A species-specific determinant on β_2-microglobulin required for Ly49A recognition of its MHC class I ligand

Motoaki Mitsuki¹², Naoki Matsumoto¹ and Kazuo Yamamoto¹

¹Department of Integrated Biosciences, Graduate School of Frontier Sciences, the University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8562, Japan
²Glyco-chain Functions Laboratory, Supra-biomolecular System Group, RIKEN Frontier Research System, 2-1 Hirosawa, Wako-shi, Saitama 351-0198, Japan

Keywords: C-type lectin, cytotoxicity, inhibitory receptor, H-2 antigen, NK cell

Abstract

The mouse inhibitory NK cell receptor Ly49A recognizes the mouse MHC class I molecule H-2D^k. The present study focuses on the species specificity of β_2-microglobulin (β_2m), an invariant component of MHC class I, in the interaction between Ly49A and H-2D^k. Transfection of the β_2m-defective mouse cell line R1E/TL8x.1 with human (h) β_2m induced cell-surface expression of H-2D^k, but failed to protect the cells from killing by Ly49A⁺ NK cells. In contrast, the cells transfected with mouse (m) β_2m were protected from killing by Ly49A⁺ NK cells. These data indicate that Ly49A distinguishes mβ_2m from hβ_2m when it recognizes the H-2D^k complexes. To identify the species-specific determinant of β_2m required for Ly49A recognition of H-2D^k, we prepared a panel of mβ_2m mutants and tested the H-2D^k that included each of the β_2m mutants for its capacity to engage Ly49A on NK cells. Ly49A failed to functionally recognize the H-2D^k that included the mβ_2m with K3R and Q29G mutations. Moreover, Ly49A was able to recognize the H-2D^k that included the hβ_2m with R3K and G29Q mutations. These data indicate that Lys3 and Gln29 consist of the central part of the species-specific determinant of β_2m required for Ly49A recognition of H-2D^k. The two residues are conserved in the mouse and the rat, in which NK cells use Ly49 family molecules as the receptors specific for MHC class I. These results suggest functional importance of β_2m in NK cell recognition of target cells.

Introduction

NK cell recognition of target cells involves MHC class I molecules on the target cells (1). NK cells express inhibitory receptors specific for MHC class I molecules. The receptors transmit inhibitory signals to prevent NK cell killing of target cells expressing appropriate MHC class I ligands (2). The MHC class I-specific inhibitory NK cell receptors are classified into two groups by their structural features. One group of the receptors that belong to the C-type lectin superfamily includes mouse and rat Ly49 and CD94/NKG2 receptors, which are used in various species including mouse, rat and human. Another group of the receptors that belong to the Ig superfamily includes human killer cell Ig-like receptors (KIR) (3,4).

Mouse Ly49A, a member of Ly49 family, is a homodimer of type II transmembrane proteins (5,6), and recognizes the mouse MHC class I molecules H-2D^d, D^a (7,8) and D^p (9). A MHC class I molecule is a complex of the three non-covalently associating components: a heavy chain unique to each MHC class I molecule, an invariant β_2-microglobulin (β_2m) and a peptide of 8-9 amino acids. Ly49A recognition of the MHC class I molecules has been most extensively studied on that of H-2D^d. Ly49A recognizes a conformation of H-2D^d that depends on peptide binding (10,11), but does not depend on carbohydrate moieties of the H-2D^d heavy chain (12). The crystal structure of the Ly49A/H-2D^d complex revealed two Ly49A binding sites on a single molecule of H-2D^d, termed site 1 and site 2 (13). Site 1 includes the N-terminus of the α1 α-helix and the C-terminus of the α2 α-helix, while site 2 spans all the three structural domains of H-2D^d: α1/α2 and α3 domains, and β_2m. Of the two Ly49A binding sites on H-2D^d, site 2 is the functional binding site for Ly49A that leads to inhibition of NK cell cytotoxicity (14) and is also a major Ly49A binding site.
Ly49A recognition of its MHC class I ligand H-2Dk
detectable in physical binding assays (14,15). Two research
groups reported that efficient recognition of mouse MHC class I
ligands by Ly49A (14,16) and Ly49C (16) requires associ-
ation of the MHC class I heavy chains with mouse (m) β2m, and
that Ly49A and Ly49C are unable to bind MHC class I
molecules that include human (h) β2m as their subunits.
Nevertheless, the molecular details of the species-specific
determinant of β2m required for recognition of the MHC class I
ligands by Ly49 family receptors have not been experimentally
addressed.

In the present study, we constructed an experimental
system that enabled us to evaluate the ability of various β2m
mutants to make complexes with heavy chains of the MHC
class I H-2Dk and further evaluate the capacity of the H-2Dk
complexes to engage Ly49A. Using this system, we demon-
strate that the interaction between Ly49A and H-2Dk depends
on the species from which β2m that constitutes H-2Dk is
derived. We further demonstrate that residues 3 and 29 of β2m
define the species specificity of β2m in Ly49A recognition of H-
2Dk using various mutants of mβ2m and hβ2m. The results help
our understanding of the Ly49–MHC class I interactions, as
well as providing an insight into molecular evolution of β2m.

Methods

Mice
C57BL/6J mice were purchased from Nippon CLEA (Tokyo,
Japan); 8- to 17-week-old female mice were used for the
experiments.

Cells
A mouse H-2k lymphoma R1.1 and its β2m-defective mutant
R1E/TL8.x.1 (R1E), both of which are negative for Fcy
receptors, were obtained from the ATCC (Manassas, VA),
and were maintained in RPMI 1640 medium (Sigma, St Louis,
MO) supplemented with 10% heat-inactivated FBS, 2 mM
L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin,
50 μM 2-mercaptoethanol and 25 mM HEPES. Ly49A+ or
Ly49A- IL-2-activated NK cells were prepared from C57BL/6J
mouse splenocytes as described (12).

Antibodies
All mAb were purified using Protein A- or Protein G-affinity
chromatography from hybridoma culture supernatants. 15-5-
5S (anti-H-2Dk), S19.8 (anti-mβ2m), BBM1 (anti-hβ2m) and
MAR18.5 (anti-κ light chain) were obtained from ATCC. A1
(anti-Ly49A) was a gift from Dr Wayne M. Yokoyama
(Washington University School of Medicine, St Louis, MO).
Fluorescein-conjugated goat anti-mouse IgG F(ab')2 was
purchased from ICN Biomedicals (Irvine, CA).

Site-directed mutagenesis
Point mutations were introduced by sequential PCR steps as
described by Cormack (17). All single mutations were
amplified with overlapping primers encoding mutations, external 5’
primers (mβ2m EcoRI; 5’-GGAATTCAGTGCGTCCTT-3’,
mβ2m BglII; 5’-TCAGATCTGCTGGCGTCCTATTGTC-3’,
mβ2m SalI; 5’-CCAGTCGCAGATATCCATGAGTC-3’) and exter-
nal 3’ primers (mβ2m SalI 5’-CAGTGCACCATGATGTGA-
TCAC-3’, hβ2m SacII; 5’-CTGGAGCTCCACCGCGGT-3’) using KOD-plus-DNA polymerase (Toyobo, Osaka, Japan)
and mβ2m (C57BL/6J strain) or hβ2m cDNA (generous gifts
dr R. K. Ribaudo, Molecular Applications Group, Silver
Spring, MD) as templates. As external 5’ primers, the mβ2m
EcoRI primer was used to generate mK3R, mQ6K and mP47E
mutants, and the mβ2m BglII primer was used to generate the
other mouse mutant β2m. The mutant β2m cDNA fragments
were subcloned into the Smal site of pBluescript II SK
(Stratagene, La Jolla, CA) and sequences were confirmed
by a LS-2000 sequencer (Li-COR, Lincoln, NE). The mutant
β2m cDNA fragments were then cloned into the pApuro
expression vector (18).

Stable transfection of cells
R1E cells (4 × 105) were transfected in 0.5 ml of RPMI 1640
medium with 10–20 μg of linearized plasmids harboring
mutant β2m by electroporation using a BTX-600 Electro Cell
Manipulator (Harvard Apparatus, Holliston, MA) with the
following setting: 0.4-cm gap, 1200 μF, 300 V and 720 Ω.
The transfectants were selected and maintained in the same
medium as used for R1.E cells that was supplemented with 0.5
μg/ml of puromycin (Sigma).

Flow cytometry analysis
Cells (2–3 × 106) were stained with 10 μg/ml of the indicated
primary antibodies for 30 min at room temperature and then
with fluorescein-conjugated goat anti-mouse IgG F(ab')2 for
15 min on ice. The stained cells were analyzed using a FACS
Calibur with CellQuest software (BD Biosciences, San
Jose, CA).

Cell cytotoxicity assays
Specific killing of targets by NK cells was determined by
standard 4-h 51Cr-release assays as described (12). In brief,
cells were labeled with 50 μCi of [51Cr]sodium chromate
(Amersham Biosciences, Tokyo, Japan) for 90 min at 37°C.
The cells were washed and then incubated with Ly49A+ or
Ly49A- NK cells at the indicated E:T ratios in 96-well U-bottom
microplates (BD Biosciences) at 37°C for 4 h. Radioactivities
released into supernatants were scintillation-counted using a
TopCount scintillation counter (Perkin-Elmer, Boston, MA).
Specific cytotoxicity was calculated as previously described
(12).

Results

Failure of Ly49A to recognize H-2Dk that includes hβ2m
To investigate whether Ly49A recognizes the H-2Dk
that contains hβ2m as a subunit, we transfected the R1.E cell line,
which is a β2m-deficient mutant of Fcy receptor-negative
mouse lymphoma R1.1 (H-2k), with hβ2m or mβ2m cDNAs. The
transfectants were assayed for expression of H-2Dk
and hβ2m or mβ2m (Fig. 1A). R1.E cells transfected with both hβ2m or
mβ2m cDNAs expressed H-2Dk on the cell surface, while
untransfected R1.E cells did not express H-2Dk on the surface.
These results indicate that hβ2m forms a complex with the H-
2Dk heavy chain and induces H-2Dk expression on the cell
surface. We next tested the capacity of the H-2Dk that
Ly49A recognition of its MHC class I ligand H-2D<sup>e</sup> 199

Fig. 1. Recognition of the H-2D<sup>e</sup> molecules that contain mβ<sub>2m</sub> or hβ<sub>2m</sub> as subunits by Ly49A. (A) R1.1 cells, R1.E cells and R1.E transfecants (mβ<sub>2m</sub> or hβ<sub>2m</sub>) were stained with the anti-H-2D<sup>e</sup> mAb 15-5-5S (bold lines), anti-hβ<sub>2m</sub> or anti-mβ<sub>2m</sub> mAb (filled histograms) or the isotype-matched control mAb MAR18.5 (dotted lines) and then analyzed by flow cytometry. For staining for β<sub>2m</sub>, the R1.E transfecant with hβ<sub>2m</sub> was stained with the anti-hβ<sub>2m</sub> mAb BBM1, while the other cells were stained with the anti-mβ<sub>2m</sub> mAb S19.8. Mean fluorescence intensities (MFI) for anti-H-2D<sup>e</sup> staining are shown in the upper right corner of each panel. Of note, the R1.1 cells were transfected with a K58A mutant of mβ<sub>2m</sub> (Fig. 2A). However, H-2D<sup>e</sup>-specific cytotoxicity was not shown), indicating that low-level expression of H-2D<sup>k</sup> with MFI = 9.3, R1.E cells transfected with the wild-type mβ<sub>2m</sub> that expressed lower levels of H-2D<sup>e</sup> (MFI = 7.4) could be protected from killing by Ly49A<sup>+</sup> NK cells (data not shown), suggesting that low-level expression of H-2D<sup>e</sup> with MFI values as low as 7.4 is sufficient to protect the cells from killing by Ly49A<sup>+</sup> NK cells, if the H-2D<sup>e</sup> contained wild-type mβ<sub>2m</sub>. These results suggest that the functional interaction between Ly49A and H-2D<sup>e</sup> requires the side-chain of mβ<sub>2m</sub> Lys58.

Expression of the H-2D<sup>e</sup> that contained mβ<sub>2m</sub> mutants with the human type residues

In order to elucidate the mβ<sub>2m</sub> residues that determine the species specificity of β<sub>2m</sub> required for Ly49A recognition of H-2D<sup>e</sup>, we prepared a panel of mβ<sub>2m</sub> mutants, in which individual residues were substituted with the corresponding residues found in hβ<sub>2m</sub>. mβ<sub>2m</sub> and hβ<sub>2m</sub> both consist of 99 amino acid residues, but their amino acid sequences differ from each other by ~30% (Fig. 3). Ly49A recognizes the complexes of the H-2D<sup>d</sup> heavy chains and mβ<sub>2m</sub> or rat β<sub>2m</sub>, but fails to recognize those of the H-2D<sup>e</sup> heavy chains and hβ<sub>2m</sub> or bovine β<sub>2m</sub> (12,19). Considering the similar involvement of β<sub>2m</sub> in Ly49A recognition of H-2D<sup>d</sup> and D<sup>e</sup>, we chose residues of mβ<sub>2m</sub> that satisfied the following two criteria to introduce mutations: (i) the residues that are conserved in mouse and rat, but not in human and bovine β<sub>2m</sub>, and (ii) the residues that are exposed to the molecular surface of H-2D<sup>e</sup> (20,21) (Fig. 3). We prepared mutants of mβ<sub>2m</sub> in which mβ<sub>2m</sub> residues that satisfied the above criteria were replaced with the corresponding human residues.

We transfected R1.E cells with the mβ<sub>2m</sub> mutant cDNAs and assayed for cell-surface expression of H-2D<sup>e</sup> (Fig. 2A). R1.E cells transfected with most of the mβ<sub>2m</sub> mutant cDNAs contained hβ<sub>2m</sub> or mβ<sub>2m</sub> as a subunit to engage Ly49A and to protect R1.E cells from killing by Ly49A<sup>+</sup> IL-2-activated NK cells (Fig. 1B). Untransfected R1.E cells were efficiently killed by either Ly49A<sup>+</sup> and Ly49A<sup>+</sup> NK cells, and addition of an anti-Ly49A antibody did not affect NK cell cytotoxicity. R1.E cells transfected with mβ<sub>2m</sub> cDNA were protected from killing by Ly49A<sup>+</sup> NK cells and the protection was reversed by addition of an anti-Ly49A antibody, suggesting that Ly49A recognizes the H-2D<sup>k</sup> that includes mβ<sub>2m</sub>, in agreement with the previous study (7). Importantly, R1.E cells transfected with hβ<sub>2m</sub> cDNA were killed efficiently by Ly49A<sup>+</sup> NK cells despite the cell-surface expression of the H-2D<sup>e</sup> that included hβ<sub>2m</sub>. These data indicate that Ly49A recognizes the H-2D<sup>e</sup> that includes mβ<sub>2m</sub> and transmits negative signals to inhibit NK cell cytotoxicity, but is unable to recognize the H-2D<sup>e</sup> that contains hβ<sub>2m</sub>. These results also suggest that Ly49A recognition of H-2D<sup>e</sup> involves a species-specific determinant of β<sub>2m</sub> that also takes part in Ly49A recognition of H-2D<sup>k</sup> (14,16).

Previously, Wang et al. (15) examined the physical interaction between the recombinant soluble forms of Ly49A and H-2D<sup>d</sup> with BIAcore to demonstrate a pivotal role of Lys58 of mβ<sub>2m</sub> in the interaction between Ly49A and H-2D<sup>d</sup>. To investigate the role of Lys58 of mβ<sub>2m</sub> in the functional interaction between Ly49A and H-2D<sup>e</sup>, we established R1.E cells stably transfected with a K58A mutant of mβ<sub>2m</sub>. Transfection of R1.E cells with the mutant mβ<sub>2m</sub> induced cell-surface expression of H-2D<sup>e</sup> as in the case of the cells transfected with the wild-type mβ<sub>2m</sub> (Fig. 2A). However, H-2D<sup>e</sup> that contained the K58A mutant of mβ<sub>2m</sub> failed to protect the cells from killing by Ly49A<sup>+</sup> NK cells. Even though R1.E transfected with the K58A mutant of mβ<sub>2m</sub> expressed low levels of H-2D<sup>e</sup> (MFI = 7.4), R1.E cells transfected with the wild-type mβ<sub>2m</sub> that expressed lower levels of H-2D<sup>e</sup> (MFI = 7.4) could be protected from killing by Ly49A<sup>+</sup> NK cells (data not shown), indicating that low-level expression of H-2D<sup>e</sup> with MFI values as low as 7.4 is sufficient to protect the cells from killing by Ly49A<sup>+</sup> NK cells, if the H-2D<sup>e</sup> contained wild-type mβ<sub>2m</sub>. These results suggest that the functional interaction between Ly49A and H-2D<sup>e</sup> requires the side-chain of mβ<sub>2m</sub> Lys58.

Expression of the H-2D<sup>e</sup> that contained mβ<sub>2m</sub> mutants with the human type residues

In order to elucidate the mβ<sub>2m</sub> residues that determine the species specificity of β<sub>2m</sub> required for Ly49A recognition of H-2D<sup>e</sup>, we prepared a panel of mβ<sub>2m</sub> mutants, in which individual residues were substituted with the corresponding residues found in hβ<sub>2m</sub>. mβ<sub>2m</sub> and hβ<sub>2m</sub> both consist of 99 amino acid residues, but their amino acid sequences differ from each other by ~30% (Fig. 3). Ly49A recognizes the complexes of the H-2D<sup>d</sup> heavy chains and mβ<sub>2m</sub> or rat β<sub>2m</sub>, but fails to recognize those of the H-2D<sup>e</sup> heavy chains and hβ<sub>2m</sub> or bovine β<sub>2m</sub> (12,19). Considering the similar involvement of β<sub>2m</sub> in Ly49A recognition of H-2D<sup>d</sup> and D<sup>e</sup>, we chose residues of mβ<sub>2m</sub> that satisfied the following two criteria to introduce mutations: (i) the residues that are conserved in mouse and rat, but not in human and bovine β<sub>2m</sub>, and (ii) the residues that are exposed to the molecular surface of H-2D<sup>e</sup> (20,21) (Fig. 3). We prepared mutants of mβ<sub>2m</sub> in which mβ<sub>2m</sub> residues that satisfied the above criteria were replaced with the corresponding human residues.

We transfected R1.E cells with the mβ<sub>2m</sub> mutant cDNAs and assayed for cell-surface expression of H-2D<sup>e</sup> (Fig. 2A). R1.E cells transfected with most of the mβ<sub>2m</sub> mutant cDNAs...
expressed H-2D\textsuperscript{k} on the cell surface at levels comparable to that of H-2D\textsuperscript{k} on the R1.E cells transfected with wild-type m\textbeta\textgamma2m. However, two mutants of m\textbeta\textgamma2m (K3R and Q6K) did not induce cell-surface expression of H-2D\textsuperscript{k} (unpublished data). The m\textbeta\textgamma2m mutant-transfected clones with expression of H-2D\textsuperscript{k} comparable to the wild-type m\textbeta\textgamma2m transfectant were chosen and used in the following assays.

Ly49A failed to recognize the H-2D\textsuperscript{k} that contained m\textbeta\textgamma2m with K3R and Q29G mutations

To investigate the functional recognition of the H-2D\textsuperscript{k} that included each of the m\textbeta\textgamma2m mutants by Ly49A, we tested the R1.E cells transfected with a panel of m\textbeta\textgamma2m mutants for their protection from killing by Ly49A\textsuperscript{+} NK cells (Fig. 2B).

Expression of the single m\textbeta\textgamma2m mutants T75K, E89K and T92I as well as that of the wild-type m\textbeta\textgamma2m protected R1.E cells from killing by Ly49A\textsuperscript{+} NK cells and the protection was reversed in the presence of an anti-Ly49A antibody. In contrast, Q29G mutation partially impaired the protective activity of H-2D\textsuperscript{k}. Gln29 of m\textbeta\textgamma2m is in the close proximity of Lys3, of which substitution by Arg ablated ability of m\textbeta\textgamma2m to induce cell-surface expression of H-2D\textsuperscript{k}. Considering the possibility that Arg3 is incompatible with Gln29 in the milieu of m\textbeta\textgamma2m, but is compatible with Gly29, which is found in h\textbeta\textgamma2m, we produced a Q29G/K3R double mutant of m\textbeta\textgamma2m and transfected R1.E cells with the mutant. As we expected, the Q29G/K3R mutant of m\textbeta\textgamma2m was able to induce cell-surface expression of H-2D\textsuperscript{k} (Fig. 2A). The Q29G/K3R transfectants of
R1.E cells were also assayed for killing by Ly49A + NK cells (Fig. 2B). Importantly, the H-2D<sup>k</sup> that included m<sub>b<sup>2m</sup></sub> with K3R and Q29G mutations failed to protect the cells from killing by Ly49A + NK cells. These data suggest that Lys3 and Gln29 of m<sub>b<sup>2m</sup></sub> are required for Ly49A recognition of H-2D<sup>k</sup>.

Ly49A recognizes the H-2D<sup>k</sup> that includes h<sub>b<sup>2m</sup></sub> mutants with R3K and G29Q mutations

To further validate the above results, we designed an inverse experiment in which residues of h<sub>b<sup>2m</sup></sub> were substituted by the corresponding residues found in m<sub>b<sup>2m</sup></sub> and the H-2D<sup>k</sup> that contained each of the h<sub>b<sup>2m</sup></sub> mutants were tested for functional recognition by Ly49A. We introduced either one or both of R3K and G29Q mutations into h<sub>b<sup>2m</sup></sub>, and stably transfected R1.E cells with these h<sub>b<sup>2m</sup></sub> mutant cDNAs. Transfection of R1.E cells with any of the h<sub>b<sup>2m</sup></sub> mutant cDNAs induced cell-surface expression of H-2D<sup>k</sup> comparable to the wild-type h<sub>b<sup>2m</sup></sub> transfectant (Fig. 4A). The wild-type as well as mutant h<sub>b<sup>2m</sup></sub> transfectants were tested for killing by Ly49A + NK cells (Fig. 4B). Expression of the R3K/G29Q double mutant of h<sub>b<sup>2m</sup></sub> protected R1.E cells from killing by Ly49A + NK cells, while that of the R3K and G29Q single mutants of h<sub>b<sup>2m</sup></sub> failed to protect the cells. These data clearly indicate that simultaneous introduction of the R3K and G29Q mutations is sufficient to give h<sub>b<sup>2m</sup></sub> a capacity to constitute the H-2D<sup>k</sup> complex competent to functionally engage Ly49A.

**Discussion**

In the present study, we demonstrated that Ly49A was unable to recognize the H-2D<sup>k</sup> that included h<sub>b<sup>2m</sup></sub> as a subunit, but was able to recognize the H-2D<sup>k</sup> that included m<sub>b<sup>2m</sup></sub> in
functional assays. Furthermore, we found that the introduction of a K58A mutation into mβ2m, which significantly impairs physical interaction between Ly49A and H-2D\textsuperscript{d} (15), also impaired the capacity of H-2D\textsuperscript{d} to engage Ly49A in functional assays. These results clearly indicate that Ly49A recognizes the H-2D\textsuperscript{d} surface that includes β2m and the recognition depends on the species of β2m that constitute H-2D\textsuperscript{d}, as was shown for Ly49A recognition of another MHC class I ligand H-2D\textsuperscript{d} (14,16). Similarly, the physical interaction between Ly49C and H-2K\textsuperscript{d} also depends on the source of the β2m subunit (16). Once established that Ly49A recognition of H-2D\textsuperscript{d} depends on the species of β2m, we next sought to identify the species-specific determinant on mβ2m required for Ly49A recognition of H-2D\textsuperscript{d}. Our findings that Q29G single mutation of mβ2m partially impaired Ly49A recognition of H-2D\textsuperscript{d}, and simultaneous introduction of the K3R and Q29G mutations into mβ2m completely abrogated Ly49A recognition of H-2D\textsuperscript{d}, indicate that the two residues are essential for efficient Ly49A recognition of H-2D\textsuperscript{d} in the context of other residues of mβ2m. In the inverse experiment, in which residues of hβ2m were substituted by those found in mβ2m, Ly49A functionally recognized the H-2D\textsuperscript{d} that contained the hβ2m with R3K and Q29Q mutations, demonstrating that the two mutations are sufficient to endow hβ2m an ability to substitute mβ2m in Ly49A recognition of H-2D\textsuperscript{d}. These results clearly indicate that Lys3 and Gln29 determine the species specificity of β2m in Ly49A recognition of its ligand H-2D\textsuperscript{d}. These results also illustrate for the first time that the mβ2m residues Lys3, Gln29 and Lys58, the last two of which have been shown to contribute to the physical interaction between Ly49A and H-2D\textsuperscript{d} (15), play a critical role in the functional interaction between Ly49A and its MHC class I ligand.

The current findings can be interpreted in light of the crystal structure of the Ly49A/H-2D\textsuperscript{d} complex (13). Ly49A appears to contact similar residues of β2m when it binds H-2D\textsuperscript{d} and H-2D\textsuperscript{a}, since both bindings were sensitive to replacement of the mβ2m subunits with hβ2m, and also to K58A mutation on the mβ2m subunits as shown in this and the previous studies (14–16). In the Ly49A/H-2D\textsuperscript{d} complex the side-chains of Lys3, Gln29 and Lys58 of β2m form hydrogen bonds with the residues of Ly49A (Fig. 5). The structure of the Ly49A/H-2D\textsuperscript{d} complex can nicely explain our conclusion that Lys3 and Gln29 determine the species specificity of β2m in the interaction between Ly49A and H-2D\textsuperscript{d}, suggesting that our current findings can be extended to the interaction between Ly49A and H-2D\textsuperscript{a}.

Comparison of amino acid sequences of β2m from various species revealed that Lys3 and Gln29 are conserved in some animals of the family Muridae such as the house mouse, Mus musculus, and the Norway rat, Rattus norvegicus. The genomes of these animals bear multiple Ly49 genes (22–24). In particular, mouse NK cells are known to use Ly49 family receptors to monitor MHC class I expression. In contrast, in other species such as primates and bovine, each of their genomes bears multiple KIR genes, but has a unique gene homologous to Ly49 (2,4,25–28), which ultimately turned into a pseudogene in higher primates including the human. Moreover, human NK cells use KIR to monitor MHC class I expression, instead of Ly49. Thus, there appears to be a correlation between the conservation of Lys3 and Gln29 of β2m and the expression of multiple Ly49 receptors specific for MHC class I molecules, at least among the animals listed above. We propose that the selective pressure to maintain the effective interactions between Ly49 family molecules and MHC class I molecules had conserved Lys3 and Gln29 of β2m in the house mouse and the Norway rat. Conversely, acquisition of Lys3 and Gln29 by ancestral β2m in a common ancestor of the house mouse and the Norway rat might have enabled ancestral Ly49 to interact with MHC class I with an affinity high enough to monitor MHC class I expression;
subsequently, Ly49 family molecules might have rapidly expanded and evolved to those seen currently in the house mouse and the Norway rat, *Cricetulus griseus* (Swiss-Prot accession no. Q9WV24), and the hispid cotton rat, *Sigmodon hispidus* (Swiss-Prot accession no. Q8C1Q3), both of which also belong to the family Muridae, have sequences of intermediate type with Arg3 and Gin29. Whether NK cells in these animals use Ly49 family receptors or KIR to monitor MHC class I expression is an interesting question that remains to be answered.

Our current results together with the previous findings [14–16] demonstrated the inhibitory Ly49 receptors Ly49A and Ly49C use β2m as a part of the interface when they bind their MHC class I ligands. Interestingly, the activating receptor mouse Ly49D recognizes mouse H-2Dd and also a xenogeneic MHC class I molecule from the Chinese hamster (29), of which β2m has K3R substitution. The inhibitory receptor Ly49G2 also recognizes a structure(s) expressed on Chinese hamster ovary cells (30), even though the nature of the structure, including whether the structure is Chinese hamster MHC class I or not, remains elusive. Moreover, the other activating receptor mouse Ly49H recognizes the virally encoded xenogeneic MHC class I-like molecule m157, which does not associate with the β2m subunit (31). Thus, activating members of the Ly49 family and possibly an inhibitory member of the Ly49 family may not use β2m as a part of the interface when they recognize their xenogeneic ligands. Whether Ly49D uses β2m as a part of the interface when it recognizes H-2Dd, also, remains elusive.

The present study together with the previous studies [14–16] has also shown that β2m constitutes a part of the interface between MHC class I molecules and the NK cell receptors Ly49A and Ly49C. During the preparation of this manuscript, it was reported that the binding site for LIR-1, an inhibitory receptor of the Ig superfamily expressed on human leukocytes, on its MHC class I ligand also include the surface of β2m (32). Moreover, human (33) and mouse (34) CD8αα homodimers contact β2m residues when they bind classical MHC class I molecules. In particular, Lys58 of β2m plays a critical role in the interaction between human MHC class I and human CD8 (35). These studies, including ours, highlight the importance of β2m as a critical part of the interfaces between the MHC class I molecules and the immune receptors.

In conclusion, Ly49A recognition of H-2Dd includes species-specific residues of β2m, and the specificity is determined by Lys3 and Gin29 of β2m. These results contribute to our understanding of the molecular mechanism underlying Ly49A recognition of its MHC class I ligands, but also suggest the functional importance of β2m in NK cell recognition of target cells.

Acknowledgements

We thank Drs W. M. Yokoyama and R. K. Ribaudo for reagents. This study was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan (12672107 and 15590057) (N. M.), and a grant from the Kato Memorial Bioscience Foundation (N. M.).

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>β2m</td>
<td>β2-microglobulin</td>
</tr>
<tr>
<td>h</td>
<td>human</td>
</tr>
<tr>
<td>KIR</td>
<td>killer cell Ig-like receptor</td>
</tr>
<tr>
<td>m</td>
<td>mouse</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>R1.E</td>
<td>R1/E/TL8x.1</td>
</tr>
</tbody>
</table>

References


