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

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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) paralogues 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 dCAT1 inhibits Mo-MLV infection, but that of mCAT1 does not. Compared with mCAT1, dCAT1 possesses an Ile-to-Val substitution at position 214 and a Gly insertion at position 236 in the ECL3. To determine the residues responsible for the loss of dCAT1 receptor function, mutants of mCAT1 were constructed. The mCAT1/insG receptor (with a Gly residue inserted at mCAT1 position 236) had greatly reduced Mo-MLV receptor function compared with mCAT1. Treatment of mCAT1/insG-expressing cells with tunicamycin, an N-linked glycosylation inhibitor, increased the transduction titre. In addition, the reduced susceptibility to Mo-MLV observed with mCAT1/insG-expressing cells correlated with impaired binding of Mo-MLV. These results show that a single amino acid insertion confers mCAT1 receptor properties on dCAT1 and provide 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 its 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 (Asn 223 and Asn 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; Tailor et al., 2003). Previous studies have shown that the integrity of the $^{235}$YGE$^{237}$ 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 murine CAT1 parologue (mCAT1) receptors retain full Mo-MLV receptor function (Kubo et al., 2002; Wang et al., 1996). Hamster CAT1 (hCAT1) orthologues fail to function as the Eco-MLV receptor but can be rendered functional after treatment with tunicamycin, an inhibitor of N-linked glycosylation (Miller & Miller, 1992; Wilson & Eiden, 1991). In addition, the rat CAT1 orthologue (rCAT1) and the Mus dunni CAT1 paralogue (dCAT1) function poorly as Mo-MLV receptors due to glycosylation-dependent inhibition (Eiden et al., 1993, 1994; Kubo et al., 2002; Tavoloni & Rudenholz, 1997). The ECL3 of rCAT1 is 2 aa longer than the ECL3 of mCAT1 (due to deletion of the Lys residue at position 222 and insertions of Ser, Pro and Leu 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).

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M. 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 & Wehrly, 1985; Lander & Chattopadhyay, 1984). It has been shown previously that pretreatment of MDTF cells with tunicamycin renders MDTF cells susceptible to Mo-MLV (Eiden et al., 1994). Compared with the mCAT1 protein, the dCAT1 protein possesses a substitution of Ile for Val at position 214 and a Gly insertion at position 236 in the ECL3, in addition to a substitution of Asn for Asp at position 373 and a substitution of Thr for Ala 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 M. dunni cells. The results observed in this study showed that the Gly insertion impairs the mCAT1 receptor function, and provide important insights into CAT1 gene evolution as the ecotropic virus receptor.

METHODS

Expression plasmids. The 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 haemagglutinin (HA) tagging: 5'-TTACTGCAGACAGATTTGCTCAGCGCGATG-3' and 5'-TCATGCGTAATCCGGAAACATCTAGCAGGTTATTTGCACTGGTCCAAGTTGCTGTCAGGAGTCTT-3'. The latter antisense primer contains the HA epitope sequence as reported previously (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 vesicular stomatitis virus G protein (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'-CTATAGGGCGAATTCCGATCCTTGCGGCC-3') and mutation primers. The sequences of the mutation primers used to construct mCAT1/insG and mCAT1/I214V were 5'-AAACCCTCCCTCACCCCCGTATTTCACGTTTGT-3' and 5'-GAGCTGCCAGTTTTTAACGGAGCCTTTCACGAA-3', respectively. The BamX primer has a nucleotide substitution that disrupts the unique BamHI site of the pTarget vector. These primers were synthesized by Nissinbo Inc. The PCR amplified fragments of approximately 900 bp, which in turn provided PCR primers for amplification of the

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 (mCAT1/WT) and mCAT1 mutants were constructed to contain a C-terminal HA epitope tag. ○, Substitution mutation; □, insertion mutation. ECL3 is indicated by grey shading. (b) Comparison of the ECL3 amino acid sequences of mCAT1, dCAT1 and mCAT1 mutants. Filled boxes, N-linked glycosylation sites; shaded box, Mo-MLV Env-binding domain. (c) Comparison of the ECL3 amino acid sequences of CAT1 proteins from various animals. Filled boxes, N-linked glycosylation sites; shaded box, Mo-MLV Env-binding domain in mCAT1.
complete plasmid containing the desired mutations. The PCR product was treated with BanHI to digest the template plasmid and transformed into Escherichia coli DH5α 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 expressing both Mo-MLV gag-pol and the lacZ vector (Cosset et al., 1995), human embryo kidney 293T cells and MDTF cells (Lander & Chattopadhyay, 1984) were cultured in Dulbecco’s modified Eagle’s medium (Sigma) at 37 °C in 5% CO₂. MDTF cells were provided from the 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 3 μg Mo-MLV gag-pol, 3 μg CAT1-expressing retroviral vector and 3 μg YSV-G expression plasmid (Chang et al., 1999) using 30 μl TransIT LT1 reagent (Mirus). Cells were washed 24 h after transfection and cultured for 24 h in fresh medium. The culture supernatant of the transfected cells was inoculated into NP2 cells, which were subsequently selected using puromycin (10 μg ml⁻¹). 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 using reverse transcriptase (Takara) from total RNA samples (1 μg). Semi-quantitative PCR was performed to detect CAT1 and GAPDH mRNAs. The sequences of the primers for the CAT1 RT-PCR were 5'-TCAAGCGTGCG-AAAGGCCCTGGAG-3' and 5'-TGCCCGCTGACAGGACACCACAGG-3', and for the GAPDH RT-PCR were 5'-AGGTGGGAGTCAACGGATTTTGT-3' and 5'-GTGGGCCATGAGGTTCCACAC-3', and were synthesized by GeneNet.

**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 & Amanuma, 2003). Target cells (2 × 10⁶) were plated onto a 60 mm culture dish and cultured for 24 h in the presence or absence of tunicamycin (400 or 800 ng ml⁻¹). Cells were washed with medium to remove tunicamycin and inoculated with the retroviral vector in the presence of polybrene (4 μg ml⁻¹). After an additional 24 h culture in fresh medium, cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Wako); cells that stained blue were counted for an estimation of transduction titre.

**Immunofluorescence microscopy.** HA-tagged CAT1-expressing NP2 cells were cultured on four-well culture slides (Miles) in the presence or absence of tunicamycin (800 ng ml⁻¹) for 24 h. Cells were fixed with cold methanol and incubated with a mouse anti-HA antibody (Covance), followed by indocarbocyanine (Cy3)-conjugated anti-mouse IgG antibody (Sigma). Cells were observed using a confocal fluorescence microscope (Leica).

**Western immunoblotting.** HA-tagged CAT1-expressing cells (2 × 10⁶) were seeded and cultured for 48 h. Cells were then cultured for an additional 24 h in the presence or absence of tunicamycin (400 or 800 ng ml⁻¹). Cell lysates were subjected to 7.5% SDS-PAGE followed by Western blotting using mouse anti-HA antibody and horseradish peroxidase-conjugated anti-mouse IgG antibody (Bio-Rad).

**Virus binding.** A virus-binding assay was performed as reported previously (Lavillette et al., 2000). Cells (5 × 10⁶) were incubated with viral stock for 30 min at 4 °C, after which unbound virus was removed by two washes with 0.2% BSA in ice-cold PBS; virus–cell complexes were incubated sequentially at 4 °C with goat anti-gp70 antisera (diluted 1:500) and phycoerythrin (PE)-conjugated donkey anti-goat antisera (diluted 1:100; Jackson Laboratories). Cells were then fixed in 1% formalin (Sigma). Fluorescence intensity was analysed by flow cytometry (Coulter or Becton Dickinson). Parental NP2 cells and NP2/mCAT1 mutants were incubated in virus-free medium and served as negative controls.

**Statistical analysis.** Differences between groups of data were determined by 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 to ecotropic Mo-MLV infection, but this resistance can be counteracted by treatment with tunicamycin, an N-linked glycosylation inhibitor. To determine 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 dCAT1 protein expression, dCAT1 was C-terminally tagged with the HA epitope. Unfortunately, the HA-tagged dCAT1 protein was not detected by Western blotting 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 was not expressed. Therefore, untagged dCAT1-expressing NP2 cells were constructed.

The transduction titre of the Mo-MLV vector in the untagged dCAT1-expressing NP2 cells was less than 10% of that in untagged and tagged mCAT1-expressing cells (Fig. 2a and c), although mRNA levels of dCAT1 and mCAT1 were similar (Fig. 2b). Serial dilution of the template first-strand cDNA induced lower levels of PCR product, indicating that these PCR conditions were semi-quantitative. In addition, tunicamycin treatment (400 ng ml⁻¹) of the dCAT1-expressing cells enhanced the transduction titre approximately tenfold (Fig. 2c), as in MDTF cells (Eiden et al., 1994). These results indicated that dCAT1 is a determinant for the lower susceptibility of MDTF cells to Mo-MLV infection and for the glycosylation-mediated loss of receptor function.

**Functional comparison of various mCAT mutant proteins**

The dCAT1 protein has an Ile-to-Val substitution at position 214 and a Gly 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 blotting using anti-HA antibody, similar to hCAT1 (Kubo et al., 2004). Therefore, we constructed mutants of HA-tagged mCAT1 (Kubo et al., 2002). The C-terminal HA tagging of mCAT1 did not affect its 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 mutants mCAT1/I214V, mCAT1/insG, mCAT1/N373D and mCAT1/T590A were constructed (Fig. 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**

Wild-type mCAT1 (mCAT1/WT)-, mCAT1/insG- and mCAT1/I214V-expressing cells were pretreated with tunicamycin (0, 400 or 800 ng ml\(^{-1}\)) for 24 h to measure the effects of receptor glycosylation on Mo-MLV vector infection. Relative transduction titres compared with those obtained on untreated mCAT1-expressing cells are shown in Fig. 3(a). Transduction titres of the Mo-MLV vectors in mCAT1/insG-expressing cells were tenfold lower than those in mCAT1/WT-expressing cells (\(P<0.001\)), as with

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**Fig. 2.** Relative susceptibilities of mCAT1- and dCAT1-expressing cells. (a) NP2 cell pools expressing the C-terminally HA-tagged mCAT1 (mCAT1/WT), untagged WT mCAT1 [mCAT1 HA(−)] and dCAT1 were inoculated with the Mo-MLV vector. Transduction titres are relative to those in mCAT1/WT-expressing cells. This experiment was repeated three times and results are shown as means ± SD. (b) Expression levels of mCAT1/WT, mCAT1 HA(−) and dCAT1 were analysed by semi-quantitative RT-PCR. To confirm the quantitativeness of the RT-PCR, first-strand cDNA prepared from mCAT1/WT-expressing cells was serially diluted twofold and PCR was performed using the diluted cDNA as template. The PCR products were subjected to agarose gel electrophoresis. (c) Transduction titres in tunicamycin-treated mCAT1/WT- and dCAT1-expressing cells. Cells were treated with 0 (open bar), 400 (shaded bar) or 800 (filled bar) ng tunicamycin ml\(^{-1}\) for 24 h. Transduction titres relative to those in untreated mCAT1/WT-expressing cells are indicated. This experiment was repeated three times and results are shown as means ± SD.

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**Fig. 3.** Relative susceptibility of each mCAT1 mutant-expressing cell pool to Mo-MLV infection and expression of C-terminally HA-tagged mCAT1 mutants in NP2 cells. (a) mCAT1/WT-, mCAT1/I214V- and mCAT1/insG-expressing NP2 cell pools were treated with tunicamycin for 24 h. Transduction titres are shown for untreated cells (filled bars) and for cells treated with 400 ng (shaded bars) or 800 ng (open bars) tunicamycin ml\(^{-1}\) and are relative to those in untreated mCAT1/WT-expressing cells. This experiment was repeated three times and results are shown as means ± SD. (b) Cell lysates were prepared from tunicamycin-treated (400 or 800 ng ml\(^{-1}\)) and untreated HA-tagged mCAT1 mutant-expressing cell pools. Cell lysates were electrophoresed on 7.5% polyacrylamide gels and Western blotting was performed using an anti-HA antibody. Molecular mass standards are indicated on the left.
dCAT1-expressing cells (Fig. 2), even though the expression levels of the mCAT1/WT and mCAT1/insG proteins were similar (Fig. 3b). The titres of the Mo-MLV vectors in mCAT1/insG-expressing cells were increased following treatment with tunicamycin in a dose-dependent manner \((P<0.05)\), as with dCAT1-expressing cells (Fig. 2). Transduction efficiency of the Mo-MLV vectors in mCAT1/I214V-expressing cells was comparable to those in mCAT1/WT-expressing cells. Transduction efficiencies of the Mo-MLV vectors in mCAT1/I214V-expressing cells were unaffected by tunicamycin treatment, as was found for mCAT1/WT-expressing cells. Protein expression levels of the mCAT1 mutants were detected at levels similar to those of mCAT1/WT as assessed by Western blotting using an anti-HA antibody (Fig. 3b). Tunicamycin treatment decreased the apparent molecular mass of the CAT1 proteins (~50 kDa), confirming that tunicamycin treatment inhibited CAT1 protein glycosylation. These results indicated that inhibition of Mo-MLV infection by the Gly insertion resulted at least in part from receptor glycosylation, as transduction levels of tunicamycin-treated mCAT1/insG-expressing cells were lower than those of mCAT1-expressing cells.

mCAT1/N373D- and mCAT1/T590A-expressing NP2 cells were as susceptible to Mo-MLV infection as were mCAT/WT-expressing cells (data not shown). Expression levels of both the mCAT1/N373D and mCAT1/T590A proteins were comparable to those of mCAT1/WT, as determined by Western blotting using an anti-HA antibody. These results indicated that the Asp and Ala residues at positions 373 and 590, respectively, as well as the Val 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 assess further the glycosylation-dependent reduction in Mo-MLV titre in cells expressing dCAT1, we constructed the mCAT1/M3insG mutant (Fig. 1a). Two conserved N-linked glycosylation sites (Asn 223 and Asn 229) are present in the ECL3 of both mCAT1 and dCAT1. The mCAT mutant mCAT1/M3, lacking both of these N-linked glycosylation sites, was provided by Dr D. Kabat (Wang et al., 1996). Site-directed mutagenesis was performed to insert a codon encoding Gly at position 236 of mCAT1/M3. Cells expressing mCAT1/WT were as susceptible to Mo-MLV vector transduction as cells expressing mCAT1/M3 (Fig. 4a). The transduction efficiencies of Mo-MLV vectors determined in cells expressing mCAT1/M3insG were two- to threefold higher than those found in mCAT1/insG-expressing cells, yielding results similar to those seen after tunicamycin treatment of mCAT1/insG-expressing cells \((P<0.05)\). This finding confirmed the observation that glycosylation of the mCAT1/insG protein inhibits Mo-MLV infection, but that glycosylation of mCAT1/WT does not. Therefore, glycosylation of the receptors from different animals per se does not always diminish receptor function. The molecular mass of the mCAT1/M3insG protein was lower than that of either the mCAT1/WT or mCAT1/insG protein, and that of the unglycosylated mCAT1/WT detected in tunicamycin-treated cells was similar to those of the mCAT1/M3 and mCAT1/M3insG (Fig. 4b). Presumably, this was attributable to elimination of the N-linked glycosylation sites in these proteins.

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

mCAT1/M3insG-expressing and tunicamycin-treated mCAT1/insG-expressing cells were less susceptible to Mo-MLV infection than mCAT1/WT-expressing cells (Figs 3 and 4). This suggested that the mCAT1/insG protein has an additional factor that inhibits Mo-MLV infection, in addition to receptor glycosylation. As the Gly 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 & Amanuma, 2003) or parental TELCeB6 cells (Cosset et al., 1995) (no Env virus, negative control) were exposed to target cells at 4 °C. mCAT1/WT-, mCAT1/I214V- and mCAT1/M3-expressing cells, which are all equally susceptible to Mo-MLV infection, showed efficient binding of the virus (Fig. 5a). mCAT1/insG-, mCAT1/M3insG- (Fig. 5a) and dCAT1-expressing cells (Fig. 5b), which were more resistant to Mo-MLV infection than mCAT1/WT-expressing cells, showed much lower binding of the virus than mCAT1/WT-expressing cells. This result suggested that binding of the Mo-MLV vectors to both mCAT1/insG and mCAT1/M3insG was impaired, indicating that the Gly 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 affected susceptibility to Mo-MLV infection, we analysed the cellular distribution of the mutant proteins by immunofluorescence microscopy using an anti-HA antibody. Each mCAT1 mutant was detected on the cell surface and in the cytoplasm, as seen for mCAT1/WT-expressing cells (Fig. 6). In addition, the cellular localization of each mCAT1 mutant was unaffected by tunicamycin treatment (400 ng ml⁻¹), indicating that neither the mutations nor tunicamycin treatment had any 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 (²³⁵YGE²³⁷ in the ECL3) (Albritton et al., 1993; Yoshimoto et al., 1993). Susceptibility of hCAT1 to Eco-MLV infection is completely suppressed by a sugar-chain linkage to the receptor, although the virus-binding domain is preserved (Wilson & 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 hCAT1 and rCAT1, it does not inhibit Eco-MLV infection. We previously reported that the extra amino acids in rCAT1 (compared with mCAT1) were associated with inhibition of Eco-MLV infection by glycosylation at these residues (Kubo et al., 2004). As the hCAT1 orthologue 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 hCAT1 and rCAT1.

The MDTF cell line expresses a paralogue of 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 (Fig. 1b and c). The mechanism of inhibition of Mo-MLV infection by dCAT1 glycosylation appears to be different from those used by both rCAT1 and hCAT1. Thus, we attempted to clarify the mechanisms of viral infection control by glycosylation of dCAT1.

In this study, we constructed several mCAT1 mutants (Fig. 1b). Transduction titres of the Mo-MLV vectors in mCAT1/I214V-expressing cells were comparable to those expressing mCAT1/WT. 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 mCAT1/WT (Figs 3b and 6). These results suggested that the Gly residue at position 236 in the ECL3

**Fig. 5.** Specific binding of the Mo-MLV Env glycoprotein to the CAT1 protein. mCAT1 mutant-expressing cells were incubated with Mo-MLV particles, followed by goat anti-Env surface glycoprotein and finally with PE-conjugated donkey anti-goat antiserum, each for 1 h at 4 °C. The fluorescence intensities of the cells were analysed by a flow cytometer from Coulter (a) or Becton Dickinson (b). Filled and open areas indicate mCAT1 mutant-expressing cells incubated with Env-free viral particles (no Env) as a negative control and with Mo-MLV Env-containing particles, respectively.

**Fig. 6.** Cell-surface expression of mCAT1 mutants. To exclude the possibility that altered cellular localization of the mCAT1 mutants affected susceptibility to Mo-MLV infection, we analysed the cellular distribution of the mutant proteins by immunofluorescence microscopy using an anti-HA antibody. Each mCAT1 mutant was detected on the cell surface and in the cytoplasm, as seen for mCAT1/WT-expressing cells (Fig. 6). In addition, the cellular localization of each mCAT1 mutant was unaffected by tunicamycin treatment (400 ng ml⁻¹), indicating that neither the mutations nor tunicamycin treatment had any effect on mCAT1 cellular localization.

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of dCAT1 is responsible for the failure of dCAT1 to function efficiently as a receptor for Mo-MLV. It has been reported that the Val residue of dCAT1 is a 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 mCAT1/WT-expressing cells, indicating that the Val residue did not affect Mo-MLV infection. Further analysis is required to determine why these inconsistent results have been observed.

Titres of Mo-MLV vectors in mCAT1/insG-expressing cells increased following treatment with tunicamycin (Fig. 3a). The transduction titres of the Mo-MLV vectors in the mCAT1/M3insG-expressing cells were two- to threefold higher than cells expressing mCAT1/insG (Fig. 4a). These results suggested that the Gly residue introduced at position 236 induces a conformational change in the dCAT1 protein that inhibits viral infection by glycosylation.

mCAT1/insG- and mCAT1/M3insG-expressing cells showed much lower binding of virus than mCAT1/WT- and mCAT1/M3-expressing cells. The dCAT1 protein has a Gly residue within the putative virus-binding site of mCAT1. As viral entry is initiated by binding of the viral envelope protein to the receptor at the target cell surface, this result indicated that the inserted Gly residue inhibits Mo-MLV infection by impairing the interaction between the Mo-MLV envelope and the CAT1 receptor. It has been reported previously that the Gly insertion in dCAT1 is responsible for the lower susceptibility of MDTF cells to Mo-MLV infection (Bae et al., 2006). This study determined the mechanism by which the Gly insertion into position 236 of mCAT1 makes it less susceptible to 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 suggested that dCAT1 glycosylation does not suppress the Env–dCAT1 interaction, but affects the folding of the mCAT1/insG protein as the Eco-MLV receptor (Helenius & Aebi, 2001, 2004). However, mCAT1/M3insG-expressing cells were two to three times more susceptible to Mo-MLV infection than mCAT1/insG-expressing cells (Fig. 4a), and this extent of difference in 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 suggest a hypothesis of co-evolution of Eco-MLV and CAT1. rCAT1 is a less efficient receptor for Eco-MLV than mCAT1 (Kubo et al., 2002; Takase-Yoden & Watanabe, 1999). We hypothesize that the rCAT1 protein might, over a number of years, have gained extra residues for protection from Eco-MLV infection (Kubo et al., 2004). Hamster cells are completely resistant to Mo-MLV infection as a result of CAT1 glycosylation (Wilson & Eiden, 1991). hCAT1 has extra amino acids as does rCAT1, and is also 4 aa longer than rCAT1 (Fig. 1c). Therefore, the gain of these residues in hCAT1 accompanies a loss of Mo-MLV receptor function. Although hCAT1 is completely resistant to Mo-MLV infection, it has been reported that
neuropathogenic and hamster-adapted variants of ecotropic Friend MLV infect hamster cells efficiently (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 defences 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). Although dCAT1 does not have the extra residues present in rCAT1 and hCAT1, it is resistant to Mo-MLV. It appears that dCAT1 has evolved from a mCAT1-type receptor independently of both rCAT1 and hCAT1, whereas human and mink CAT1 proteins have evolved to combine features of both hamster and M. dunni receptors. Finally, human and mink CAT1 proteins have 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 likely that a viral receptor protein directly acquires resistance to viral infection by mutations in its virus-binding domain. However, the CAT1 protein shows a complex evolutionary pathway. CAT1 consists of multiple membrane-spanning proteins and its expression requires an unknown complex mechanism, as many of the CAT1 mutants are not expressed (Kubo et al., 2004). It has been reported that knockout of the cat1 gene in mice is lethal, indicating a critical role for 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 such as 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 the 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 ASC1T, Pit1 and Pit2 receptors have all been reported to be inactivated by glycosylation in some types of cell and hence fail to facilitate entry by the feline endogenous retrovirus RD114, gibbon ape leukemia virus and amphotropic MLV, respectively. Thus, glycosylation-mediated blocks in receptor function extend not only to other orthologues of CAT1 such as rCAT1 (Kubo et al., 2004) but also to other gammaretroviral receptors.

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