Intracellular Cysteine Residues in the Tail of MHC Class I Proteins Are Crucial for Extracellular Recognition by Leukocyte Ig-Like Receptor 1


*J Immunol* 2007; 179:3655-3661; doi: 10.4049/jimmunol.179.6.3655
http://www.jimmunol.org/content/179/6/3655

References
This article cites 28 articles, 11 of which you can access for free at: http://www.jimmunol.org/content/179/6/3655.full#ref-list-1

Subscription
Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Intracellular Cysteine Residues in the Tail of MHC Class I Proteins Are Crucial for Extracellular Recognition by Leukocyte Ig-Like Receptor 1

Raizy Gruda,* Hagit Achkout,* Noam Stern-Ginossar,* Roi Gazit,* Gili Betser-Cohen,* Irit Manaster,* Gil Katz,* Tsufit Gonen-Gross,* Boaz Tirosh,† and Ofer Mandelboim2*

The activity of NK cells is regulated by activating receptors that recognize mainly stress-induced ligands and by inhibitory receptors that recognize mostly MHC class I proteins on target cells. Comparing the cytoplasmic tail sequences of various MHC class I proteins revealed the presence of unique cysteine residues in some of the MHC class I molecules which are absent in others. To study the role of these unique cysteines, we performed site specific mutagenesis, generating MHC class I molecules lacking these cysteines, and demonstrated that their expression on the cell surface was impaired. Surprisingly, we demonstrated that these cysteine residues are crucial for the surface binding of the leukocyte Ig-like receptor 1 inhibitory receptor to the MHC class I proteins, but not for the binding of the KIR2DL1 inhibitory receptor. In addition, we demonstrated that the cysteine residues in the cytoplasmic tail of MHC class I proteins are crucial for their egress from the endoplasmic reticulum and for their palmitoylation, thus probably affecting their expression on the cell surface. Finally, we show that the cysteine residues are important for proper extracellular conformation. Thus, although the interaction between leukocyte Ig-like receptor 1 and MHC class I proteins is formed between two extracellular surfaces, the intracellular components of MHC class I proteins play a crucial role in this recognition. The Journal of Immunology, 2007, 179: 3655–3661.

The MHC class I molecules are membrane glycoproteins expressed at varying levels on the surfaces of all mammalian somatic cells. Antigenic peptides presented by MHC class I molecules are mainly derived from proteolysis of proteins in the cytosol (1). MHC class I assembly and export from the endoplasmic reticulum (ER) is a complex process subject to a set of quality control mechanisms. This orchestrated process acts concertedly to ensure the proper assembly of MHC class I proteins and their expression on the cell surface (2).

NK cells are lymphocytes that provide innate immunity by killing virally infected or tumor cells independently of prior specific Ag stimulation (3). Reduced or absent cell surface expression of class I MHC molecules is one factor that renders a cell susceptible to NK cell cytotoxicity or whether the intracellular portion also plays a role in NK cell inhibition has been little studied.

In this study, we investigated the function of unique cysteine residues in the cytoplasmic tail (CT) of MHC class I alleles. This investigation revealed a crucial role for these cysteine residues in the ER egress of MHC class I proteins, in palmitoylation of these molecules, in the expression of the MHC class I molecules on the cell surface, and in the LIR1-mediated inhibition of NK cell cytotoxicity.

Materials and Methods

Cells, Abs, and fusion proteins

The cell line used in this work was the human B lymphoblastoid MHC class I-negative 721.221 (221) cell line. Primary NK cells were isolated from PBL using the human NK isolation kit according to the manufacturer’s instructions and the AutoMACS instrument (Miltenyi Biotec). LIR1 and KIR2DL1-positive NK clones were identified by flow cytometry using the anti-LIR1 mAb HPF1 and the anti-KIR2DL1 mAb HP3E4.

The Abs used in this work were BB7.1 (IgG1) directed against HLA-B7; W6/32 (IgG2a) directed against MHC class I, mem123 (IgG3), mem75, mem189 (IgG1), A14/F12 (IgG1), and A1.4 (IgG1), all directed against MHC class I (obtained from V. Horejsi, Prague, Czech Republic); the mAb HP3E4 (IgM) directed against the KIR2DL1; the mAb HPF1 (IgG1) directed against CD85J/LIR1; and the 12E7 (IgG1) directed against the CD99 which was used as a control mAb.

The production and purification of the LIR1-Ig and KIR2DL1-Ig fusion proteins were previously described (8).

Generation of 721.221 cells expressing mutated HLA-B7 and HLA-Cw6 proteins

For generation of the mutated MHC class I proteins, we amplified two overlapping fragments of the gene by PCR. The upstream fragment was...
amplified using a gene-specific 5’ end primer (including the HindIII restriction site) and an internal 3’ primer bearing the mutation. The downstream fragment was amplified using an internal 5’ primer bearing the mutation and a gene-specific 3’ end primer (including EcoRI restriction site). Next, both purified fragments were mixed together with the 5’ end primer and the 3’ end primer to generate the mutated full-gene cDNA. All different mutants of the same HLA-B7 gene were generated using appropriate edge primers and different internal primers. The various cDNAs were then cloned into the pcDNA3 mammalian expression vector and stably transfected into the 721.221 cell line. All transfecteds were periodically monitored for expression by staining with the BB7.1 mAb. The 5’ HLA-B7 end primer was CCACAAGCTTGGCCACATGCTGGT CAGTGGGCCCGCAGAAC and the 3’ HLA-B7 end primer was GGAATTCTCTAAGCTTTGAAGACACATCGAGA. For HLA-B7 C309W, the 5’ internal primer was TCTCGTGTATGTTGAGAGAAGAAGT and the 3’ internal primer was ACTCTTCTCCATCATCACAGCA. For HLA-B7 C326S, the 5’ internal primer was GTGCGTGCTCGAGCAGCT and the 3’ internal primer was AGGGTCGACGAGCGACA. For HLA-B7 C326S, the cdNA of HLA-B7 C309W was used with the internal primers for HLA-B7 C326S. The mutated HLA-Cw6 C309W molecule was generated in a similar way.

Measurement of MHC class I internalization
Cells were resuspended at 10⁵ in 100 µl of RPMI 1640/10% FCS and incubated on ice for 30 min with 0.1 µg of W6/32 mAb. Cells were then washed three times with RPMI 1640/10% FCS and incubated at 37°C in 5% CO₂. After various times of incubation, aliquots of cells were removed, washed three times with RPMI 1640/10% FCS and incubated at 37°C in 5% CO₂. After various times of incubation, aliquots of cells were removed, fixed by the addition of FACS medium (0.1% azide/1% BSA/PBS), and kept on ice. After an appropriate number of aliquots had been taken, cells were labeled with an excess of FITC-labeled goat anti-mouse IgG for 30 min and subsequently washed in FACS medium before analysis by flow cytometry.

Cytotoxicity assays
The cytotoxicity activity of LIR1 and KIR2DL1 NK clones against various targets was assessed in 5-h [³⁵S]methionine release assays as described previously (9). In experiments in which mAbs were included, the final concentration was 10 µg/ml. In all cytotoxicity assays performed, spontaneous release did not exceed 20% of the maximal labeling.

Pulse-labeling chase
Cells (5 × 10⁶/time point) were preincubated in methionine/cysteine-deficient DMEM (1 × 10⁶ cells/ml) for 1 h at 37°C, pulse labeled with 0.25 mCi of [³⁵S]methionine/cysteine (Amersham Pharmacia Biotech) for 15 min, then washed three times in a 10-fold excess of medium containing methionine/cysteine, and chased in RPMI 1640/10% FCS medium (1 × 10⁶ cells/ml). At the end of each time point, cells were washed four times with cold PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂.

Analysis of protein acylation
In brief, 1 × 10⁶ cells were resuspended in 2 ml of RPMI 1640 culture medium supplemented with 10% dialyzed FBS and 10 mM sodium pyruvate and subsequently labeled with 250 µCi of [³⁵S]methionine/cysteine (Amersham Pharmacia Biotech) in 1 ml of medium. The cells were labeled overnight and subsequently lysed, immunoprecipitated, and protein acylation was analyzed by gel electrophoresis and autoradiography.

Immunoprecipitation
Cells were detergent solubilized on ice in lysis buffer (PBS containing 150 mM NaCl, 50 mM Tris (pH 7.6), 0.5% Nonidet P-40, 9 mM iodoacetamide, 5 mM EDTA, 1 mM PMSF, and aprotinin). Lysates were centrifuged and supernatants were preclarred for 30 min at 4°C using protein A/G (Santa Cruz Biotechnology). Preclarred lysates were then immunoprecipitated for 2 h at 4°C using protein A/G, which were preincubated overnight at 4°C with the W6/32 mAb, and then were washed four times in lysis buffer.

Endoglycosidase H (EndoH) digestion
Proteins were digested with EndoH following the manufacturer’s instructions (NEB).

Gel electrophoresis and autoradiography
Samples were analyzed by SDS-PAGE on 10% acrylamide gels. For autoradiography, gels were fixed in 25% isopropanol/10% acetic acid for 30 min followed by treatment with Amplify solution (Amersham Pharmacia Biotech) for 30 min and then dried. Dried gels were exposed to x-ray film (Hyperfilm MP; Amersham Pharmacia Biotech) at 80°C for 24 h (³⁵S) or for 60 days (³H).

Results
The cysteine residues in the MHC class I protein tails are important for their expression on the cell surface
The contribution of the CT of the MHC class I molecules to their expression on the cell surface has been previously demonstrated (10). Hence, we were interested to see whether significant amino acid differences exist between the CTs of the different MHC class I proteins. Alignment of the HLA-A3, -B7, and -Cw6 proteins revealed that unique cysteine residues are present in the CT of the HLA-B7 and -Cw6, but are absent in the A3 protein (Fig. 1A). The number of cysteine residues in the CT of the different MHC class I varies; all HLA-A proteins lack cysteine residues (except for one in position 340, highlighted in bold in Fig. 1A), all HLA-B proteins contain either one or two cysteines (positions 309 and 326, highlighted in bold in Fig. 1A), while all HLA-C contain three or four cysteines (positions 309, 321, 326, and in several alleles) and, 340, highlighted in bold in Fig. 1A).

The steady-state expression of MHC class I molecules on the cell surface is a result of equilibrium between the rate of newly presented molecules and their internalization rate. To test whether the cysteine residues in the tail of the MHC class I molecules are important for their expression on the cell surface, we replaced the cysteines in the HLA-B7 tail with the corresponding residues of the HLA-A3 protein. Thus, Cys³⁰⁹ was mutated to Trp (C309W), Cys³²⁶ was mutated to Ser (C326S), and we have also generated a HLA-B7 protein in which the two cysteines were mutated (C309W and C326S).

The HLA-Cw6 proteins contain three cysteines in their CT (at positions 309, 321, and 340). Because Cys³⁴⁰ appears both in the HLA-A3 and in the HLA-Cw6 (see Fig. 1A), and because Cys³²¹ is not present in HLA-B7 (see Fig. 1A), we decided to mutate only Cys³⁰⁹ to Trp. The mutated alleles were transfected into 721.221 cells lacking endogenous MHC class I molecules and their cell surface expression was measured by staining with the BB7.1 or W6/32 mAb (Fig. 1, B–D). The MHC class I down-regulation of the HLA-B7 and HLA-C was measured in comparison to the mutated alleles. All mutated molecules were down-regulated from the cell surface more rapidly in comparison to the wild-type (WT) molecules (Fig. 2). A linear decrease in the expression of HLA-B7 was observed and, after 90 min, only ~75% of the mutated HLA-B7 proteins were left on the cell surface in comparison to 90% of the WT protein (Fig. 2A). Regarding HLA-Cw6, a rapid internalization was initially observed and, after 10 min, ~85% of the mutated proteins were left on the cell surface. Later, a slow gradual decrease was observed. After 90 min, 60% of the mutated HLA-Cw6 protein was expressed on the cell surface in comparison to 75% of the WT protein (Fig. 2B).

The cysteine residues in the tail of the MHC class I proteins are crucial for LIR1 function
The LIR1 inhibitory receptor, which is expressed on NK cells, binds MHC class I molecules with a broad specificity and transfers an inhibitory signal via the ITIMs located in its intracellular domain (11). We tested the recognition of the WT HLA-B7 and the mutated HLA-B7 by the LIR1 using the LIR1-Ig fusion protein. The LIR1-Ig fusion protein bound to the 721.221 cells that express the WT HLA-B7 molecules (Fig. 3A), but to our great surprise, the recognition of the HLA-B7 molecules was completely abolished when the cysteine residues in the CT were...
mutated (Fig. 3A). This difference cannot be attributed to the low stability of the mutated HLA-B7 proteins because the binding assay was performed on ice in the presence of azide and under these conditions the MHC class I molecules are stable on the cell surface (data not shown). Furthermore, it cannot be attributed to the levels of MHC class I proteins, since all HLA-B7 proteins are expressed on the cell surface at comparable levels (Fig. 1).

We further analyzed whether the reduced LIR1-Ig recognition affects the killing by NK cells using LIR1-positive NK clones (insets in Fig. 3, B–D). Killing assays were performed against 721.221 cells and against 721.221 cells that express the WT and mutated HLA-B7 proteins. As a positive control, we used 721.221 cells that express the HLA-G proteins, which bind the LIR1 receptor in high affinity. Both HLA-B7 and HLA-G inhibited the killing by the LIR1-positive NK clones, while the mutated HLA-B7 molecules did not (Fig. 3, B–D). This inhibition was specific because blocking the MHC molecules with the W6/32 mAb (which recognizes the HLA proteins at the LIR1 binding site) abolished the inhibition, while the control mAb 12E7 did not (Fig. 3, B–D).

The intracellular cysteines are not important for KIR2DL1 function

We next tested the binding of the LIR1-Ig fusion protein to the WT and to the mutated HLA-Cw6 and found that the recognition of the mutated HLA-Cw6 was also dramatically reduced (Fig. 4A). Please note that the binding level of the LIR1-Ig fusion protein to HLA-Cw6 was lower than the binding to HLA-B7 (mean fluorescence intensity (MFI) = 26 and 130, respectively; cf Figs. 3 and 4). Despite the fact that low levels of LIR-1 binding were still evident in the WT HLA-Cw6, they were not sufficient to inhibit the killing by LIR1-positive NK clones (data not shown). Therefore, we decided to study the recognition of the HLA-Cw6 vs the mutated HLA-Cw6 by the KIR2DL1 inhibitory receptor. Such an
investigation would also provide us with valuable information regarding the specificity of the LIR1-defective recognition of the mutated HLA-B7 proteins. Using the KIR2DL1-Ig fusion protein, we demonstrated that KIR2DL1 recognizes equally well HLA-Cw6 and HLA-Cw6 C309W proteins (Fig. 4B). Killing assays using KIR2DL1-positive NK clones against 721.221 expressing the WT or the mutated HLA-Cw6 molecules demonstrated that both molecules were able to inhibit the killing in a similar efficiency (Fig. 4C). The inhibition was specific because blocking of the KIR2DL1 receptor with the HP3E4 mAb abolished the inhibition, whereas a control Ab had no effect (Fig. 4C).

Mutated MHC class I molecules are not palmitoylated

The above results indicate that the cysteine residues in the tail of the MHC class I proteins are important for their expression on the cell surface and for their recognition by the LIR1 inhibitory receptor. What could be the mechanisms responsible for these phenomena? To answer this question, we decided first to investigate the biochemical properties of the mutated proteins.

S-palmitoylation is a posttranslational modification in which the 16-carbon fatty acid palmitate is covalently conjugated to cysteine residues through a thioester linkage (12). It has been previously demonstrated that some MHC class I molecules are palmitoylated while others are not, and it was suggested that the cysteine residues in the transmembrane region are fatty acylated (13). We therefore tested whether WT and cysteine-mutated MHC class I molecules are lipid modified. HLA-B7 molecules (mutated and WT) were
preincubated with radiolabeled palmitic acid and immunoprecipitated with W6/32. As can be seen in Fig. 5, the WT HLA-B7 was palmitoylated, while the mutated HLA-B7 molecules were not (Fig. 5, upper panel), despite the fact that the mutated HLA-B7 proteins were expressed to a similar level (Fig. 5, lower panel).

Mutations in cysteine residues in the CT of MHC class I molecules alter their biosynthesis and maturation

The cysteine residues in the tail of the MHC class I molecules may also have an effect on the biosynthesis and maturation of the MHC class I molecules. To analyze the biosynthesis and maturation, WT and mutated cells were radiolabeled with [35S]methionine for 15 min and then chased for 30–60 min. After the chase period, the cells were lysed, immunoprecipitated using the anti-MHC class I mAb W6/32, treated with or without EndoH, and analyzed on 10% SDS-PAGE.

As shown in Fig. 6, the WT and the mutated HLA-B7 molecules were all EndoH sensitive at time 0; however, marked differences were observed from 30 min on. The WT HLA-B7 proteins were completely EndoH resistant after 30 min of the pulse, indicating that the molecules trafficked into the Golgi and acquired the complex-type glycosylation. In marked contrast, the mutated HLA-B7 molecules showed nearly identical rates of trafficking that were significantly slower than that of the WT HLA-B7. Approximately 50% of the mutated HLA-B7 molecules were still EndoH sensitive even 60 min after the synthesis (Fig. 6). These results indicate that the egress of HLA-B7 molecule was severely impaired in the absence of the cysteine residues.

The intracellular cysteines affect the extracellular conformation of MHC class I proteins

Crystallography experiments demonstrated that the LIR1 inhibitory receptor binds the MHC class I molecule at the α3 domain, which is adjacent to the CT (14, 15), whereas the KIR2DL1 receptor recognizes the MHC class I proteins at the α1 and α2 domains (14, 16). Therefore, it might be possible that the mutations in the cysteine residues of the CT will alter the conformation, most likely at the nearby α3 domain, thus preventing the binding of the LIR1 receptor without affecting the binding of KIR2DL1.

To test this hypothesis, we examined the binding of a panel of mAb against MHC class I molecules to the WT HLA-B7 and to the mutated HLA-B7 molecules. Several mAb such as A14/F12, A1.4, BB7.1, and mem123 bound the WT and mutated alleles at a similar level, indicating that the expression levels of the different alleles are similar among the various transfectants (Fig. 7). Interestingly, however, the binding of other mAb such as mem75 and mem189 to the mutated HLA-B7 alleles was dramatically lower (Fig. 7), implying that the cysteine mutations had an influence on the level of cell surface expression of the HLA-B7 molecule.
conformation of the MHC molecules, as recognized by these particular Abs.

Discussion
The presence of MHC class I molecules at the cell surface is a function of generating a complete complex composed of H chain, L chain, and peptide in the groove (17). In addition, the cell surface expression of the MHC class I proteins can vary due to the balance between the rate of newly presented molecules and their internalization rates (18–20).

MHC class I molecules spontaneously internalize via coated pits and the endocytosed Ags are transferred from endosomes to the trans-Golgi reticulum and trans-Golgi cisternae, suggesting recycling of these Ags back to the cell surface (18–20). The contribution of the CT of the MHC class I molecules to their expression on the cell surface has been previously demonstrated (10). It was shown that the internalization of the MHC class I molecules requires a specific portion of the CT (21), which also includes the cysteine residues addressed here. We show a direct role of the cysteine residues in the CT of the HLA-B and HLA-C molecules that contribute to the expression of the MHC class I molecules on the cell surface (Fig. 2).

What is the mechanism responsible for the reduced expression of the mutated MHC class I proteins? During assembly and folding, newly synthesized MHC class I molecules undergo extensive quality control in the ER performed by an array of chaperones and assisting proteins (22). We show that the absence of the cysteine residues results in an impaired egress of the MHC class I proteins (23, 24). Thus, the complexes which leave the ER might be less stable, which may facilitate their down-regulation (as shown in Fig. 2).

We also show that the tail cysteines of the MHC class I molecules are subjected to S-palmitoylation. S-palmitoylation is the reversal addition of palmitate to proteins on cysteine residues via a thioester linkage (12). The structural features required for palmitoylation are poorly defined but cysteines that are close to membrane-interacting domains seem to be preferred. Palmitoylation increases the hydrophobicity of proteins and contributes to their membrane anchoring (12). Palmitoylation also influences the trafficking of some transmembrane proteins; it affects endocytosis, recycling, protein stability, and transport from the ER to the plasma membrane (23, 24). In addition, many palmitoylated proteins, including Src family kinases, associate with lipid rafts (25). Thus, the instability of the mutated MHC class I molecules presented here may also be explained by the lack of palmitoylation (Fig. 5).

The presence of MHC class I molecules on the cell surface is a main factor determining the fate of the cell once encountering NK cells. Reduced or absent cell surface expression of class I MHC molecules may render a cell susceptible to NK cell-mediated lysis, depending on the presence of activating ligands (4). MHC class I molecules are recognized by a broad spectrum of receptors on NK cells that mediate inhibitory signals that prevent the killing (26). In this study, we demonstrated that the cysteine residues in the CT of the MHC class I molecules are crucial for the recognition of the MHC class I molecules by the LIR1 receptor. It has been previously demonstrated (27) that the COOH-terminal portion of HLA-C is a determinant in inhibition of NK cell cytotoxicity and, in particular, the cysteine residue at position 309. However, the molecular mechanism responsible for this phenomenon and the receptors that are involved were not characterized.

In this study, we show that upon mutating the cysteine residues in the CT of MHC class I molecules the recognition of the MHC class I molecules by the LIR1 inhibitory receptor (Fig. 3A) and LIR1-mediated inhibition of NK clones (Fig. 3, B–D) were abolished.

The KIR2DL1 is another inhibitory receptor belonging to the KIR family, which recognizes MHC class I molecules and mediates inhibition of NK cells (5, 6). In contrast to the LIR1 receptor, the cysteine residues in the CT of the MHC class I molecules do not play a role in the recognition of the MHC class I molecules by the KIR2DL1 (Fig. 4, B and C). Concordantly with these results, it has been shown that NK clones that were EB6/HP5E4 positive (i.e., KIR2DL1 positive) recognize HLA-CW6 independently of the CT (27).

It is unlikely that the impaired ER egress of MHC class I molecules or the lack of S-palmitoylation or the consequent instability of MHC class I on the cell surface are responsible for the lack of inhibition by LIR, since these alterations should also affect KIR2DL1 function. Thus, other explanations should account for this phenomenon. Crystallographic experiments demonstrated that the LIR1 receptor binds the α3 domain and the β2-microglobulin of the MHC class I molecule (14, 15), while the KIR2DL1 receptor binds to the α1–α2 domains (14, 16). We therefore hypothesized that being adjacent to the CT, the conformation of the α3 domain may be effected when mutating the cysteine residues in the CT, whereas the conformation of the far α1–α2 domains is not. Thus, the recognition by the LIR1 and not by the KIR2DL1 may be disrupted. Indeed, by using a panel of mAb against MHC class I molecules, we were able to demonstrate that the conformation of the HLA-B7 molecule was altered as a result of mutating the cysteine residues in the CT (Fig. 7).

Since the first crystal structure of a protein was solved (28), much has developed in this field and three-dimensional interactions between many proteins have been demonstrated. When resolving interactions between membrane proteins by crystallographic methods, in most cases, the CT is not taken into account. In this study, we proved that cysteine residues in the CT play an inseparable part in determining these interactions. Indeed, nuclear magnetic resonance analysis of the LIR1-MHC class I complex indicates that upon complex formation, conformational changes are observed (15). Our results suggest that when the MHC class I proteins are expressed at the cell surface, the cysteine residues in the CT are important for proper MHC class I assembly and for egress from the ER, for S-palmitoylation of MHC class I molecules, and for a correct conformation at the cell surface. In the absence of the cysteine residues, MHC class I expression on the cell surface and recognition by the LIR1 receptor are impaired.

Acknowledgments
We thank Dr. Shoshana Israel and Shoshana Batat for MHC haplotyping. We thank Dr. Vaclav Horejsi for providing the anti-MHC class I Abs.

Disclosures
The authors have no financial conflict of interest.

References


