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Role of Conserved Glycosylation Site Unique to Murine Class I MHC in Recognition by Ly-49 NK Cell Receptor

Rebecca H. Lian,* J. Douglas Freeman,* Dixie L. Mager,*† and Fumio Takei‡‡

The recognition of class I MHC molecules on target cells by the Ly-49 family of receptors regulates NK cytotoxicity. Previous studies have suggested that carbohydrates are involved in the recognition of class I MHC by Ly-49, although their precise role remains unclear. Here, we examined the role of asparagine-linked carbohydrates of the murine class I MHC in the binding to Ly-49A and Ly-49C. We have generated H-2Dd mutants that lack the highly conserved glycosylation sites at amino acid residues 86 in the α1 domain and 176 in the α2 domain, respectively. These mutant Dd cDNAs were transfected into leukemic cell lines, and the binding of the transfected cells to COS cells expressing Ly-49A or Ly-49C, as well as their susceptibility to lysis by Ly-49A+ NK cells, was examined. Only the mutation of the α2 domain glycosylation site significantly reduced the binding of Dd to Ly-49A and Ly-49C. Cells expressing Dd with the mutation at this site were partially resistant to killing by Ly-49A+ NK cells. These results suggest that, while carbohydrates linked to residue 176 seem to function as a part of the ligand structure for the Ly-49 family of NK receptors, there are additional structural features involved in this recognition. This glycosylation site is highly conserved among murine class I MHC but is not found among those of other species, suggesting that its role is unique to the murine immune system. It further suggests that murine class I MHC and Ly-49 gene families may have evolved in concert. The Journal of Immunology, 1998, 161: 2301–2306.

In many systems, expression of class I MHC molecules on target cells inversely correlates with their susceptibility to NK cell lysis (1–4). The “missing self” hypothesis states that NK cells distinguish between normal and aberrant cells by recognizing and killing targets that lack self class I MHC (1, 5). Recent findings of MHC-specific inhibitory receptors on NK cells have provided the molecular basis for this hypothesis. In mice, the Ly-49 family of type II transmembrane proteins containing C-type lectin domains has been identified to be NK receptors for class I MHC. Of the nine Ly-49 molecules (Ly-49A-I) thus far identified, Ly-49A and Ly-49C are the best characterized. Ly-49A has been shown by Ab inhibition studies to bind specifically to the H-2Dd and H-2Kb haplotypes, resulting in the inability of Ly-49A− NK cells to kill targets of these haplotypes in vitro and in vivo (6–8). The interaction between Ly-49A and class I MHC has also been demonstrated by the adhesion of Ly-49A+ lymphoma cells to purified and immobilized Dd and Kb (9). Similarly, Ly-49C binds certain class I MHC molecules, including H-2Dd, Kd, and Kb (10, 11), and functions as an inhibitory receptor for NK cells (12). Binding studies using various blocking mAbs have suggested that Ly-49 binds to the α1/α2 domains of class I MHC (13).

Ly-49 contains a putative carbohydrate recognition domain (14–16), and previous studies have shown that Ly-49A and -C bind sulfated polysaccharides (17, 18). Furthermore, treatment of cells expressing class I MHC with tunicamycin to inhibit glycosylation (17) or with fucosidase (18) inhibited binding of class I MHC to Ly-49A or -C. These results suggested an involvement of carbohydrates in the recognition of class I MHC. However, the precise role of carbohydrates on class I MHC in its interaction with the Ly-49 family of NK inhibitory receptors has not yet been determined. The murine class I MHC molecules have two conserved asparagine-N-linked glycosylation sites, amino acid residues at positions 86 in the α1 domain and 176 in the α2 domain. In this study, we report that the glycosylation at residue 176, but not residue 86, is required for the binding of class I MHC to Ly-49A and Ly-49C. We also report that other structural features are likely to be involved in this interaction. Interestingly, unlike residue 86, which is invariably conserved among all class I MHC molecules thus far sequenced (19–22), the glycosylation site at residue 176 is conserved only among murine class I MHC and not those of other species. These findings suggest that the Ly-49 family of murine NK inhibitory receptors appear to have evolved simultaneously with murine class I MHC molecules.

Materials and Methods

Abs and flow cytometry

The 5E6 (anti-Ly-49C and -I) and YE1/48 (anti-Ly-49A) mAbs have been described (23, 24). The 34-5-8S, 34-2-12S, and 34-4-20S hybridomas (anti-Dd) were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Flow cytometric analysis was done as described (11). Briefly, 5 × 10⁶ cells were incubated for 30 min on ice with the appropriate hybridoma supernatants, followed by two washes with HBSS (containing 2% FCS) and an incubation with an FITC-conjugated secondary Ab. After a final rinse with HBSS/FBS, cells were stained with propidium iodide for detection of nonviable cells. Flow cytometric analysis was subsequently performed on the FACScan (Becton Dickinson, Mountain View, CA).

Site-directed mutagenesis

The cDNA encoding Dd was isolated by RT-PCR from the murine B lymphoma line A20. The Dd mut1 in which serine 88 is replaced by glycine was constructed using overlapping polymerase chain reaction (PCR) technology. The Dd mut1 was rescued from the mutant with an additional glycosylation site at amino acid residue 88 that has been used in vivo to assess the role of the glycosylation site in vivo. The murine class I MHC molecules used in these studies were generated by a two-step PCR using Pfu I polymerase (Stratagene). The cDNA encoding Dd was isolated by RT-PCR from the murine B lymphoma line A20. The Dd mut1 in which serine 88 is replaced by glycine was constructed using overlapping PCR technology. The Dd mut1 was rescued from the mutant with an additional glycosylation site at amino acid residue 88 that has been used in vivo to assess the role of the glycosylation site in vivo.

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Ly-49 and GLYCOSYLATION SITE OF CLASS I MHC

Results

Expression of N-linked glycosylation-deficient H-2 Dd

To determine the role of the conserved N-linked carbohydrates of class I MHC in the recognition of class I MHC by Ly-49, two glycosylation-deficient mutants of Dd were generated. The Dd mut1 lacks the glycosylation site at residue 86 due to a replacement of serine by glycine at residue 88. In the Dd mut2, residue 176 is deficient in glycosylation due to a substitution of threonine at residue 178 by glycine. The wild-type and mutant Dd cDNA clones were subsequently transfected into the murine erythroleukemic cell line GM979, the rat basophilic leukemia line RBL-1, and the murine lymphoma cell line C1498, and transfectants expressing Dd were established. Flow cytometric analysis of the transfected cells using the conformation-dependent anti-Dd mAb 34-5-8 showed that wild-type Dd and both mutant Dd were expressed at comparable levels (Fig. 1A). No differential binding of other mAb that recognize Dd, including 34-4-21, 34-2-12, and 34-4-20, to wild-type and mutant Dd was observed (data not shown), indicating that the mutations did not significantly disrupt the conformation of Dd. To verify that the expressed mutant Dd proteins were indeed deficient at one glycosylation site, Dd was immunoprecipitated from the transfected cells using the 34-5-8 mAb and analyzed by SDS-PAGE. The mutant Dd had lower apparent molecular mass than that of the wild-type (Fig. 1B), presumably because lack of glycosylation at the mutated sites would reduce the overall mass. Treatment of immunoprecipitated wild-type Dd with N-glycosidase F for 6 h to cleave off N-linked carbohydrates yielded three bands in SDS-PAGE, corresponding to undigested, partially digested, and fully digested Dd (Fig. 1B, upper panel). The apparent size of the two mutant Dd corresponded to the partially digested wild-type molecule, and treatment with N-glycosidase F reduced the sizes of mutant Dd to that of the fully digested wild-type Dd. Furthermore, prolonged treatment with the enzyme subsequently resulted in complete deglycosylation of all the Dd molecules to its lowest molecular mass (Fig. 1B, lower panel). These results confirmed that the glycosylation of Dd was indeed disrupted by the mutations as expected.

Binding of mutant Dd to Ly-49A and Ly-49C

Binding of mutant Dd to Ly-49 was determined by adhesion of Dd-transfected cells to Ly-49-transfected COS cells. Flow cytometric analysis showed high levels of Ly-49A and Ly-49C expression on the transfected COS cells (Fig. 2A). GM979 transfected with wild-type Dd readily bound to Ly-49A-transfected COS cells (Fig. 2B). Similarly, GM979 transfected with Dd mut1 also bound to Ly-49A-transfected COS cells, and no significant effect of this mutation was observed in the binding assay. In contrast, Dd mut2-transfected GM979 failed to bind to Ly-49A-transfected COS cells. This was not due to the expression level of this mutant Dd, because flow cytometric analysis (Fig. 1A) showed the level of this mutant Dd to be slightly higher than that of Dd mut1. None of the GM979 lines bound to control COS cells transfected with vector alone, whereas untransfected GM979 cells did not bind to Ly-49A-transfected COS cells, indicating that the binding in this assay is mediated by Dd and Ly-49A. Quantitative analysis using 35Cr-labeled transfected GM979 cells in the presence and absence of YE1/32 (anti-Ly-49A mAb) also confirmed the binding of wild-type Dd and Dd mut1 to Ly-49A (Fig. 2C). The binding of Dd mut2 was significantly lower even in the absence of the blocking Ab.

To examine the binding of mutant Dd to Ly-49C, the rat leukemia line RBL-1 was used, since endogenous class I MHC (H-2") on untransfected GM979 cells bound to Ly-49C-transfected COS cells. RBL-1 expressing wild-type Dd and Dd mut1 bound avidly

GGGGCGCAT-3' and 5'-CCCTGGTTGGTGAACCGGACC-3' (phosphorylated). The downstream PCR to generate a DNA fragment encoding the C-terminal portion of Dd was performed using primers 5'- CGCGGGCCGCTCTTCAACACT-3' (phosphorylated) and 5'-TT GAATTCATGTGTTAGTCTGGTGTGATAGGG-3'. The two PCR fragments were blunt-end ligated and used as a template for the second round of PCR that used the primers 5'-CTGGATCCCATGATGGGGCGAT-3' and 5'-TTGAATTCATGTGTTAGTCTGGTGTGATAGGG-3'. The Dd mut2 in which threonine in residue 178 is replaced by glycine was also generated in the same manner. The upstream PCR to generate the mutated DNA fragment was done using primers 5'-CTGGATCCCATGATGGGGCGAT-3' and 5'-CCCGAGTCTCCGCCTTCGAGATCT-3' (phosphorylated), and the downstream PCR was done using primers 5'-CTGGATCCCATGATGGGGCGAT-3' (phosphorylated) and 5'-TTGAATTCATGTGTTAGTCTGGTGTGATAGGG-3'. The second round of PCR was done using the same primers as those for the first mutation. The DNA fragments from the second round PCR were digested with BamHI and EcoRI, subcloned into pBluescript, and sequenced.

Transfection

The cell lines COS-1, RBL-1, GM979, and C1498 were obtained from ATCC and cultured in DMEM supplemented with 5% FCS. Ly-49A and -C cDNAs that had been cloned into the pAX142 expression vector were transfected into COS cells using Lipofectamine (Canadian Life Technologies, Burlington, ON) according to the manufacturer's protocols. Transfection of RBL-1, GM979, and C1498 cells were performed by electroporation at 0.45 kV and 125 μF, and stable transfectants resistant to G418 (Canadian Life Technologies) were subsequently selected for high expression by limiting dilution, panning, or cell sorting.

Immunoprecipitation

GM979 transfectants were surface biotinylated and lysed with lysis buffer (1% Triton X-100 lysis buffer containing 1% BSA, 150 mM NaCl, and 0.1% Na2HPO4, in 10 mM Tris-HCl, pH 7.5) as described (25). The clarified lysates were incubated overnight at 4°C with anti-Dd (34-5-8)-coupled beads (50 μl of a 1:1 slurry). The beads were then washed four times with lysis buffer (without BSA and Na2HPO4), and bound proteins were eluted with 100 μl 2× SDS sample buffer containing 50 mM DTT and boiling for 5 min. The eluted proteins were subsequently analyzed by SDS-PAGE and transferred onto nitrocellulose for Western blotting. Following incubation with peroxidase-conjugated streptavidin, proteins were detected using the ECL (Amersham, Arlington Heights, IL) chemiluminescence method.

N-glycosidase F treatment

Following immunoprecipitation as above, Dd bound to anti-Dd-mAb-coupled beads were washed and resuspended with PBS, treated with two units of N-glycosidase F (Boehringer Mannheim Canada, Laval, QC) for 6 h or 18 h at 37°C, and finally eluted with 100 μl 2× SDS sample buffer.

Binding assays

Twenty-four hours after transfection, COS cells were trypsinized and re-plated on 6-cm dishes (Falcon, Oxnard, CA). Forty-eighty hours later, Dd transfectants were added to COS cells. For the adhesion of RBL-1 transfectants, dishes containing COS cells were pretreated with heat-inactivated BSA (0.1% in PBS) for 1 h at 37°C to reduce nonspecific binding. Adhesion assays were performed for 2 h at 37°C or 15 min at room temperature for GM979 and RBL-1 cells, respectively. The plates were subsequently rinsed six times with prewarmed media and photographed on a Nikon Diaphot microscope. For quantitative assays, GM979 cells were radiolabeled with 35Cr (Mandel Scientific, Guelph, ON), and equivalent amounts of radioactivity were subsequently added to COS cells. After the unbound cells were removed by rinsing with prewarmed media, the remaining bound cells were lysed with 10% Triton X-100 and counted for radioactivity.

NK cell isolation

Spleens from C57BL/6 mice were homogenized and passed through a nylon wool column (Polysciences, Warrington, PA). Flow-through cells were then further enriched for NK cells using the immunomagnetic separation method, StemSep (StemCell Technologies, Vancouver, BC). The NK cells were subsequently cultured for 3 days in 1000 U/ml of murine IL-2 before purification of the Ly-49A subpopulation using the MACS separation method (Miltenyi Biotec, Auburn, CA). After 6 additional days of culture in the presence of IL-2, the Ly-49A NK cells were used in standard 3Cr release cytotoxicity assays.
zymatically treated with and GM979 cells. (A)efore, glycosylation at residue 176 but not residue 86, of Dd is important for the binding to Ly-49C as well as Ly-49A in this assay system.

**Cytotoxicity of mutant Dd by Ly-49A+ NK cells**

The binding of Dd on target cells to Ly-49A on NK cells has been shown to inhibit cytotoxicity of Ly-49A+ NK cells (7). Therefore, we tested whether the very low level of binding of Dd mut2 to Ly-49A results in defective inhibition in NK cytotoxicity. For this study, we were unable to use RBL-1 or GM979 cells, because the former labeled poorly with chromium and the latter cells were NK-resistant. Therefore, we transfected wild-type and mutant Dd into the murine leukemic cell line C1498, which is highly sensitive to NK cytotoxicity. The expression levels of Dd on the transfectants were comparable (Fig. 4A). In cytotoxicity assays, untransfected C1498 cells were readily lysed by Ly-49A+ NK cells. C1498-expressing wild-type Dd or Dd mut1 were resistant to Ly-49A+ NK cells, whereas Dd mut2-transfected C1498 cells were only partially resistant to Ly-49A+ NK cells (Fig. 4B). In four independent experiments, the cytotoxic killing of Dd mut2-transfected C1498 was consistently higher than that of wild-type Dd or Dd mut1 transfectants, while no statistically significant difference was seen between wild-type Dd and Dd mut1. The cytotoxicities were restored to the level of control untransfected C1498 cells in the presence of mAb to Ly-49A (YE1/48) or F(ab')2 fragment of anti-Dd (34-5-8), demonstrating that the resistance of these cells to NK killing is due to the specific recognition of Dd by Ly-49A. These results indicate that the recognition of Dd mut2 by Ly-49A is less efficient than that of wild-type Dd or Dd mut1.

**Discussion**

The results presented in this report have demonstrated that the conserved glycosylation site at residue 176 of Dd is involved in the interaction with the murine NK receptors Ly-49A and -C. In cell adhesion assays using Dd-transfected leukemic cells and Ly-49A-transfected COS cells, the mutant Dd (Dd mut2) lacking this glycosylation site did not significantly bind to Ly-49A or -C, whereas wild-type Dd and the mutant Dd (Dd mut1) lacking the other conserved glycosylation site at residue 86 readily mediated cell adhesion. In cytotoxicity assays, Dd mut2 only partially inhibited cytotoxicity of Ly-49A+ NK cells. The level of this inhibition was significantly lower than that of wild-type Dd or Dd mut1, which almost completely inhibited cytotoxicity of Ly-49A+ NK cells. The difference seen in the two assays is probably due to low affinity interaction between Dd and Ly-49A or -C. It is likely that binding of Dd-transfected cells to Ly-49-transfected COS cells requires a large number of Ly-49 molecules binding to Dd, allowing high enough avidity interaction to maintain cell-cell binding during the washing procedure. In contrast, inhibition of NK cytotoxicity may require much less receptor (Ly-49A)-ligand (Dd) interaction. Thus, while the affinity of Dd mut2 for Ly-49A seems too low to mediate cell-cell adhesion, it is sufficient to partially inhibit cytotoxicity of Ly-49A+ NK cells. It should be noted that the expression levels of Dd and the two mutant Dd on the transfected cells in these studies were comparable, and the mutations did not seem to affect the conformation of Dd as determined by the binding of conformation-dependent mAbs. Therefore, these results suggest that carbohydrates linked to residue 176, but not residue 86, modulate the affinity of Dd for Ly-49A.

The view that asparagine 176-linked carbohydrates on Dd plays a role in the interaction with Ly-49 is consistent with previous findings. Ly-49A and -C have been shown to bind certain polysaccharides (16–18). Furthermore, binding of class I MHC to Ly-49C is also inhibited by treatment of class I MHC-positive cells to both Ly-49A+ and Ly-49C+ COS cells whereas Dd mut2 showed no significant binding above background (Fig. 3). In all cases, the binding assays were scored in a blind fashion, and the results were consistent in three independent experiments. Therefore, glycosylation at residue 176 but not residue 86, of Dd is

[FIGURE 1. Analysis of wild-type and mutant Dd on transfected RBL-1 and GM979 cells. (A) Dd-transfected cells were stained with the 34-5-8 (anti-Dd) mAb plus FITC-conjugated anti-mouse Ig secondary Ab and analyzed by flow cytometry. (a) untransfected GM979, (b) GM979 transfected with wild-type Dd, (c) GM979 transfected with Dd mut1, (d) GM979 transfected with Dd mut2, (e) untransfected RBL-1, (f) RBL-1 transfected with wild-type Dd, (g) RBL-1 transfected with Dd mut1 (h) RBL-1 transfected with Dd mut2. (B) Dd-transfected GM979 cells were cell-surface biotinylated, immunoprecipitated with the anti-Dd mAb, 34-5-8, and enzymatically treated with N-glycosidase F for 6 h (upper panel) or 18 h (lower panel) before SDS-PAGE and Western blot analysis. The biotinylated proteins were subsequently detected by probing with streptavidin conjugated to horseradish peroxidase.]
with tunicamycin (17) or fucosidase (18). These results indicate that carbohydrates on class I MHC are involved in the recognition by Ly-49. On the other hand, it is unlikely that the binding of class I MHC to Ly-49 is mediated solely by carbohydrates. It has been shown that recognition of class I MHC by Ly-49 depends on the conformation of the former since Ly-49A is unable to recognize class I MHC lacking bound peptides (13). Therefore, Ly-49 most probably recognizes a combination of carbohydrates and the peptide backbone structure of class I MHC. This is consistent with our previous finding that the binding of Ly-49 to class I MHC involves not only the carbohydrate recognition domain of Ly-49 but also a portion of the stalk region immediately adjacent to the carbohydrate recognition domain (14, 16). The involvement of carbohydrates of class I MHC in the binding to Ly-49 implies that NK cytotoxicity may be regulated not only by the expression level of class I MHC on target cells but also by the pattern of glycosylation of class I MHC.

While this manuscript was under review, Matsumoto et al. reported similar studies with different results (26). In their studies, mutations at the conserved glycosylation sites of Dd had no detectable effects on inhibition of NK cytotoxicity or binding of Dd-transfected C1498 cells to Ly-49A-transfected Chinese hamster ovary cells. The reasons for the discrepancies are unknown at this time, but they may be due to differences in cells used, mutations (substitutions of asparagines with glutamines in their studies vs substitution of serine or threonine with glycine in this study), or expression levels of Dd on target cells. Our studies have shown that mutation of the glycosylation site at residue 176 reduces the binding of Dd to Ly-49, but the binding is sufficient to induce partial inhibition of cytotoxicity of Ly-49A

NK cells. It is conceivable that, if the expression level of this mutant Dd on target cells is high enough, it can protect the targets from cytotoxicity.

It is of interest that glycosylation at residue 176, but not residue 86, of class I MHC is involved in the binding to Ly-49. Residues

FIGURE 2. Adhesion of mutant Dd to Ly-49A and Ly-49C. (A) 72 h following transfection, COS cells were analyzed for Ly-49A and Ly-49C expression using the mAbs YE1/48 and SE6, respectively. The solid histograms represent COS cells transfected with Ly-49 cDNAs, and open histograms are cells transfected with the vector alone (pAX142). (B) Dd-transfected GM979 cells were incubated with Ly-49A-transfected COS cells for 2 h. Unbound cells were removed by gently swirling the media and removing it. The wash procedures were repeated six times. The remaining bound cells were photographed. (C) GM979 cells were labeled with 51Cr and incubated with COS cells in the presence or absence of YE1/32 anti-Ly-49A mAb. After removal of unbound cells by rinsing, bound cells were lysed with 10% Triton X-100, and cell lysates were then counted for radioactivity.
86 and 176 are located at the end of the α1 and α2 domains, respectively, and are outside of the peptide binding groove. The residue 86 glycosylation site is invariably conserved among all class I MHC thus far sequenced (19, 21). In contrast, the residue 176 glycosylation site is conserved only among murine class I MHC (Fig. 5). A survey of various class I MHC sequences in the data base showed that this glycosylation site in the α2 domain is conserved among all classical murine class I MHC including K^d, K^k, K^I, D^d, D^b, and D^f but is not found in any others examined. In addition to the class I MHC sequences listed in Figure 5, those of human (10 HLA-A, 30 HLA-B, 7 HLA-C), six rat, 13 chimpanzee, 5 orangutan, 14 gorilla, 3 baboon, 3 gibbon, 11 rhesus monkey, 6 cat, 3 rabbit, 3 cow, 5 horse, 2 cheetah, 2 squirrel, 2 frog, and 4 fish were examined. The glycosylation site at residue 176 was not found among any of the nonmurine class I MHC. This indicates that this glycosylation site in the α2 domain likely arose after the divergence of mice from other species and has become fixed in all murine class I MHC genes.

Carbohydrates on residue 86 are thought to be important for the binding of class I MHC to the chaperone calnexin during the assembly of class I MHC α-chain with β2 microglobulin and peptides (19, 27, 28). On the other hand, the functional role of carbohydrates on residue 176 of murine class I MHC has not yet been described. Since it is conserved among murine class I MHC and not found among others, its function should be uniquely attributed to mice. The results in this study show that carbohydrates on residue 176 function as a part of the ligand structure for the murine NK receptor Ly-49. Therefore, it is possible that murine class I MHC conserved this glycosylation site during evolution to maintain the function to serve as a ligand for the Ly-49 family. The complex and highly polymorphic Ly-49 family likely evolved in response to the rapid evolution of murine class I MHC. The lack of this N-linked glycosylation in class I MHC of other species suggests that the murine Ly-49 family diverged from other NK receptors with respect to how they recognize class I MHC on target cells. Although Ly-49 has also been found in rats (29, 30), direct interaction between rat Ly-49 and rat class I MHC has not been demonstrated. The absence of glycosylation sites in the α2 domain of rat class I MHC suggests that binding specificities of rat Ly-49 may be quite different from murine Ly-49. Moreover, efforts to isolate homologous human Ly-49 genes using mouse probes have
We thank Dr. V. Kumar for the 5E6 hybridoma.

FIGURE 5. Amino acid sequences of class I MHC of various species. The sequences were randomly selected from the GENBANK data base. Two of the rat class I MHC shown are RT1.Ab (Ab) and RT1.Ac (Ac).

not been successful. While a human Ly-49 gene family may exist, our results support the hypothesis that it may be significantly diverged from mouse Ly-49, reflecting the rapid evolution of genes involved in immune functions.

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