane domain of the Ly-49W-activating receptor fused to the ectodomain of the inhibitory Ly-49G^{B6} receptor on the RNK-16 rat NK cell leukemia, as described previously [25]. This approach provides a means to study Ly-49 inhibitory receptor interaction with its ligands, where recognition is detected as a direct, positive NK cell response, instead of inhibition by the receptor of undefined activating receptor functions [25]. Significant expression of the chimeric Ly-49W/G^{B6} receptor on a RNK-16 transfectant clone 2G2 was demonstrated by staining with Cwy-3, CK-1, and 4D11 antibodies (**Fig. 6A**). No staining of untransfected RNK-16 cells with any of the three antibodies was detected (data not shown). Ly-49G^{B6} recognizes H-2D^{d} [25], and expression of the Ly-49W/G^{B6} chimera on RNK-16 cells results in cytotoxicity toward DBA/2 strain (H-2^{d}) but not C57BL/6 (H2^{b}) Con A blasts [25] (Fig. 6B, upper panel). We compared the ability of CK-1, Cwy-3, and 4D11 antibodies to block cytotoxicity mediated by the Ly-49W/G^{B6} chimeric receptor. As shown in Figure 6B, CK-1 and 4D11 were effective at inhibiting recognition by the chimeric receptor bearing the Ly-49G^{B6} ectodomain; however, the Cwy-3 antibody was completely unable to interfere, even at high antibody concentrations (Fig. 6B, upper and lower panels). The inhibition of recognition by CK-1 was specific, as OX-8, an IgG1 isotype control that binds to CD8α molecules on the surface of RNK-W/G^{B6} effector cells, did not affect recognition, whereas CK-1 significantly inhibited cytolysis (Fig. 6B, lower panel). As the CK-1 and Cwy-3 antibodies bind the Ly-49G^{B6} ectodomain at independent sites, these data indicate that CK-1 but not Cwy-3 binds a site, such as 4D11, which is important for ligand interaction. Moreover, as we have mapped the residues responsible for CK-1 binding to the polymorphic loop between the β4 and β5 strands, this indicates that this loop is involved in ligand recognition by Ly-49G^{B6}.

DISCUSSION

Antibodies cross-reactive for several Ly-49 family members and multiple alleles of a given Ly-49 gene have been generally

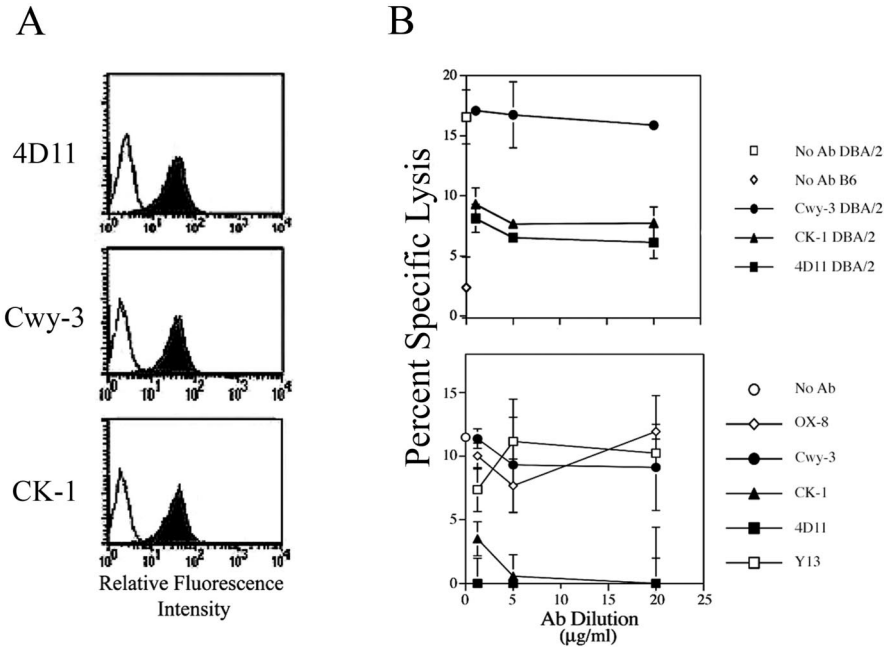

Fig. 6. Epitope dependence of Ly-49G interaction with ligand. (A) Expression of the Ly-49W/G^{B6} chimera on RNK-16 transfectant 2G2, detected with Cwy-3, CK-1, and 4D11 antibodies (filled) and isotype controls BB7 and Y13, respectively (unfilled). (B) Effect of Cwy-3, CK-1, and 4D11 antibodies (Ab) on the inhibition of Ly-49G2^{B6} recognition of target cells bearing its MHC I ligand. Lysis of DBA/2 Con A blasts by RNK.WG^{B6} clone 2G2 was measured with no antibody or in the presence of anti-Ly-49G antibodies (Cwy-3, CK-1, or 4D11, upper and lower panels) or control antibodies (OX-8 or Y13–238, respectively, lower panel), at the indicated concentrations. C57BL/6 Con A blasts were negative control target cells (upper panel). The FcR of effector cells was blocked by incubation with rat IgG (40 μg/ml) prior to addition of the indicated antibodies. Lysis was determined in a standard 4-h ^{51}Cr-release assay using an E:T ratio of 12.5:1. Antibodies were at the indicated concentrations during the assay. Data represent the mean of triplicate wells, and error bars represent the respective standard deviations.

useful in the study of Ly-49 receptor functions [23, 32]. As it has become apparent that the Ly-49 family contains 24 or more members [14, 33, 34], antibodies that identify a particular Ly-49 member or a narrow subset of receptors are needed to attribute expression or function to specific receptors. We generated antibodies that discriminate between Ly-49G alleles and Ly-49G-related Ly-49 family members by taking advantage of known, polymorphic differences between inbred mouse strains [14]. We demonstrated that CK-1, similar to the Cwy-3 antibody, recognizes the C57BL/6 but not BALB/c allele product of Ly-49G. It is more important that CK-1 can discriminate Ly-49M from Ly-49W, activating receptors that are similar to Ly-49G in their ectodomains. This level of discrimination is high, as Ly-49W and Ly-49M share 96% amino acid identity. The Cwy-3 antibody and AT8 antibodies have been used together to characterize the functionality of NK cell subsets expressing specific Ly-49G allele(s) isolated from F_1 animals [35]. The CK-1 antibody provides additional discrimination between Ly-49G-related receptors and may be useful in discerning the specificities and functions of NK cell subsets in a broader range of mouse strains that express two Ly-49G alleles and/or Ly-49G-related activating receptors. However, as CK-1 recognizes Ly-49G2^{NOD} (data not shown) and Ly-49M^{NOD}, it may be difficult to discriminate between these receptors on ex vivo NK cells from NOD mice. The significance of this limitation depends on the relative frequency of Ly-49G2 and Ly-49M expression on NOD strain NK cells, which remains to be determined.

We have identified the polymorphic amino acid residues of Ly-49G that confer binding specificity for the CK-1 and Cwy-3 antibodies. We modeled the location of the amino acids on Ly-49G in three dimensions, based on the known crystal structure of the related Ly-49A receptor [36]. The CK-1 antibody requires the presence of two aspartic acids, D249 and D251, which reside on a loop between the β4 and β5 strand of Ly-49G^{B6}, and likely, on other CK-1-recognized receptors such as Ly-49M for interaction (**Fig. 7A**). The β4–β5 loop is highly polymorphic within the Ly-49 family, and at least 11 different sequences have been identified. The Cwy-3 antibody requires an asparagine (e.g., Ly-49G^{B6}) or valine (e.g., Ly-49W) but not an aspartic acid (Ly-49G$^{BALB/c}$) at position 159. The N- or V159 is located on the β1 strand (Fig. 7A), a significant distance from the CK-1 epitope but near the Ly-49 dimer interface, which is consistent with our observation that CK-1 and Cwy-3 can bind Ly-49G^{B6} simultaneously. Like Ly-49W1^{NOD}, Ly-49L$^{BALB/c}$ contains a Val residue at position 159, yet it is poorly recognized if at all by Cwy-3. This may stem from the presence of a number of nonconservative amino acid substitutions within the Ly-49L receptor dimer interface in the CRD, which may alter the conformation of the receptor and as a consequence, eliminate the Cwy-3 epitope or the accessibility of Cwy-3 for V159. The amino acids required for CK-1 or Cwy-3 recognition are predicted to be solvent-exposed (Fig. 7A); thus, they are unlikely to influence the folding or conformation of the receptor nor are they in positions to alter the Ly-49 dimer interface. Rather, the required amino acids are likely to interact directly with the antibodies and contribute to the respective antibody-combining sites. We did not map the common epitope recognized by the pan-specific 4D11 antibody; however, this epitope is not identical to those recognized by CK-1 or Cwy-3, as mutations that eliminated CK-1 or Cwy-3 binding did not affect binding by 4D11.

Mutagenesis and co-crystal structures have provided insight into how Ly-49A and -C bind their high-affinity class I ligands, H-2D^d and K^b, respectively [36, 39, 40]. However, there have been no studies that define specific sites on Ly-49G, which mediate class I recognition. Ly-49A and -G are within the same Ly-49 subfamily [25, 29], and D^d is a shared ligand for both receptors. Thus, the co-crystal of Ly-49A bound to D^d may serve as an appropriate model for interaction of MHC I with Ly-49G or Ly-49G-related activating receptors. The Ly-49A-D^d cocrystal indicates two distinct areas of potential interaction, termed site 1 and site 2, and these sites are suggested when modeling Ly-49G^{B6} binding to D^d (Fig. 7B). Site 1 primarily involves a region on one end of the α1 and α2 domains of the MHC I molecule, whereas site 2 is a larger contact region beneath the peptide-binding groove and includes a large cleft formed by portions of the α1, α2, and α3

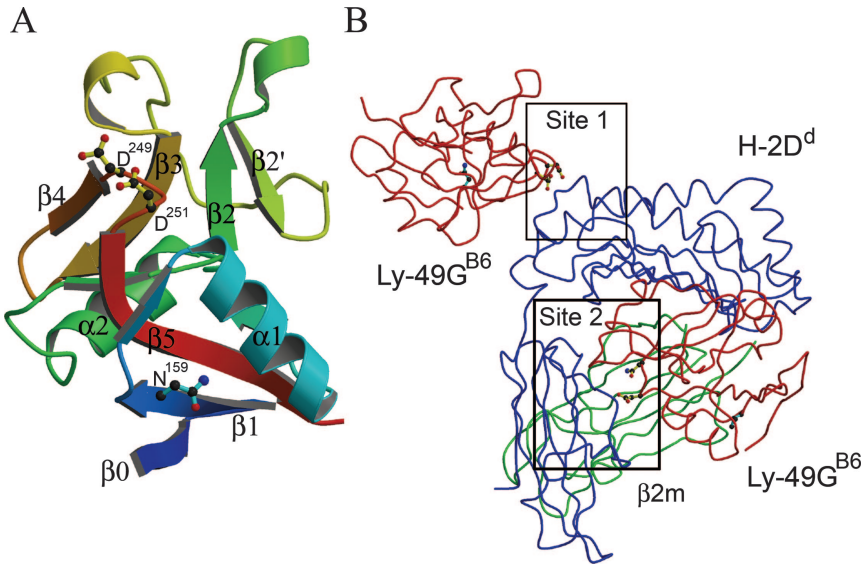

Fig. 7. Location of Ly-49G residues that determine CK-1 and Cwy-3 binding and their interaction with H-2D^d. (A) Model of the Ly-49G^{B6} monomer, based on the Ly-49A crystal structure {protein database (PDB) ID #1QO3; ref. [36]}. Residues D249, D251 (CK-1 epitope), and N159 (Cwy-3 epitope) are shown in ball-and-stick form with yellow and blue bonds, respectively. (B) Interaction of Ly-49G^{B6} with D^d, based on Ly-49A-D^d complex (PDB ID #1QO3; ref. [36]). H-2D^d heavy chain, β2-microglobulin (β2m), and the Ly-49G^{B6} monomers are shown in blue, green, and red, respectively, and the interactions at sites 1 and 2 are boxed. Ly-49G^{B6} residues determining CK-1 (yellow) and Cwy-3 (blue) specificity are shown in ball-and-stick form. Images were created using the programs MOLSCRIPT [37] and Raster3D [38].

domains of the MHC I heavy chain and β2m. The site 2 region has been shown to be important for Ly-49A and -C interactions with class I ligands; this may also be the case for Ly-49G. Focusing on site 2, co-crystal structures of Ly-49A and -C with their respective ligands indicate that Ly-49A and -C share a group of conserved residues within the Ly-49 family, which include S236, R239, and D/E241, forming a patch that interacts with class I ligands. Ly-49A has multiple, additional contacts with class I and β2m at site 2, beyond those observed with Ly-49C, particularly with residue Q29 of β2m [36]. These additional contacts are mediated largely by variable residues within the Ly-49 family, particularly of the β4–β5 loop [41]. The epitope recognized by Cwy-3 maps to a site on Ly-49G^{B6} that is distant from potential sites of class I interaction (Fig. 7B) and is consistent with our observation that this antibody is unable to block a functional interaction of Ly-49G^{B6} with D^{d}. It should be mentioned that Cwy-3 can block Ly-49G^{B6} interaction with a class I ligand but only when a large, multivalent molecule such as protein G is used as a secondary reagent, presumably enhancing steric hindrance [25]. In contrast, CK-1 binds residues of the Ly-49G^{B6} β4–β5 loop, which are predicted to interact directly with class I at site 2 and site 1 (Fig. 7B), and we have shown that CK-1 is quite effective at disrupting functional MHC I interaction with the Ly-49G^{B6} ectodomain. As the β4–β5 loop is distinct from the conserved 236–241 patch and used by Ly-49A but not Ly-49C for MHC I interaction, our results with CK-1 suggest that Ly-49G binds D^{d} in a manner similar to Ly-49A but not Ly-49C. We have also shown that the β4–β5 loop is important in determining the class I allele specificity of Ly-49G-related activating receptors by mutagenesis [41]. The effectiveness of CK-1 in blocking responses and the mapping of its epitope to the β4–β5 loop are complementary to results obtained with Ly-49 mutagenesis and cocrystal structures [36, 39, 40] and emphasize the importance of the β4–β5 loop in MHC I recognition by Ly-49A and -G receptors.

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