

Mechanisms underlying glycosylation-mediated loss of ecotropic receptor function in murine MDTF cells, and its implication for receptor evolution

Hiroaki Yoshii,^{1,2} Haruka Kamiyama,¹ Hiroshi Amanuma,³ Kazunori Oishi,^{2,4} Naoki Yamamoto,^{1,5} and Yoshinao Kubo¹

¹Department of AIDS Research, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan

²Department of Preventive and Therapeutic Research for Infectious Diseases, Institute of Tropical Medicine, Nagasaki University, Nagasaki, Japan

³Molecular Cell Science Laboratory, RIKEN, Saitama, Japan

⁴International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

⁵AIDS Research Center, National Institute of Infectious Diseases, Tokyo, Japan

Correspondence Yoshinao Kubo yoshinao@net.nagasaki-u.ac.jp

Summary; 188 words. Text; 4734 words. Six figures.

Running title: Ecotropic MLV receptor of MDTF cells

SUMMARY

A *Mus dunni* tail fibroblast (MDTF) cell line is highly resistant to infection by ecotropic Moloney murine leukemia virus (Mo-MLV). The cationic amino acid transporter type 1 (CAT1) paralogs of murine NIH 3T3 and MDTF cells (mCAT1 and dCAT1, respectively) contain two conserved N-linked glycosylation sites in the third extracellular loop (ECL3, the putative Mo-MLV binding site). Glycosylation of the dCAT1 inhibits Mo-MLV infection, but that of the mCAT1 does not. Compared to mCAT1, dCAT1 possesses an isoleucine-to-valine substitution at position 214 and a glycine insertion at position 236 in the ECL3. To determine the residues responsible for the loss of dCAT1 receptor function, we constructed mutants of the mCAT1. The mCAT1/insG receptor (wherein a glycine residue is inserted at mCAT1 position 236) has greatly reduced Mo-MLV receptor function compared to mCAT1. Treatment of mCAT1/insG-expressing cells with tunicamycin, an N-linked glycosylation inhibitor, increased the transduction titer. In addition, the reduced susceptibility to Mo-MLV observed with mCAT1/insG-expressing cells correlates with impaired binding of Mo-MLV. These results show a single amino acid insertion confers mCAT1 receptor properties to dCAT1 and provides an important insight into the co-evolution of virus-host interactions.

INTRODUCTION

The entry of ecotropic murine leukemia virus (Eco-MLV) into host cells is initiated by the interaction between the viral envelope glycoprotein (Env) and the receptor, cationic amino acid transporter type 1 (CAT1) (Albritton et al., 1989; Kim et al., 1991; Wang et al., 1991). The CAT1 protein is a 14-transmembrane multi-spanning molecule comprised of seven extracellular and six cytoplasmic loops with two conserved N-linked glycosylation sites (asparagines 223 and 229). The third extracellular loop (ECL3) appears to be critical for ecotropic retrovirus receptor function (Albritton et al., 1989; Overbaugh et al., 2001; Sommerfelt, 1999; Taylor et al., 2003). Previous studies have shown that the integrity of the YGE₂₃₅₋₂₃₇ motif in ECL3 is essential for MLV receptor function (Albritton et al., 1993; Yoshimoto et al., 1993).

Susceptibility to Eco-MLV is restricted to murine and certain rat cells. Glycosylated mCAT1 receptors retain full Mo-MLV receptor function (Kubo et al., 2002; Wang et al., 1996). Hamster CAT1 orthologs fail to function as Eco-MLV receptor but can be rendered functional after treatment with tunicamycin, an inhibitor of N-linked glycosylation (Miller and Miller, 1992; Wilson and Eiden, 1991). In addition, the rat CAT1 ortholog (rCAT1) and the *Mus dunni* CAT1 paralog (dCAT1) function poorly as Mo-MLV receptors due to glycosylation-dependent inhibition (Eiden et al., 1993, 1994; Kubo et al., 2002; Tavoloni and Rudenholz, 1997). The ECL3 of rCAT1 is two amino acids longer than the ECL3 of mCAT1 (a deletion of the lysine residue at position 222 and

insertions of serine, proline, and leucine at positions 226–228 compared with mCAT1). We previously reported that the extra amino acids in rCAT1 (compared with mCAT1) are associated with the inhibition of Eco-MLV infection by rCAT1 glycosylation (Kubo et al., 2004).

Mus dunni tail fibroblast (MDTF) cells are permissive for all four classes of MLV: ecotropic, xenotropic, polytropic, and amphotropic, with the notable exception of the ecotropic Moloney murine leukemia virus (Mo-MLV) (Chesebro and Wehrly, 1985; Lander and Chattopadhyay, 1984). It has been previously shown that pretreatment of MDTF cells with tunicamycin renders MDTF cells susceptible to Mo-MLV (Eiden et al., 1994). Compared to the mCAT1 protein, the dCAT1 protein possesses a substitution of isoleucine for valine at position 214 and a glycine insertion at position 236 in the ECL3, in addition to a substitution of asparagine for aspartic acid at position 373 and a substitution of threonine for alanine at position 590 (Eiden et al., 1993) (Fig. 1A). To determine which amino acid changes are responsible for the low susceptibility of dCAT1 to Mo-MLV infection, we constructed mutants of the mCAT1 receptor and elucidated the mechanism by which Mo-MLV infection is blocked in Mus dunni cells. The result observed in this study showed that the glycine insertion impairs the mCAT1 receptor function, and provides important insights into the CAT1 gene evolution as the ecotropic virus receptor.

METHODS

Expression plasmids. cDNA clones of mCAT1 and dCAT1 were kindly provided by Dr. J. M. Cunningham (Harvard Medical School, USA) (Albritton et al., 1989) and Dr. M. Eiden (NIAID, USA) (Eiden et al., 1993). A plasmid encoding the mCAT1/M3 mutant was kindly provided by Dr. D. Kabat (Oregon Health Science University, USA) (Wang et al., 1996). PCR was performed using these cDNA clones as templates with the following primers for C-terminal HA tagging: 5'-TTACTGCAGACAGATTTGCTCAGCGCGATG-3' and 5'-TCATGCGTAATCCGGAACATCGTACGGGTATTTGCACTGGTCCAAGTTGCTGTCAGGAGTCTT-3'. The latter antisense primer contains the HA epitope sequence as previously reported (Kubo et al., 2002). The PCR product was cloned into the pTarget vector (Promega) by TA cloning. The EcoRI-NotI fragment containing the HA-tagged CAT1 sequence was subcloned into the pMXpuro retrovirus vector (Onishi et al., 1996). A VSV-G expression plasmid (pHEF-VSVG) was obtained from Dr. L. Chang through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH, USA (Chang et al., 1999).

Mutagenesis. PCR-mediated mutagenesis was performed on the HA-tagged mCAT1- and mCAT1/M3-expressing plasmids using 5'-phosphorylated BamX (5'-CTATAGGGCGAATTCCGATCCTTGCTAGCC-3') and mutation primers. The sequences of the

mutation primers used to construct mCAT1/insG and mCAT1/I214V were as follows:

5'-AAACCCTCCCTCACCCCGTATTTACGTTTGT-3' and

5'-GAGCTGCCAGTTTTTAACGGAGCCTTTCACGAA-3'. The BamX primer has a nucleotide

substitution that disrupts the unique BamHI site of the pTarget vector. These primers were

synthesized by Nissinbo Inc. (Japan). The PCR amplified approximately 900 base-pair fragments,

which in turn provided PCR primers for the amplification of the complete plasmid containing the

desired mutations. The PCR product was treated with BamHI to digest the template plasmid and

was then transformed into *E. coli* DH5 α competent cells (TAKARA). The EcoRI-NotI fragment

containing the HA-tagged CAT1 mutant sequence was subcloned into the pMXpuro retroviral

vector. The nucleotide sequence of the mutant was confirmed by sequencing (Genenet and

Applied Biosystems).

Cells. Human glioma NP2 cells (Soda et al., 1999), human rhabdomyosarcoma TELCeB6 cells

that express both Mo-MLV gag-pol and the LacZ vector (Cosset et al., 1995), human embryo

kidney 293T cells, and *Mus dunni* tail fibroblast MDTF cells (Lander and Chattopadhyay, 1984)

were cultured in Dulbecco's modified Eagle's medium (Sigma, USA) at 37 °C in 5% CO₂. MDTF

cells were provided from American Type Culture Collection (ATCC). The culture media were

supplemented with 8% fetal bovine serum (Biofluids). CAT1-expressing NP2 cells were

constructed as follows: Human embryo kidney 293T cells were transfected with Mo-MLV gag-pol (3 µg), CAT1-expressing retroviral vector (3 µg), and VSV-G expression plasmids (3 µg) (Chang et al., 1999) using the TransIT LT1 reagent (30 µL) (Mirus). The cells were washed 24 hr after transfection and then cultured for 24 hr in fresh medium. The culture supernatant of the transfected cells was inoculated into NP2 cells that were subsequently selected by puromycin (10 µg/mL). The puromycin-resistant cell pool was utilized in this study.

Semi-quantitative RT-PCR. Total RNA samples were isolated from NP2 cells expressing the HA-tagged wild type mCAT1 and untagged dCAT1. First strand cDNA was synthesized by a reverse transcriptase (TAKARA) from the total RNA samples (1 µg). Semi-quantitative PCR was performed to detect CAT1 and GAPDH mRNAs. Nucleotide sequences of primers for the CAT1 RT-PCR were 5'-TCAAGCGTGGCAAGAGCCTGGAG-3' and 5'-TGCCCCTGACAGGACACCAGAGAA-3'. Nucleotide sequences of primers for the GAPDH RT-PCR were 5'-AGGTCGGAGTCAACGGATTTGGT-3' and 5'-GTGGGCCATGAGGTCCACCAC-3'. These primers were synthesized by Genenet Co. LTD.

Transduction assay. The ecotropic retroviral vector encoding the LacZ gene was generated by stable transfection of TELCeB6 cells, which express the Mo-MLV gag-pol genes and the

LacZ-containing retroviral vector genome (Cosset et al., 1995), using the wild-type Mo-MLV Env expression plasmid (Kubo and Amanuma, 2003). Target cells (2×10^5) were plated onto a 60-mm culture dish and cultured for 24 hr in either the presence or absence of tunicamycin (400 or 800 ng/mL). The cells were washed with medium to remove tunicamycin and then inoculated with the retroviral vector in the presence of polybrene (4 μ g/mL). After an additional 24 hr culture in fresh medium, the cells were stained with 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) (Wako); the cells stained blue were counted for an estimation of transduction titer.

Immunofluorescence microscopy. The HA-tagged CAT1-expressing NP2 cells were cultured on 4-well culture slides (Miles) in either the presence or absence of tunicamycin (800 ng/mL) for 24 hr. The cells were fixed with cold methanol and incubated first with a mouse anti-HA antibody (Covance) and then with an indocarbocyanine (Cy3)-conjugated anti-mouse IgG antibody (Sigma). The cells were observed using a confocal fluorescence microscopy (Leica).

Western immunoblotting. The HA-tagged CAT1-expressing cells (2×10^5) were plated and cultured for 48 hr. The cells were cultured for an additional 24 hr in either presence or absence of tunicamycin (400 or 800 ng/mL). Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (7.5%) and subsequently to Western immunoblotting

using the mouse anti-HA antibody and a horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Rad).

Virus binding. This virus-binding assay was performed as reported (Lavillette et al., 2000).

Cells (5×10^5) were incubated with viral stock for 30 min at 4 °C, after which unbound virus was removed by two washes with ice-cold 0.2% bovine serum albumin in phosphate-buffered saline; virus-cell complexes were incubated sequentially at 4 °C with goat anti-gp70 antiserum (1:500) and phycoerythrin (PE)-conjugated donkey anti-goat antiserum (1:100; Jackson Laboratories). The cells were then fixed in 1% formalin (Sigma). Fluorescence intensity was analyzed by flow cytometry (Coulter or Becton Dickinson). Parent NP2 cells and NP2/mCAT1 mutants were incubated in virus-free media and served as negative controls.

Statistical analysis. Differences between groups of data were determined by the Student's t-test.

Statistical significance was set at $p < 0.05$ for all tests.

RESULTS

Characterization of dCAT1 in human NP2 cells

MDTF cells are resistant for ecotropic Mo-MLV infection, and the resistance is counteracted by treatment with tunicamycin, an N-linked glycosylation inhibitor. To know whether the ecotropic receptor of MDTF cells or another cellular factor is responsible for this event, dCAT1-expressing human NP2 cells were constructed. To detect the dCAT1 protein expression, dCAT1 was C-terminally tagged with the HA epitope. Unfortunately, the HA-tagged dCAT1 protein was not detected by Western immunoblotting using an anti-HA antibody, and NP2 cells transduced by the HA-tagged dCAT1-encoding retrovirus vector were not susceptible to the Mo-MLV vector infection, indicating that the HA-tagged dCAT1 cannot be expressed. Therefore, untagged dCAT1-expressing NP2 cells were constructed.

Transduction titer of Mo-MLV vector in the untagged dCAT1-expressing NP2 cells was less than 10 % of that in untagged and tagged mCAT1-expressing cells (Figs. 2A and C), although mRNA levels of the dCAT1 and mCAT1 were similar (Fig. 2B). Serial dilution of the template first strand cDNA induced lower levels of PCR products, indicating that these PCR conditions were semi-quantitative. In addition, tunicamycin treatment (400 ng/ml) of the dCAT1-expressing cells enhanced transduction titer about 10 times (Fig. 2C), like MDTF cells (Eiden et al. 1994). These results indicate that the dCAT1 is determinant for the lower susceptibility of MDTF cells to the

Mo-MLV infection and for the glycosylation-mediated loss of receptor function.

Functional comparison of various mCAT mutant proteins

The dCAT1 protein has an isoleucine-to-valine substitution at position 214 and a glycine insertion at position 236 in the ECL3, compared with the mCAT1 sequence (Fig. 1A) (Eiden et al., 1994). The C-terminally HA-tagged dCAT1 protein was not detected by Western immunoblotting using the anti-HA antibody as was hamster CAT1 (Kubo et al., 2004). Therefore, we constructed mutants of HA-tagged mCAT1 (Kubo et al, 2002). The C-terminal HA tagging of the mCAT1 did not affect the receptor function (Fig. 2A).

To identify the residues that restrict the ability of dCAT1 to function as a Mo-MLV receptor, we constructed HA-tagged mCAT1 mutants and tested each for receptor function following their expression in human glioma NP2 cells (Soda et al., 1999). The following mutants (mCAT1/I214V, mCAT1/insG, mCAT1/N373D, and mCAT1/T590A) were constructed (Figs. 1A and B) and their sequences confirmed by sequencing. We engineered the expression of each mCAT1 mutant in NP2 cells using a previously described procedure (Kubo et al., 2004).

mCAT1/insG-expressing cells are resistant to Mo-MLV

mCAT1-, mCAT1/insG-, and mCAT1/I214V-expressing cells were pretreated with

tunicamycin (0, 400, and 800 ng/mL) for 24 hr to measure the effects of receptor glycosylation on Mo-MLV vector infection. Relative transduction titers compared to those obtained on untreated mCAT1-expressing cells are shown in Fig. 3A. Transduction titers of the Mo-MLV vectors in the mCAT1/insG-expressing cells were 10 times lower than those in the mCAT1-expressing cells ($p < 0.001$) like the dCAT1-expressing cells (Fig. 2), even though the expression levels of the mCAT1 and mCAT1/insG proteins were similar (Fig. 3B). The titers of the Mo-MLV vectors in mCAT1/insG-expressing cells were increased following treatment with tunicamycin in a dose-dependent manner ($p < 0.05$) like the dCAT1-expressing cells (Fig. 2). Transduction efficiency of the Mo-MLV vectors in mCAT1/I214V-expressing cells was comparable to those in the mCAT1-expressing cells. Transduction efficiencies of the Mo-MLV vectors in mCAT1/I214V-expressing cells were unaffected by tunicamycin treatment as was found for the mCAT1-expressing cells. Protein expression levels of the mCAT1 mutants were detected at levels similar to those of mCAT1 as assessed by Western immunoblotting using the anti-HA antibody (Fig. 3B). Tunicamycin treatment decreased the apparent molecular weight of the CAT1 proteins (~50 kDa), confirming that tunicamycin treatment inhibited CAT1 protein glycosylation. These results indicate that the inhibition of Mo-MLV infection by the glycine insertion results at least in part from receptor glycosylation, because transduction of tunicamycin-treated mCAT1/insG-expressing cells were lower than those of mCAT1-expressing cells.

The mCAT1/N373D- and mCAT1/T590A-expressing NP2 cells were as susceptible to Mo-MLV infection as were mCAT1-expressing cells (data not shown). Expression levels of both the mCAT1/N373D and mCAT1/T590A proteins were comparable to those of mCAT1 as determined by Western immunoblotting using the anti-HA antibody. These results indicate that the aspartic acid and alanine residues at positions 373 and 590, respectively, as well as the valine residue at position 214 in the dCAT1 protein are not associated with the reduced susceptibility to Mo-MLV of cells expressing dCAT1.

Glycosylation of dCAT1 inhibits Mo-MLV infection

To further assess the glycosylation-dependent reduction in Mo-MLV titer in cells expressing dCAT1, we constructed the mCAT1/M3insG mutant (Fig. 1A). Two conserved N-linked glycosylation sites (asparagines 223 and 229) are present in the ECL3 of both mCAT1 and dCAT1. The mCAT mutant, mCAT1/M3, lacks both of these N-linked glycosylation sites as provided by Dr. D. Kabat (Wang et al., 1996). Site-directed mutagenesis was performed to insert a codon encoding glycine at residue position 236 of the mCAT1/M3. The cells expressing mCAT1/WT were as susceptible to the Mo-MLV vector transduction as the cells expressing mCAT1/M3 (Fig. 4A). The transduction efficiencies of Mo-MLV vectors determined on cells expressing mCAT1/M3insG were 2–3 fold higher than those examined in the

mCAT1/insG-expressing cells yielding results similar to those seen after tunicamycin treatment of the mCAT1/insG-expressing cells ($p < 0.05$). This finding confirms the observation that the glycosylation of the mCAT1/insG protein inhibits Mo-MLV infection, but the glycosylation of mCAT1 does not. Therefore, glycosylation of the receptors from different animals per se does not always diminish receptor function. The molecular weight of the mCAT1/M3insG protein was lower than those of either the mCAT1 or mCAT1/insG protein, and that of the unglycosylated mCAT1/WT detected in tunicamycin-treated cells was similar to those of the mCAT1M3 and mCAT1/M3insG (Fig. 4B). Presumably this is attributable to ablation of the N-linked glycosylation sites in these proteins.

Binding of Mo-MLV to the mCAT1/insG is impaired

The mCAT1/M3insG-expressing and tunicamycin-treated mCAT1/insG-expressing cells were less susceptible to Mo-MLV infection than were mCAT1/WT-expressing cells (Figs. 3 and 4). This suggests that the mCAT1/insG protein has an additional factor that inhibits Mo-MLV infection in addition to the receptor glycosylation. Because the glycine residue at position 236 has been inserted into the virus-binding motif in the receptor (Albritton et al., 1993; Yoshimoto et al., 1993), we tested the ability of Mo-MLV vectors to bind to mCAT1 mutant-expressing NP2 cells (Lavillette et al., 2000). Cultured supernatants of either Mo-MLV Env-expressing TELCeB6 cells (Kubo and

Amanuma, 2003) or parental TELCeB6 cells (Cosset et al., 1995) (no Env virus, negative control) were exposed to target cells at 4 °C. Both the mCAT1/WT-, mCAT1/I214V-, and mCAT1/M3-expressing cells, which are equally susceptible to Mo-MLV infection, showed efficient binding of the virus (Figs. 5A and B). The mCAT1/insG-, mCAT1/M3insG-, and dCAT1-expressing cells, which are more resistant to Mo-MLV infection than are the mCAT1-expressing cells, showed much lower binding of the virus than did the mCAT1/WT-expressing cells. This result suggests that the binding of the Mo-MLV vectors to both mCAT1/insG and mCAT1/M3insG is impaired, indicating that the glycine insertion inhibits vector binding to the receptor independently of receptor glycosylation status.

Cell surface expression of mCAT1 mutants

To exclude the possibility that altered cellular localization of the mCAT1 mutants affects susceptibility to Mo-MLV infection, we analyzed the cellular distribution of the mutant proteins by immunofluorescence microscopy using the anti-HA antibody. Each mCAT1 mutant was detected on the cell surface and in the cytoplasm as seen for mCAT1-expressing cells (Fig. 6). Also, the cellular localization of each mCAT1 mutant was unaffected by tunicamycin treatment (400 ng/□L). This result indicates that both mutations and tunicamycin treatment have no effect on mCAT1 cellular localization.

DISCUSSION

Mechanism of glycosylation-mediated loss of receptor function in MDTF cells

Human CAT1 does not function as a Mo-MLV receptor, presumably because this receptor lacks a critical motif that is part of the virus-binding domain (YGE₂₃₅₋₂₃₇ in the ECL3) (Albritton et al., 1993; Yoshimoto et al., 1993). Susceptibility of hamster CAT1 to Eco-MLV infection is completely suppressed by a sugar chain linkage to the receptor, although the virus-binding domain is preserved (Wilson and Eiden, 1991). Eco-MLV infection is suppressed in part by a sugar chain linkage in rCAT1 (Kubo et al., 2002). Although mCAT1 is glycosylated as are both hamster CAT1 and rCAT1, it does not inhibit Eco-MLV infection. We previously reported that the extra amino acids in rCAT1 (compared with mCAT1) are associated with the inhibition of Eco-MLV infection by glycosylation at these residues (Kubo et al., 2004). Because the hamster CAT1 ortholog contains the extra amino acids that are a resident part of rCAT1 (Fig. 1C), a similar glycosylation mechanism may also inhibit viral infection in both hamster CAT1 and rCAT1.

The MDTF cell line expresses a paralog of the mCAT1, dCAT, and glycosylation of dCAT1 inhibits Mo-MLV infection (Eiden et al., 1993, 1994). The dCAT1 protein does not have the extra amino acids of rCAT1, but possesses a single amino-acid insertion and a single amino acid substitution in the ECL3 compared with mCAT1. The mechanism of inhibition of Mo-MLV infection by dCAT1 glycosylation appears to be different than those used by both rCAT1 and

hamster CAT1. Thus, we attempted to clarify the mechanisms of viral infection control by the glycosylation of dCAT1.

In this study, we constructed several mCAT1 mutants (Fig. 1B). Transduction titers of the Mo-MLV vectors in mCAT1/I214V-expressing cells were comparable to those expressing mCAT1/WT. The mCAT1/insG-expressing cells were more resistant to Mo-MLV than those expressing mCAT1/WT (Fig. 3A). Protein expression levels and cellular localization of the mCAT1 mutants were similar to those of the mCAT1/WT (Figs. 3B and 6). These results suggest that the glycine residue at position 236 in the ECL3 of dCAT1 is responsible for the failure of dCAT1 to efficiently function as a receptor for Mo-MLV. It has been reported that the valine residue of dCAT1 is determinant for the lower susceptibility to Mo-MLV infection (Eiden et al., 1994). However, in our study, mCAT1/I214V-expressing cells were as susceptible as the mCAT1/WT-expressing cells, indicating that the valine residue does not affect Mo-MLV infection. Further analysis is required to know the reason why the inconsistent results were observed.

Titers of Mo-MLV vectors in mCAT1/insG-expressing cells increased following treatment with tunicamycin (Fig. 3A). The transduction titers of the Mo-MLV vectors in the mCAT1/M3insG-expressing cells were 2–3 fold higher than cells expressing mCAT1/insG (Fig. 4A). These results suggest that the glycine residue introduced at position 236 induces a conformational change in the dCAT1 protein that inhibits viral infection by glycosylation.

The mCAT1/insG- and mCAT1/M3insG-expressing cells showed much lower binding of virus than did the mCAT1/WT- and mCAT1/M3-expressing cells. The dCAT1 protein has a glycine residue within the putative virus-binding site of mCAT1. Because viral entry is initiated by the binding of the viral envelope protein to the receptor at the target cell surface, this result indicates that the inserted glycine residue inhibits Mo-MLV infection by impairing the interaction between the Mo-MLV envelope and the CAT1 receptor. It has already reported that the glycine insertion in dCAT1 is responsible for lower susceptibility of MDTF cells to Mo-MLV infection (Bae et al., 2006). This study found the mechanism by which the glycine insertion into the position 236 of mCAT1 confers it less susceptible to the Mo-MLV infection; the glycosylation-mediated loss of receptor function and the inhibition of Env protein binding to the receptor protein.

The Mo-MLV vector particles bound equally to mCAT1/insG- and mCAT1/M3insG-expressing cells (Fig. 5). This result suggests that dCAT1 glycosylation does not suppress the Env-dCAT1 interaction, but affects the folding of mCAT1/insG protein as the Eco-MLV receptor (Helenius and Aebi, 2001 and 2004). However, the mCAT1/M3insG-expressing cells were 2-3 times more susceptible to Mo-MLV infection than the mCAT1/insG-expressing cells (Fig. 4A), and this extent of the difference in the Env-receptor interaction could be undetectable by this binding assay. Therefore, we cannot exclude the

possibility that the sugar chain linked to dCAT1 physically prevents efficient interaction of Mo-MLV Env and dCAT1.

Speculation on the co-evolution of Eco-MLV and receptor CAT1

Based on both the present results and previous reports, we put forward this thesis of co-evolution of Eco-MLV and CAT1. Rat CAT1 is a less efficient receptor for Eco-MLV than mCAT1 (Kubo et al., 2002; Takase-Yoden and Watanabe, 1999). We would hypothesize that the rCAT1 protein might over the course of years obtain extra residues for protection from Eco-MLV infection (Kubo et al., 2004). Hamster is completely resistant to Mo-MLV infection by CAT1 glycosylation (Wilson and Eiden, 1991). Hamster CAT1 has the extra amino acids as does rCAT1, and is additionally three amino acids longer than rCAT1 (Fig. 1C). Therefore, the gain of these residues in hamster CAT1 accompanied a loss of Mo-MLV receptor function. Although hamster CAT1 is completely resistant to Mo-MLV infection, it has been reported that neuropathogenic and hamster-adapted variants of ecotropic Friend MLV efficiently infect hamster cells (Ishimoto, 1985; Jung et al., 2004; Masuda et al., 1996). These findings indicate that certain ecotropic murine virus variants have also evolved to counteract the host defenses that are mediated by receptor glycosylation.

The six additional residues in the ECL3 are present in both human and mink CAT1s (Kubo

et al., 2002; Yoshimoto et al., 1991) (Fig. 1C). Even though dCAT1 does not have the extra residues present in rCAT1 and hamster CAT1, it is resistant to Mo-MLV. It appears that dCAT1 evolved from a mCAT1-type receptor independently of both rCAT1 and hamster CAT1, whereas human and mink CAT1s have evolved to combine features of both hamster and *Mus dunni* receptors. Finally, human and mink CAT1 acquired complete resistance to infection by all ecotropic murine retroviral variants as a consequence of the acquired mutations in the virus-binding domain.

It is most likely that a viral receptor protein directly acquires resistance to viral infection by mutations in its virus-binding domain. However, the CAT1 protein showed a complex evolutionary pathway. CAT1 consists of multiple membrane-spanning proteins and its expression requires an unknown complex mechanism, since many of the CAT1 mutants were not expressed (Kubo et al., 2004). It has been reported that knocking the *cat1* gene out in mice is lethal, indicating a critical role of CAT1 in growth control during mouse development (Perkins et al., 1997). These characteristics of the CAT1 protein may decrease the speed of evolution and cause a more complex evolutionary pathway. One of the reasons why most retroviral receptors have multiple membrane-spanning proteins like mCAT1 (Sommerfelt, 1999) might be the reduced evolutionary rate of mutation among transporter proteins.

These investigations into glycosylation mediated inactivation of ecotropic receptor provide

novel insights into the co-evolution of host–virus interactions. All of the gammaretrovirus receptors identified to date employ glycosylated carrier facilitator proteins as receptors (Tailor et al., 2003); therefore, it is not surprising that cell-specific addition of N-linked sugars to receptor proteins has evolved as a method for restricting efficient infection by gammaretroviruses (Overbaugh et al., 2001; Tailor et al., 2000). The ASCT1, Pit1, and Pit2 receptors have all been reported to be inactivated by glycosylation in some types of cells and hence fail to facilitate entry by the feline endogenous retrovirus RD114, gibbon ape leukemia virus (GALV), and amphotropic MLV, respectively. Thus glycosylation-mediated blocks to receptor function extend not only to other orthologs of CAT1 such as rCAT1 (Kubo et al., 2004) but to other gammaretroviral receptors as well.

ACKNOWLEDGEMENTS

We thank François-Loïc Cosset for providing TELCeB6 cells, Hiroo Hoshino for providing NP2 cells, Toshio Kitamura for providing the pMX-puro retroviral vector, James M. Cunningham for providing the mCAT1 cDNA clone, and David Kabat for providing the mCAT1/M3 expression plasmid. The VSV-G expression plasmid was provided from Lung-Ji Chang through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. We thank Maribeth V. Eiden for providing the dCAT1 cDNA clone and MDTF cell line, and for critical reading and editing of this manuscript. We also thank Koya Ariyoshi, Hironori Sato, and Toshifumi Matsuyama for discussions, and Chika Tominaga, Chiho Mitani, and Haruka Kamiyama for assistance. We wish to thank Kei Miyakawa for technical assistance with flow cytometry. This study was supported by a Health Science Research Grant from the Ministry of Health, Labour, and Welfare of Japan, and a Cooperative Research Grant of the Institute of Tropical Medicine, Nagasaki University.

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Figure Legends

Fig. 1. Comparison of the amino acid sequences of mCAT1, dCAT1, and constructed mCAT1 mutants. (A) Structure of HA-tagged mCAT1 mutant proteins. Four amino-acid differences distinguish mCAT1 from dCAT1. The wild-type mCAT1 and mCAT1 mutants were constructed to contain an HA epitope tag C-terminally. Symbols: open circle, substitution mutation; open square, insertion mutation; grey square, third extracellular loop. (B) Amino acid sequences of the third extracellular loops of mCAT1, dCAT1, and mCAT1 mutants are compared. Black box, N-linked glycosylation sites; gray box, Mo-MLV Env binding domain. (C) Amino acid sequences of the third extracellular loop of CAT1 proteins from various animals are indicated. Black box, N-linked glycosylation sites; gray box, Mo-MLV Env-binding domain in mCAT1.

Fig. 2. Relative susceptibilities of mCAT1- and dCAT1-expressing cells. (A) NP2 cell pools expressing the C-terminally HA-tagged mCAT1 (mCAT1/WT), untagged mCAT1 (mCAT1/WT HA(-)), and dCAT1 were inoculated with the Mo-MLV vector. Relative transduction titers to those in the mCAT1/WT-expressing cells are indicated. This experiment was repeated three times. Error bars show standard deviations. (B) Expression levels of the mCAT1/WT, mCAT1/WT-HA(-), and dCAT1 were analyzed by semiquantitative RT-PCR. To confirm quantitiveness of the RT-PCR, first strand cDNA prepared from the mCAT1/WT-expressing cells

was serially diluted by 2 times, and PCR was performed using the diluted cDNA as template. The PCR products were subjected to agarose gel electrophoresis. (C) Transduction titers in tunicamycin-treated cells mCAT1/WT- and dCAT1-expressing cells were measured. Cells were treated with 0 (open bar), 400 (gray bar), and 800 ng/ml (closed bar) for 24 hr. Relative transduction titers to those in untreated mCAT1/WT-expressing cells are indicated. This experiment was repeated three times. Error bars show standard deviations.

Fig. 3. Relative susceptibilities of each mCAT1 mutant-expressing cell pool to Mo-MLV infection and expression of C-terminally HA-tagged mCAT1 mutants in NP2 cells. (A) The mCAT1/WT-, mCAT1/I214V-, and mCAT1/insG expressing NP2 cell pools were treated with tunicamycin for 24 hr. Closed bar, transduction titer in untreated cells; gray bar, transduction titer in tunicamycin-treated cells (400 ng/ml); open bar, transduction titer in tunicamycin-treated cells (800 ng/ml). Relative transduction titers to those in untreated mCAT1/WT-expressing cells are indicated. This experiment was repeated three times. Error bars show standard deviations. (B) Cell lysates were prepared from treated (400 and 800 ng/mL) and untreated HA-tagged mCAT1 mutant-expressing cell pools. Cell lysates were electrophoresed on 7.5% polyacrylamide gels. Western immunoblotting was performed using an anti-HA antibody. Molecular weight standards are indicated on the left side.

Fig. 4. Effects of amino acid substitutions at the N-linked glycosylation sites of CAT1 on Mo-MLV infection and on molecular weights of HA-tagged mCAT1 mutant proteins. (A) Relative transduction titers in the mCAT1 mutant-expressing cells are indicated. The mCAT1/M3insG has no glycosylation site. This experiment was repeated three times. Error bars show standard deviations. (B) Cell lysates were prepared from untreated mCAT1/WT-, mCAT1/WT- mCAT1/M3, and mCAT1/M3insG-expressing cells and tunicamycin-treated mCAT1/WT-expressing cells. The cell lysates were electrophoresed on 7.5% polyacrylamide gels. Western immunoblotting was performed using an anti-HA antibody.

Fig. 5. Specific binding of the Mo-MLV Env glycoprotein to the CAT1 protein. The mCAT1 mutant-expressing cells were incubated with Mo-MLV particles at 4 °C for 1 hr, then with goat anti-envelope surface glycoprotein (SU) at 4 °C for 1 hr, and finally with PE-conjugated donkey anti-goat antiserum at 4 °C for 1 hr. Fluorescent intensities of the cells were analyzed by a flow cytometer of Coulter (panel A) or Becton Dickinson (panel B). Closed and open areas indicate the mCAT1 mutant-expressing cells incubated with Env-free viral particles (no Env) as negative control and with Mo-MLV Env-containing particles, respectively.

Fig. 6. Cell surface expression of the mCAT1/WT and mCAT1 mutant proteins. The CAT1-expressing cells were cultured on chamber slides, fixed with cold methanol, then treated with mouse anti-HA antibody, followed by treatment with Cy3-conjugated anti-mouse IgG antibody. The cells were observed under confocal fluorescence microscopy. Fluorescence and differential interference contrast images of the same fields are shown on the left and right panels, respectively.

Fig. 1A

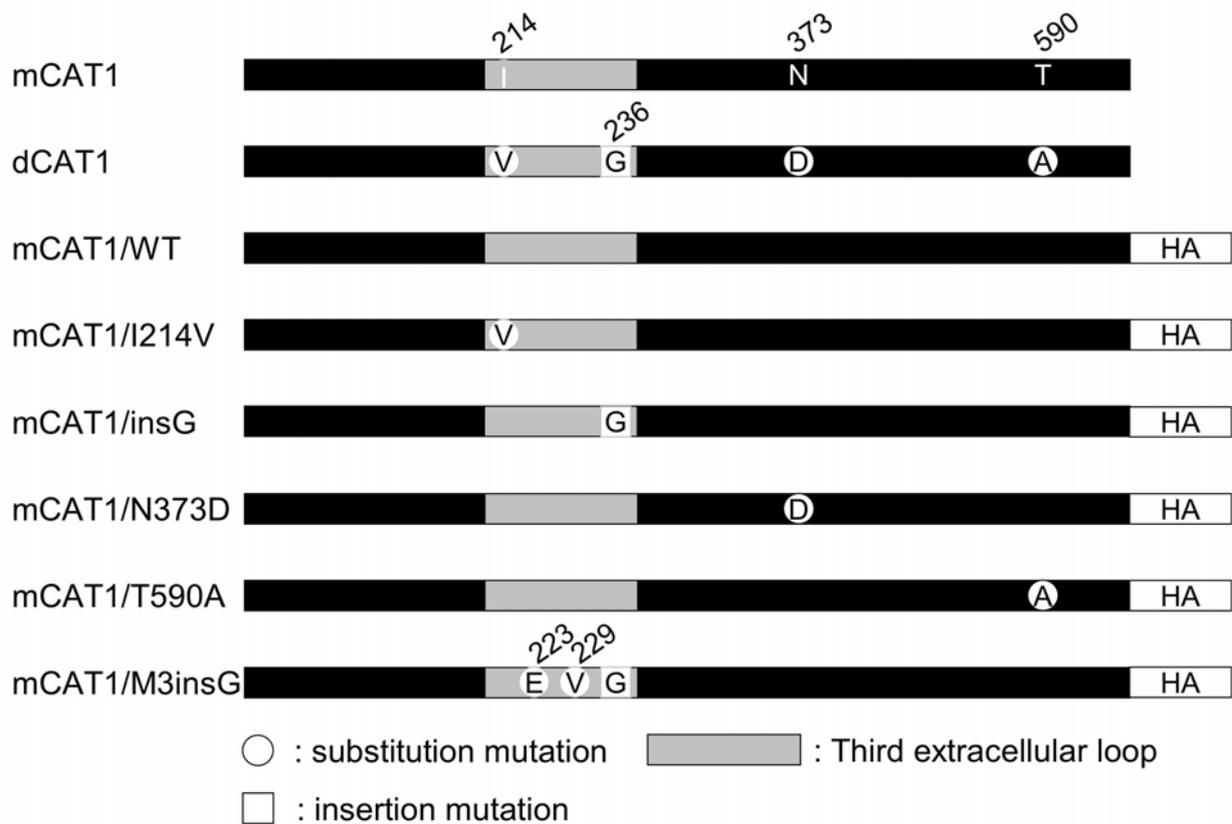


Fig. 1B

		210	220	230	240
<i>Mus musculus</i>	mCAT1	V K G S I K N W Q L T E K	N F S C N N	N D T N V K Y - G E	G G F M
<i>Mus dunni</i>	dCAT1 V G
Receptor mutants					
	mCAT1/I214V V
	mCAT1/insG G
	mCAT1/M3 E V
	mCAT1/M3insG E V G

Fig. 1C

Species	Cell line	Sequence
mouse	NIH 3T3	VKGS I KNWQLTEK - - - NFS - - - CNNNDT NVKY - GEGGF M
<i>Mus dunni</i>	MDTF	VKGSVKNWQLTEK - - - NFS - - - CNNNDT NVKYGGEGGF M
rat	F10	VKGS I ENWQLTE - - - NKSSPLCGNNDT NVKY - GEGGF M
hamster	CHO	VKGS I KNWQLTEEDFLNRSSPLCGNNDT NVKH - GEGGF M
mink	CCL64	VKGS I KNWQLTEEDFQNTSSHRCLSNDT KQGT LGAGGF M
human	Hut 78	VKGSVKNWQLTEEDFGNTSGPLCLNNDT KEGKPGVGGF M

Fig. 2A

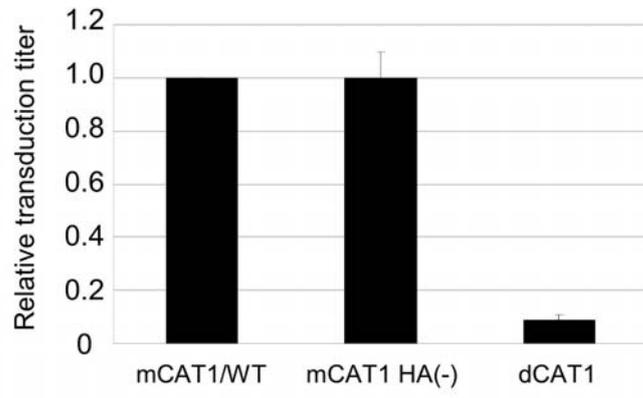


Fig. 2C

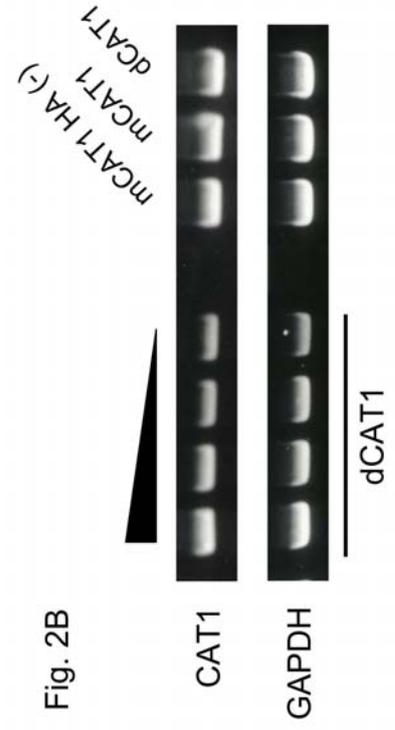
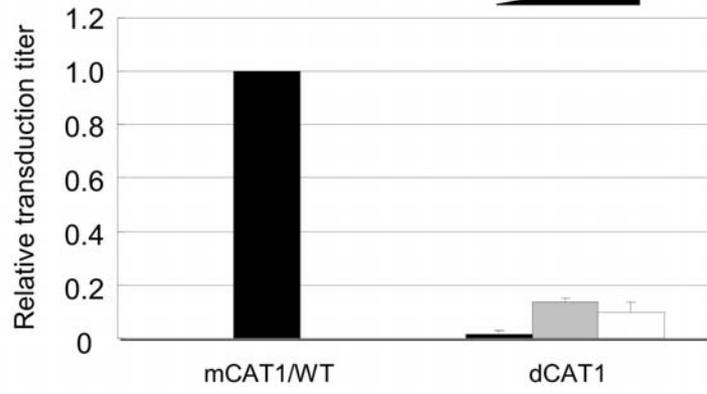


Fig. 2B

Fig. 3A

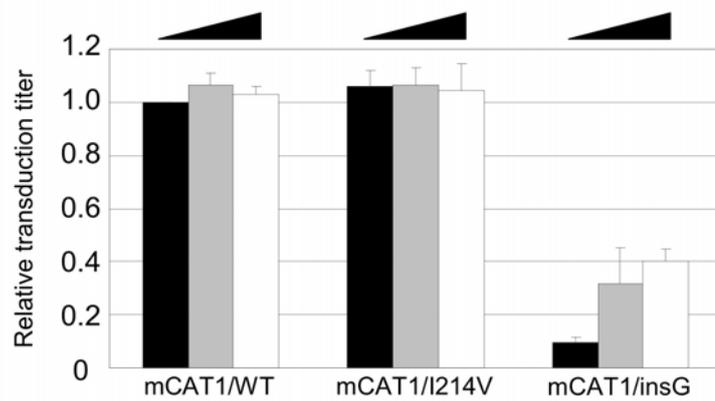


Fig. 3B

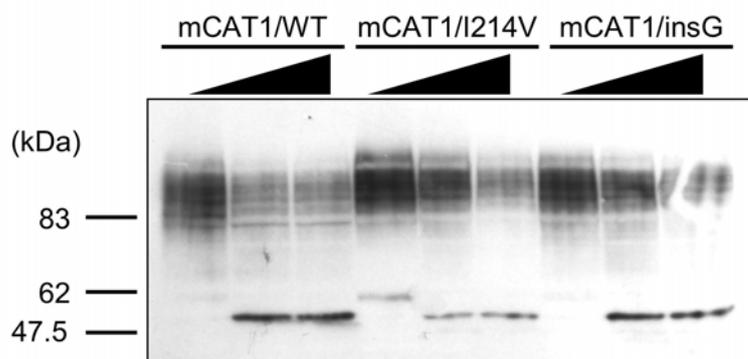


Fig. 4A

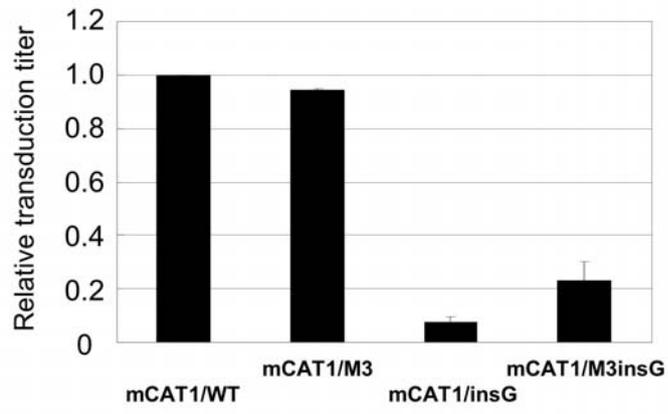


Fig. 4B

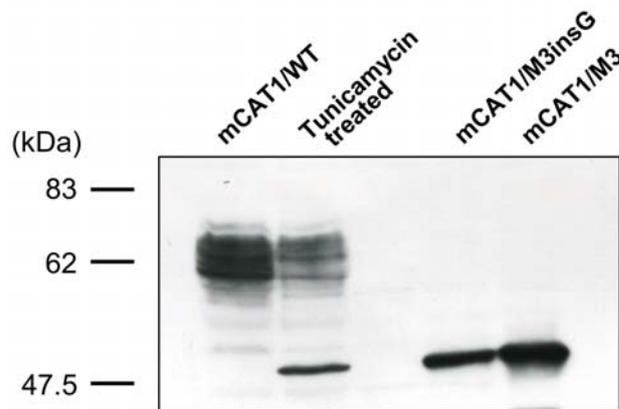


Fig. 5A

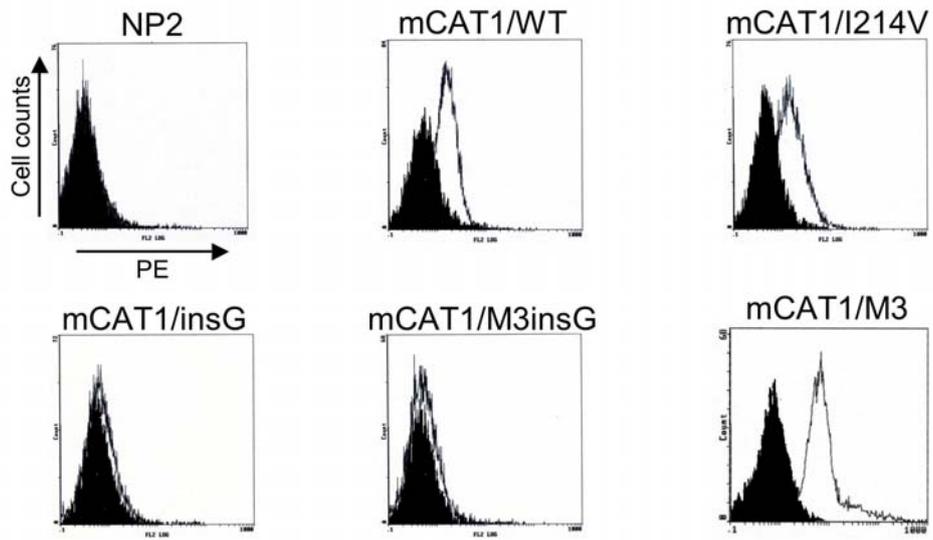


Fig. 5B

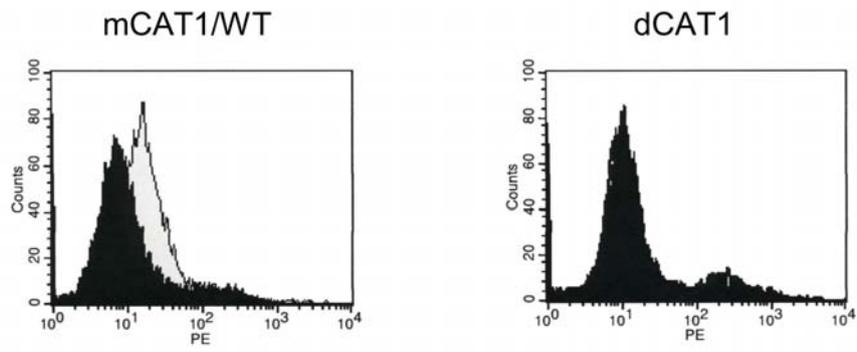


Fig. 6

