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Modulation of Ecotropic Murine Retroviruses by N-Linked Glycosylation of the Cell Surface Receptor/Amino Acid Transporter

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The cell surface receptor for ecotropic host-range (infection limited to mice or rats) murine leukemia viruses (MuLVs) is the widely expressed system y^+ transporter for cationic amino acids (CAT-1). Like other retroviruses, ecotropic MuLV infection eliminates virus-binding sites from cell surfaces and results in complete interference to superinfection. Surprisingly, infection causes only partial (ca 40 to 60%) loss of mouse CAT-1 transporter activity. The NIH/Swiss mouse CAT-1 (mCAT-1) contains 622 amino acids with 14 hydrophobic potential membrane-spanning sequences, and it is known that the third extracellular loop from the amino terminus is required for virus binding. Although loop 3 is hypervariable in different species and mouse strains, consistent with its proposed role in virus-host coevolution, loop 3 sequences of both susceptible and resistant species contain consensus sites for N-linked glycosylation. Both of the consensus sites in loop 3 of mCAT-1 are known to be glycosylated and to contain oligosaccharides with diverse sizes (J. W. Kim and J. M. Cunningham, J. Biol. Chem. 268:16316–16320, 1993). We confirmed by several lines of evidence that N-linked glycosylation occludes a potentially functional virus-binding site in the CAT-1 protein of hamsters, thus contributing to resistance of that species. To study the role of receptor glycosylation in animals susceptible to infection, we eliminated loop 3 glycosylation sites by mutagenesis of an mCAT-1 cDNA clone, and we expressed wild-type and mutant receptors in mink fibroblasts and *Xenopus* oocytes. These receptors had indistinguishable transport properties, as determined by kinetic and voltage-jump electrophysiological studies of arginine uptake in oocytes and by analyses of L-[^{3}H]arginine uptake in mink cells. Bindings of ecotropic envelope glycoprotein gp70 to the accessible receptor sites on surfaces of mink cells expressing wild-type or mutant mCAT-1 were not significantly different in kinetics or in equilibrium affinities (i.e., $K_D \approx 3.7 \times 10^{-10}$ to 7.5×10^{-10} M). However, when values were normalized to the same levels of mCAT-1 transporter expression, cells with wild-type glycosylated mCAT-1 had only approximately 50% as many sites for gp70 binding as cells with unglycosylated mCAT-1. Although infection with ecotropic MuLV had no effect on activity of the mink CAT-1 transporter that does not bind virus, it caused partial down-modulation of wild-type mCAT-1 and complete down-modulation of unglycosylated mutant mCAT-1. These results suggest that N-linked glycosylation causes wild-type mCAT-1 heterogeneity and that a significant proportion is inaccessible to virus. In part because only the interactive fraction of mCAT-1 can be down-modulated, infected murine cells conserve an amino acid transport capability that supports their viability.

The cell surface receptor for ecotropic host-range murine leukemia viruses (MuLVs) has been identified as the widely expressed system y^+ transporter for cationic amino acids termed CAT-1 (16, 35). Although all mammals have homologous CAT-1 transporters, only mice and rats are susceptible to infection by ecotropic MuLVs. The receptor of NIH/Swiss mice will be abbreviated mCAT-1. By constructing and analyzing human-mouse interspecies chimeric CAT-1 proteins, it was shown that the putative third extracellular loop from the amino terminus is essential for infection and that this loop contains a site necessary for binding the viral envelope glycoprotein gp70 (1, 38). Accordingly, substitution of a YGE sequence (amino acids 235 to 237) from mCAT-1 loop 3 for the corresponding PGV sequence of the human protein generated a functional ecotropic virus receptor (ecoR) (1, 38).

Although loop 3 sequences are hypervariable in CAT-1 proteins of different species (1, 2, 8, 31, 38; also, see below) and strains of mice (2, 7, 8), all of the known sequences contain at least one consensus site for N-linked glycosylation. In NIH/Swiss mice, loop 3 contains two NX(S/T) sites that are known to be glycosylated intracellularly (17). These occur 6 and 12 amino acids amino terminal to the YGE site implicated in virus attachment. The mCAT-1 has a large heterogeneity in apparent M_r that is eliminated by removing N-linked oligosaccharides from its loop 3 (17). This finding implies that mCAT-1 consists of diverse glycoforms (19, 27).

It has been suggested that glycosylation of loop 3 can prevent infection of resistant mammals (8, 24, 25, 37). Chinese hamster ovary (CHO) fibroblasts are normally resistant to ecotropic MuLVs, but they become approximately 5% as susceptible as mouse fibroblasts after incubation with tunicamycin, an inhibitor of N-linked glycosylation (24). CHO cell glycosylation mutants Lec 2 and Lec 8, which were selected for resistance to

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wheat germ agglutinin and have respective deficiencies in transport of CMP-sialic acid and CMP-galactose into Golgi vesicles, are also weakly susceptible to ecotropic MuLVs (8, 25). Furthermore, fibroblasts from the Asian mouse *Mus dunni* are resistant to the Moloney isolate of ecotropic MuLV although they are susceptible to other ecotropic virus isolates (8). *M. dunni* CAT-1 has a distinct loop 3 sequence with two consensus sites for N-linked glycosylation (7). Mutagenesis of the glycosylation site closest to the putative virus-binding site eliminated the block to Moloney MuLV (8). Treatment of *M. dunni* fibroblasts with tunicamycin also caused susceptibility to Moloney MuLV (8). The possibility that N-linked glycosylation of receptors is a widely used mechanism for host defense against retroviruses is supported by studies of amphotropic MuLVs (24, 25, 37) and human immunodeficiency virus type 2 (33). Similarly, N-linked glycosylation of mongoose acetylcholine receptor causes resistance to cobra toxin (3).

The previous studies did not address the role of loop 3 glycosylation in susceptible animals. This is a critical issue because mice inherit proviral copies of ecotropic MuLVs and are often highly viremic without significant disease. Furthermore, aspects of ecotropic MuLV-induced pathogenesis including hemolytic anemia (30), immunosuppression (18), and neural degeneration (22, 26, 32) have been mapped to strain-specific sequences in viral envelope glycoproteins. This finding suggests that variations in envelope glycoproteins and perhaps in their interactions with receptors can have severe pathogenic consequences. We have examined whether N-linked glycosylation of loop 3 in NIH/Swiss mouse mCAT-1 might control binding of ecotropic MuLV and/or pathologic sequelae of infection. Our results support these hypotheses.

MATERIALS AND METHODS

Cells and viruses. Mink lung CCL64 fibroblast and CHO cells were from the American Type Culture Collection (Rockville, Md.). ψ-2 ecotropic packaging cells line (21) and PA12 amphotropic packaging cells (23) were from R. C. Mulligan (Massachusetts Institute of Technology, Cambridge) and A. D. Miller (Fred Hutchinson Cancer Center, Seattle, Wash.), respectively. CEN (36) is a derivative of CCL64 fibroblasts that expressed a large quantity of recombinant mouse mCAT-1. CERD.C19 (36) is a derivative of CHO cells that expressed a relatively high level of mCAT-1. R-CCL64 and R-CEN are CCL64 and CEN cells chronically infected by Rauscher MuLV (34). CHO cells and their derivatives were grown in α minimum essential medium supplemented with 10% fetal bovine serum. Other cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum.

Helper-free ecotropic host-range virus from ψ-2 cells that encoded the neomycin phosphotransferase gene (MSV-neo) has been described elsewhere (6). Virus preparations were harvested by placing fresh culture medium on half-confluent monolayers of virus-producing cell lines for 16 to 24 h, removing the medium, and filtering it through a 0.2-μm-pore-size filter. Cells (10^5) in 25-cm^2 flasks were infected by incubation with 1 ml of virus-containing medium for 2 h at 37°C in the presence of Polybrene (8 μg/ml) (4).

mCAT-1 expression in mink cells. Wild-type and three mutant (M1, M2, and M3) mCAT-1 constructs were subcloned into the retroviral vector pSFF (4). These plasmid DNAs were transfected into 1:1 cocultures of ψ-2 and PA12 cells, and the mixture of helper-free amphotropic and ecotropic host-range viruses was recovered in the culture medium (4) and used to infect mink CCL64 fibroblasts. These infected cells were then superinfected with a helper-free ecotropic pseudotype of MSV-neo (6), and G418-resistant colonies were selected (36).

Sequence analyses of hamster and mink mCAT-1 third extracellular loop. Total RNA was isolated (5) from CHO cells or mink lung fibroblasts. Reverse transcription with random hexamer primers was followed by PCR using primers 1 (5′ GATCGGATCCTGGAACCTGATTCTCTCCTAC 3′) and 2 (5′ GATC GAATTCCATCTGGTATACCAGATTAGG 3′). Amplified PCR fragments (nucleotides 538 to 1506) (2) were subcloned and sequenced by the standard dideoxynucleotide termination method (28).

Construction of mCAT-1 mutants. Site-directed mutagenesis was used to make the mCAT-1 mutants by using two synthetic oligonucleotide primers (5′ CTCACGGAGAAAGAATT CTCCT 3′ and 5′ GTAACAACGTCGACACAA ACGTG 3′) with the Amersham in vitro mutagenesis system. Mutations were verified by sequencing.

Amino acid uptake assay. The amino acid transport assay was based on the method described by Gazzola et al. (10) and Wang et al. (34). All washes of the monolayers and measurements of amino acid uptake were done in Earle's balanced salt solution (EBSS) (1.8 mM $CaCl_2$, 5.3 mM KCl, 0.8 mM $MgSO_4$, 117 mM NaCl, 1 mM NaH_2PO_4, 5.6 mM D-glucose, 0.03 mM phenol red [pH 7.4]; Gibco BRL, Grand Island, N.Y.). Two days before the transport assay, 10^5 cells were plated in 2-cm^2 wells of a 24-well cluster dish. Cells were washed with amino acid-free EBSS and then exposed to the radioactive substrate for 0.5 min at 37°C. L-[^{3}H]arginine in EBSS with a specific activity of 50 μCi/μmol was used. After incubation with radioactive solute, cells were washed rapidly twice with 2 ml of ice-cold phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM Na_2HPO_4) and extracted with 200 μl of 5% trichloroacetic acid, and the soluble phase was then counted in a liquid scintillation spectrometer. The protein concentration of each culture was measured by the Bio-Rad protein assay (Bio-Rad, Richmond, Calif.) after the 5% trichloroacetic acid-insoluble phase of the culture was dissolved in 200 μl of 0.1 N NaOH. The rates of amino acid uptake for individual cultures were standardized to their protein concentrations and fitted by least squares to the Michaelis-Menten equation (34). The least-squares fitting analysis resulted in computer-derived K_m and V_{max} estimates and standard deviations of the estimates.

gp70 binding assay. One day before analysis, 10^5 cells were plated onto coverslips in six-well (9.5-cm^2) dishes. Cells were sequentially incubated at 37°C with purified gp70 (4 μg/ml) (12) for 2 h, with a 1:200 dilution of goat anti-gp70 antibody (12, 36) for 2 h, and with ^{125}I-protein A (0.4 μCi/ml) for 30 min. After cells were rinsed three times (37°C, 5 min) with culture medium and once with PBS, they were lysed with 0.1 N NaOH and the solution was counted in a γ counter. The radioactivity measured was standardized to the protein concentration measured by the Bio-Rad protein assay.

Electrophysiological measurements. Oocyte expression and transport assays were performed as previously described (15). cRNA was transcribed from cDNA encoding either mCAT-1 or the M3 mutant, 50 ng was microinjected into stage V-VI *Xenopus laevis* oocytes, and transport assays were performed 4 to 5 days later. The frog Ringer's solution used for recording contained 96 mM NaCl, 2 mM KCl, and 1.8 mM $CaCl_2$ and was buffered with 5 mM hemisodium *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; pH 7.5). Two-electrode voltage clamp recordings were performed at 22°C, using a GeneClamp 500 amplifier interfaced to a computer by a Digidata 1200 A/D (Axon Instruments). Recording microelectrodes contained 3 M KCl and had resistances between 0.2 and 1.0 MΩ. Data acquisition and analysis were performed with pCLAMP software (Axon Instruments). For voltage-jump experiments, oocytes were voltage clamped at a holding potential of −30 mV, and command pulses were given to potentials between −120 mV and +30 mV in 10-mV increments for 500 ms in the presence or absence of arginine. Current signals were digitized at 2 kHz and low-pass filtered at 1 kHz. The relaxation of the arginine-specific inward currents was fit by a first-order exponential function by using Clampfit software, version 6.0 (Axon Instruments).

Nucleotide sequence accession numbers. The GenBank accession numbers for the mink and hamster partial CAT-1 nucleotide sequences are U49796 and U49797, respectively.

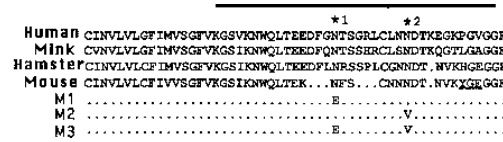

FIG. 1. Predicted amino acid sequences of the third extracellular loop regions of CAT-1 proteins of different species and the sequences of the mouse CAT-1 mutants M1, M2, and M3. Sequences of the third extracellular loop are below the solid bar. The two potential sites for N-linked glycosylation are indicated by asterisks. The YGE sequence of mouse CAT-1 that has been implicated in virus binding (1, 38) is underlined. M1, M2, and M3 are mouse CAT-1 mutants that lack either or both sites for N-linked glycosylation as a result of substituting N with E or V as indicated.

RESULTS

Sequence comparison of CAT-1 proteins from different species. Figure 1 shows alignment of amino acid sequences for extracellular loop 3 regions in the CAT-1 proteins from human, mink, rat, Chinese hamster, and NIH/Swiss mouse cells. The diversity, which is heavily clustered within putative loop 3 (under the bar), appears to be especially prevalent in susceptible species and their near relatives. Indeed, polymorphism of loop 3 sequences occurs in different strains of mice (2, 7) and in hamsters (8).

Interestingly, known loop 3 sequences all contain NX(S/T) consensus sites for N-linked glycosylation, and it has been

TABLE 1. Characteristics of wild-type and mutant mCAT-1 expressed in CCL64 mink lung fibroblasts

mCAT-1 expressed[a] (cell clone)	gp binding to cells[b] (cpm/μg of protein)	MOI[c]	K_m[d] (μM)	V_{max}[d] (nmol/min/mg of protein)	gp70/V_{max} binding	n[e]
Wild-type (CEN)	23.6 ± 0.6	0.69 ± 0.05	120 ± 18	5.5 ± 0.9	4.29 ± 0.71	7
Mutant M1 (1-A)	25.7 ± 2.0	0.68 ± 0.07	141 ± 32	5.6 ± 0.7	4.59 ± 0.67	5
Mutant M2 (2-A)	13.3 ± 0.3	0.56 ± 0.07	154 ± 38	3.3 ± 0.2	4.03 ± 0.26	6
Mutant M3 (3-A)	19.0 ± 1.1	0.63 ± 0.05	141 ± 19	2.7 ± 0.5	7.04 ± 1.36	4

[a] The wild-type and M1, M2, and M3 mutant mCAT-1 clones were expressed in CCL64 mink lung fibroblasts. Cell clones that expressed relatively high levels of gp70 binding activities were chosen.

[b] Analyzed by sequentially incubating 2×10^5 cells at 37°C with purified gp70 (4 μg of protein per ml) for 2 h, with antibody against gp70 for 2 h, and with ^{125}I-protein A (0.4 μCi/ml) for 30 min. The radioactivity bound to the cells was standardized by measuring the protein content of each culture well. Results for the cell lines CEN, 1-A, 2-A, and 3-A are means ± standard errors of the means for three culture wells. Binding of gp70 to the control CCL64 cells was below the detectable level (<0.02 cpm/μg of protein). Radioactivity was similarly undetectable when gp70 was eliminated from the incubations. With this same protocol, mouse NIH 3T3 fibroblasts bound approximately one-third as much gp70 as CEN cells.

[c] Susceptibilities of the cell clones to infection were measured by infecting the cells with a preparation of a helper-free ecotropic host-range virus that encodes human growth hormone (SFF-hGH) and by measuring the fraction of growth hormone-expressing cells 48 h later by immunofluorescence (36). The multiplicities of infection (MOI) were calculated using the equation MOI = $-(\log f)/0.44$, where f is the fraction of cells that were not infected. The cell clones that expressed wild-type or mutant mCAT-1 transporter were infected equally within experimental error.

[d] mCAT-1 transport activities were obtained by subtracting datum points for L-[^{3}H]arginine uptake into control mink CCL64 cells from the datum points for the cells that express wild-type and mutant mCAT-1 and by fitting the results to the Michaelis-Menten equation by least-squares analysis, using a computer program (Kaleidograph) (34). Results are means ± s.e.m.

[e] n, number of independent transport assays.

shown that both of these sites are glycosylated in mCAT-1 of NIH/Swiss mice (17). To study their functions, they were mutated as diagrammed in Fig. 1 to produce receptors M1, M2, and M3, which lack either or both glycosylation sites.

Analysis of gp70 binding and amino acid transport by wild-type and mutant mCAT-1 proteins. The mCAT-1 constructs were introduced into mink CCL64 fibroblasts by using a retroviral vector as described previously (15, 34, 36). Expressing cells were susceptible to infection by a helper-free ecotropic pseudotype of MSV-neo (6), and this enabled us to isolate cell clones resistant to G418 (36). All G418-resistant cell clones bound gp70 envelope glycoprotein purified from Friend ecotropic MuLV (12), and we selected for analysis clones that were high expressers by this criterion (Table 1). As indicated, cell clones with wild-type or mutant mCAT-1 were equally susceptible to infection. Wild-type and mutant mCAT-1 were also active transporters with similar Michaelis-Menten constants (K_m values) for L-arginine uptake. However, the ratio of gp70 binding to V_{max} was significantly greater for mutant M3 than for wild-type mCAT-1 or for M1 and M2 mutants.

An important aspect of our L-[^{3}H]arginine transport assays was the use of 24-well plates that contained control mink CCL64 fibroblasts in some wells and CCL64 clones that express mCAT-1 proteins in other wells. In addition, we used rapid multiwell solute addition and rinse methods (10). This ensured that conditions used to measure initial rates of amino acid uptake, including times and temperatures of incubations and rinses, were always identical for cells compared on any plate. However, because many of our studies required use of several plates, slight differences in conditions could not be avoided. Consequently, for the studies described below, we normalized measured initial velocities of transport for each cell clone relative to the data for control mink CCL64 cells that were obtained on the same multiwell culture plate. Such normalized transport velocities were highly reproducible in repetitive assays of any cell clone.

Figure 2 shows L-[^{3}H]arginine uptake into these same mink fibroblast clones and into control CCL64 mink fibroblasts that were either uninfected or infected with ecotropic Rauscher MuLV. As described previously, infection of the mink CCL64 cells that lack mCAT-1 was accomplished by using Rauscher MuLV that had been pseudotyped with an amphotropic envelope by passage through the retroviral packaging cell line PA12; these infected mink cells released ecotropic host-range virions and were uniformly positive for gp70-specific immunofluorescence (34). The results in Fig. 2A confirmed that mink cells with wild-type mCAT-1 contain y^+ transport activity above the background caused by the endogenous mink y^+ transporter. Infection with Rauscher MuLV caused only partial (ca. 40 to 60%) loss of wild-type mouse y^+ transport activity, whereas the same infection caused no loss of the endogenous mink y^+ transporter. Thus, infection with ecotropic virus down-modulates only a y^+ transporter that binds virus. Figures 2B to D show results for mink cell clones that express mCAT-1 mutants M1, M2, and M3, respectively. The fraction of mCAT-1-specific transport activity that was down-modulated by infection was greater for the mutants than for the wild type. For mutant M3, infected cells reproducibly had the same transport activity as control mink cells (Fig. 3D). Table 2 summarizes results of many independent assays.

We also measured initial rates of L-[^{3}H]arginine outflow from cells that had been preloaded to equilibrium by incubation with 1 mM L-[^{3}H]arginine as previously described (23), and these results are also summarized in Table 2. Down-modulation of M3 mutant mCAT-1 transporter appears to be complete for both uptake and outflow assays (Table 2). Consistent with the data in Table 1, the ratio of gp70 binding relative to transport activity is several times larger for mutant M3 than for the wild-type, M1, or M2 forms of mCAT-1 for both uptake and outflow assays.

The conclusion that infection causes only partial down-modulation of the wild-type transporter was verified in assays using another clone of mink CCL64 cells (clone I-21) that expresses only approximately 20% as much wild-type mCAT-1 as clone CEN. Infection caused the mCAT-1 component of arginine uptake activity in I-21 cells to be down-modulated by 53 ± 18% (n = 3). Moreover, the gp70 binding/relative V_{max} value for transport was reproducibly approximately twice as high for different cell clones expressing M3 mutant transporters as for wild-type transporters (results not shown). Therefore, these differences between wild-type and M3 mutant mCAT-1 were reproducible for cell clones that expressed different amounts of these transporters.

Kinetics of gp70 binding to wild-type and mutant mCAT-1. One interpretation of the foregoing results is that binding of a saturating concentration of gp70 onto cells can be reduced by

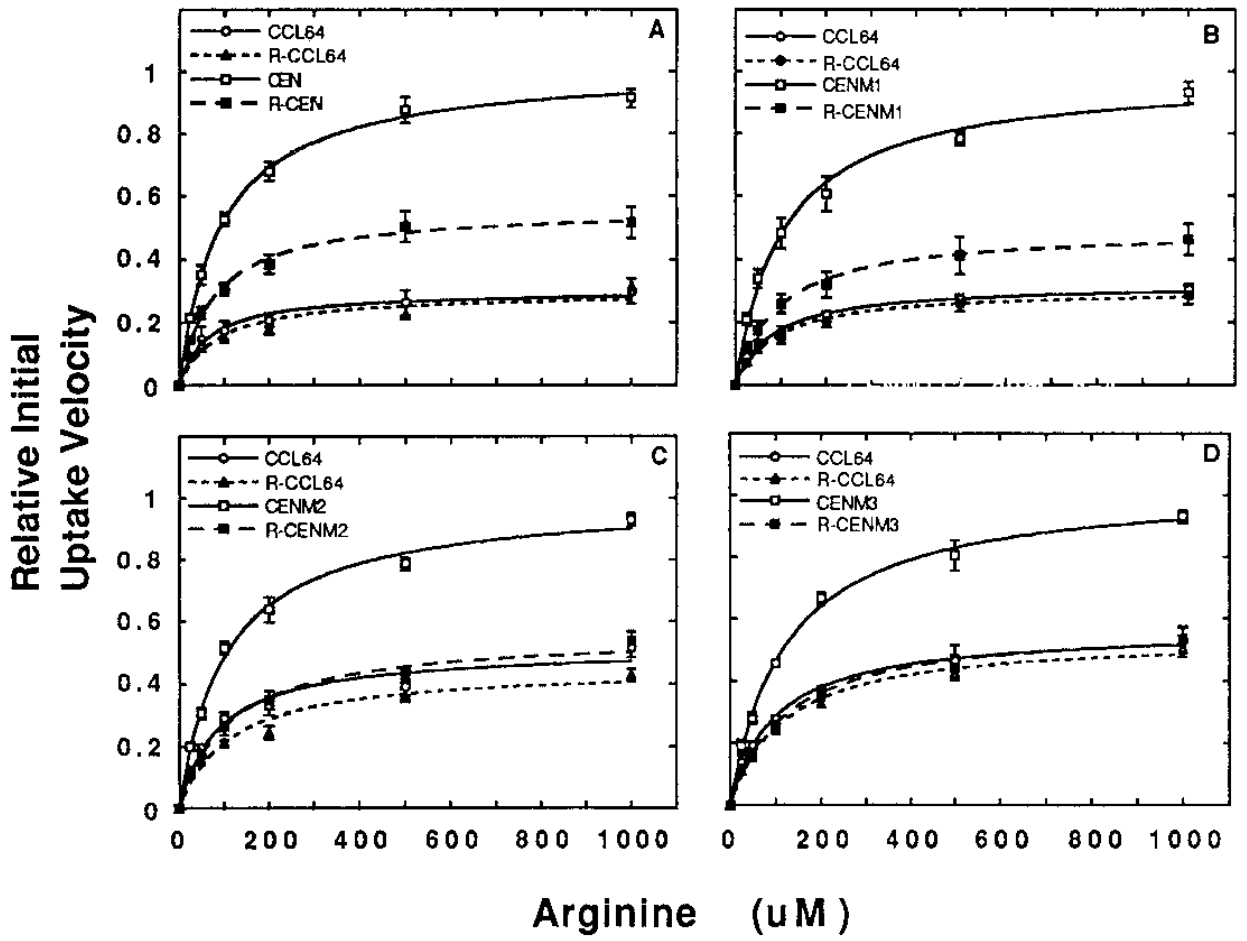

FIG. 2. Down-modulation of arginine uptake into cells caused by infection with Rauscher ecotropic MuLV. CCL64 are mink lung fibroblasts. CEN, CENM1, CENM2, and CENM3 are CCL64 derivatives that express wild-type mCAT-1 and mutants M1, M2, and M3, respectively. The cell clones that express M1, M2, and M3 used were 1-A, 2-A, and 3-A, respectively. The cells infected with Rauscher ecotropic MuLV are indicated by the prefix R-. All cells used in each panel were always assayed in the same 24-well culture plates. Results from individual culture plates were normalized by setting the V_{max} measured for the uninfected mCAT-1-expressing cell to 1.0. Results from repeated assays are presented as means ± standard errors of the means (n = 4 to 6 as recorded in Table 2). This normalization procedure helps to show all results for the mCAT-1-expressing cells on the same ordinate scale but results in small apparent differences for the control CCL64 cells in the different panels. In contrast, the results in Tables 2 and 3 were normalized by setting V_{max} for the control cells to 1.0.

glycosylation of mCAT-1, presumably because some mCAT-1 glycoforms may have relatively large or highly processed oligosaccharides that block virus-binding sites. In addition to complete blockage, however, it is conceivable that some glycoforms have reduced rates of gp70 binding or increased rates of gp70 dissociation. Although attachment of gp70 to cells is rapid, dissociation is relatively difficult to study because it is slower than the rate of endocytosis of gp70-receptor complexes, which has a half-time of approximately 4 to 5 h at 37°C (14). To compare kinetics of gp70 binding onto mink CCL64 fibroblasts that express wild-type or M3 mutant receptors, we used cell clones that express relatively low numbers of receptors and plated the cultures at low cell densities. Control experiments indicated that this was necessary to maintain gp70 in excess and to avoid decreases in gp70 concentration during the incubations that would reduce the kinetics of binding.

Figure 3A shows equilibrium binding of different quantities of gp70 that were added to monolayer cultures of two cell clones (5neo and 8) that express mutant M3 and to one clone that expresses wild-type mCAT-1 (I-21). The protein concentrations of the gp70 preparations that gave half-maximal levels of binding were 45.9 ± 4.3 and 47.6 ± 9.0 ng/ml for the two M3-expressing clones and 93.5 ± 31 ng/ml for the cells expressing wild-type mCAT-1. As described in the legend to Fig. 3, these results suggest that the gp70 dissociation constant, K_D, is approximately 3.6×10^{-10} M for mutant M3 and approximately twice as large for wild-type mCAT-1.

Figure 3B shows rates of gp70 binding to these same clones when we used an excess concentration (0.68 μg of protein per ml) of our gp70 preparation. The data are fit to a logarithmic function by least squares, consistent with the hypothesis of a single binding constant and with estimated half-times for binding of 4.7 ± 0.96 and 5.6 ± 1.1 min for the two M3-expressing clones and of 5.9 ± 1.2 min for the clone that expresses wild-type mCAT-1. These results suggest that gp70 binds at equal rates to accessible sites in M3 mutant and wild-type mCAT-1 proteins. Since the equilibrium measurements (Fig. 3A) imply a slightly lower gp70 affinity for wild-type mCAT-1, we infer that glycosylation may increase the rate of gp70 dissociation approximately twofold. However, the small differences are of uncertain significance.

Indistinguishable transporter kinetics for wild-type and M3 mutant mCAT-1. To assess whether mutations of the glycosylation sites affected the transport kinetics of mCAT-1, the two-

TABLE 2. Down-modulation of mCAT-1 transport activity in cells infected with ecotropic Rauscher MuLV

mCAT-1 expressed (cell clone)	Relative V_{max}[a] uninfected cells (infected cells)	gp70 binding/relative V_{max}[b] uninfected cells	Down-modulation[c] (%)	n[d]
Wild-type (CEN)	Import, 2.45 ± 0.47 (0.90 ± 0.23)	9.6 ± 1.9	62 ± 6	4
	Export, 2.93 ± 0.27 (1.72 ± 0.31)	8.1 ± 0.8	39 ± 8	5
Mutant M1 (1-A)	Import, 2.14 ± 0.21 (0.53 ± 0.17)	12.0 ± 1.5	74 ± 7	5
	Export, 2.20 ± 0.40 (0.72 ± 0.23)	11.7 ± 2.3	73 ± 12	6
Mutant M2 (2-A)	Import, 0.94 ± 0.12 (0.09 ± 0.09)	14.1 ± 2.8	94 ± 9	6
	Export, 2.64 ± 0.29 (0.95 ± 0.21)	5.0 ± 0.6	65 ± 6	6
Mutant M3 (3-A)	Import, 0.81 ± 0.18 (0.03 ± 0.14)	23.5 ± 5.4	103 ± 17	4
	Export, 0.82 ± 0.14 (0.05 ± 0.23)	23.2 ± 4.2	113 ± 18	5

[a] Relative V_{max} = (V_{max}, cell clone − V_{max}, CCL64)/V_{max}, CCL64. These values were measured for both import and export of L-[^{3}H]arginine from the cell clones (34). Relative V_{max} is the maximum transport activity of mCAT-1 in the CCL64 cell clone normalized to the V_{max} of control CCL64 mink fibroblasts.

[b] The gp70 binding results for each cell clone are from Table 1. Errors in the ratio are standard deviations calculated by the propagation of error method.

[c] Calculated for each assay as [(relative V_{max}, uninfected − relative V_{max}, infected)/relative V_{max}, uninfected] × 100. Results are the means and standard deviations for independent assays. Because each assay was analyzed independently before the means and standard deviations were calculated, the values for down-modulation in this column differ slightly from values that would derive from the average relative V_{max} data shown in the second column. Analysis of these results by the paired-comparisons t test indicated that down-modulation of the wild-type and M1 forms of mCAT-1 were incomplete to greater than a 95% confidence level and that down-modulation reduced the M3 mutant transport activity to a level not significantly different from that of the control CCL64 cells. The data for the M2 mutant suggested that down-modulation was incomplete.

[d] n, number of independent transport assays.

electrode voltage clamp technique was used. For these experiments, oocytes expressing either wild-type or M3 mCAT-1 were voltage clamped at a holding potential of −30 mV. Subtraction of currents recorded during voltage jumps in control Ringer's solution from those in the presence of arginine yields arginine-induced currents. Such currents are shown in Fig. 4 for an oocyte expressing either wild-type or M3 mCAT-1. Following a perturbation in membrane voltage, inward currents develop with an instantaneous component, after which there is a slow relaxation reflecting the time required for the transport rate to reach a new steady-state value. In a cyclic kinetic scheme, the slowest time constant in this process reflects the rate-limiting step in the transport cycle (20). Following a voltage jump, the relaxation of the current to a new steady-state for oocytes expressing either the wild-type or mutant transporter was well fit by a single exponential function (not shown). Analysis of these time constants suggests that the rate-limiting transport step has not been altered by the mutations, since this time constant was not significantly different in the two transporters (Fig. 4C). Additionally, the Michaelis-Menten constant of the transporter for arginine determined under voltage clamp (−60 mV) was not significantly altered by this mutation (80.9 ± 6.7 and 60.1 ± 10.6 μM for the wild type and the M3 mutant, respectively, in oocytes at 22°C), further supporting

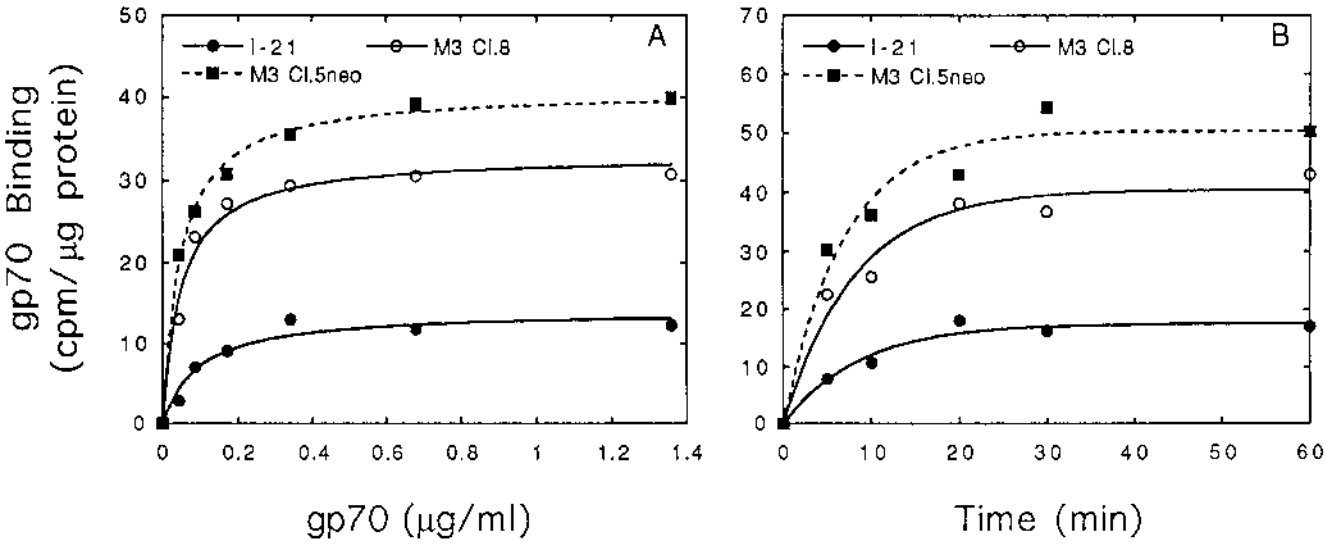

FIG. 3. The kinetics of gp70 binding to the wild-type M3 mutant mCAT-1 transporters are similar. 5neo and 8 are clones of CCL64 fibroblasts expressing the M3 mutant form of mCAT-1. I-21 expresses wild-type mCAT-1. (A) Representative equilibrium (120 min) binding experiment. The binding of a partially purified gp70 preparation to the cells was assayed as described in Materials and Methods except that the concentration of gp70 was varied (the values shown are micrograms of the total protein in the preparation). The half-maximal binding concentrations taken from a least-squares hyperbolic fit of the data ± the standard errors of the fit are 93.1 ± 29 ng/ml for I-21, 45.3 ± 4.2 ng/ml for M3 C1.5neo, and 47.1 ± 9.0 for M3 C1.8. Using these values and an estimated purity of the gp70 preparation of 40% (on the basis of electrophoresis in a polyacrylamide gel and staining of the gel with Coomassie blue dye) and an M_r of 50,000 for the protein portion of gp70, we estimated the K_D values as approximately 3.5×10^{-10} and 3.7×10^{-10} M for the M3 mutants and 7.5×10^{-10} M for wild-type mCAT-1. (B) Representative time course of gp70 binding. A 0.68-μg/ml concentration of the partially purified gp70 preparation was incubated with the cells for the times shown. The half-times ($t_{1/2}$) for binding, estimated from a least-squares fit to the equation $B = B_{max}(1 - e^{-kt})$, where $k = \ln 2/t_{1/2}$, are 5.9 ± 1.2 min for I-21, 4.7 ± 0.96 min for M3 C1.5neo, and 5.6 ± 1.1 min for M3 C1.8 cells.

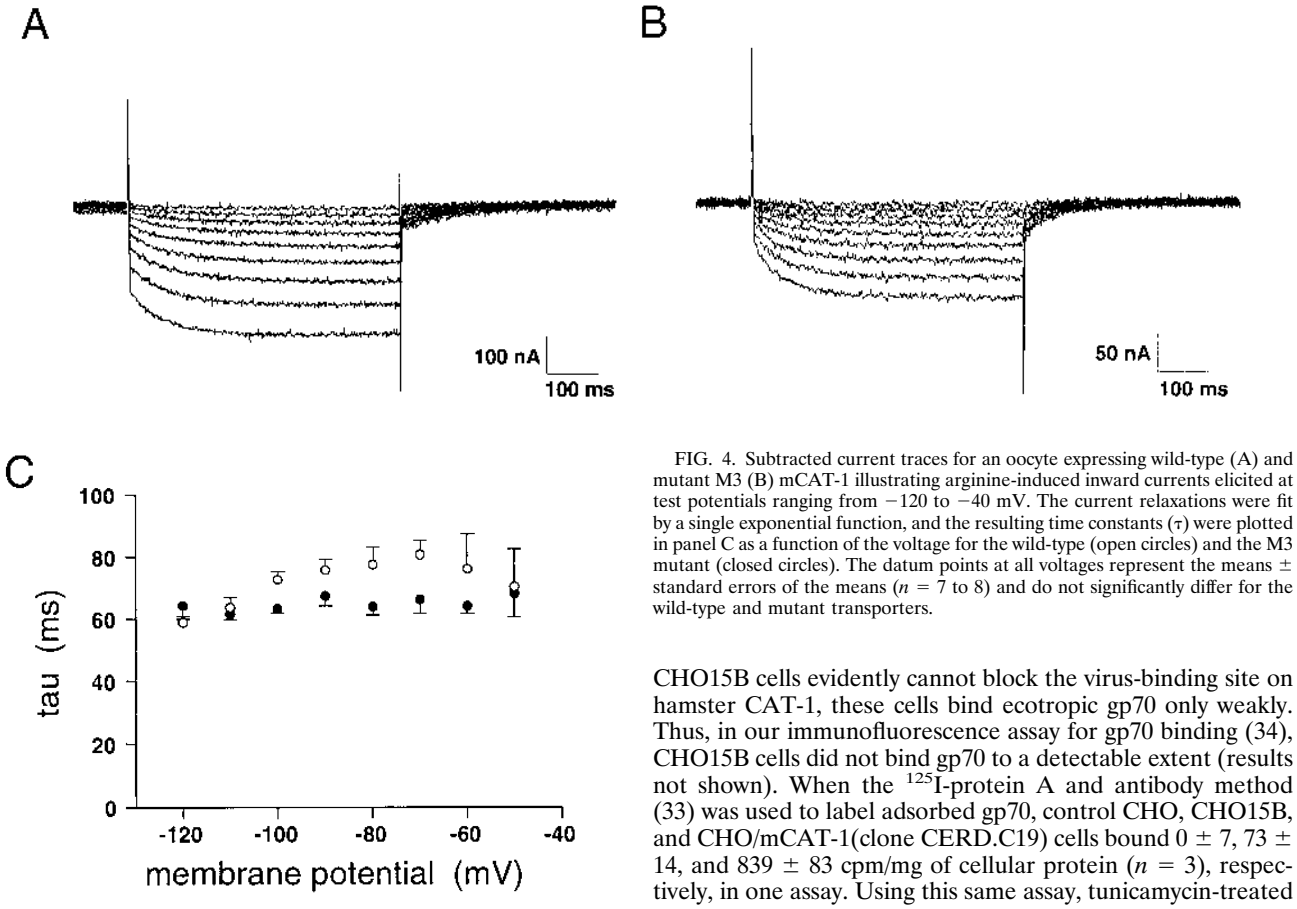

FIG. 4. Subtracted current traces for an oocyte expressing wild-type (A) and mutant M3 (B) mCAT-1 illustrating arginine-induced inward currents elicited at test potentials ranging from −120 to −40 mV. The current relaxations were fit by a single exponential function, and the resulting time constants (τ) were plotted in panel C as a function of the voltage for the wild-type (open circles) and the M3 mutant (closed circles). The datum points at all voltages represent the means ± standard errors of the means (n = 7 to 8) and do not significantly differ for the wild-type and mutant transporters.

the conclusion that removal of the glycosylation sites has not significantly altered the transporter kinetics.

Effects of N-linked glycosylation on ecotropic MuLV infection of CHO fibroblasts. In agreement with previous studies (24, 25, 37), treatment of CHO cells with tunicamycin also made them susceptible to infection by the Rauscher strain of ecotropic MuLV (Table 3). As an improved positive control for quantitatively analyzing receptor function and for monitoring damaging side effects of tunicamycin (9), we used a clone of CHO/mCAT-1 cells (clone CERD.C19) (36). This positive control is preferable to murine fibroblasts because cells of different species inherently differ in efficiencies of infection by MuLVs (36) and cell lines also differ in inhibitory effects of tunicamycin (9). As shown in Table 3 (experiments 1A and 1B), tunicamycin-treated CHO cells were approximately 7% as susceptible to infection by Rauscher MuLV as tunicamycin-treated CHO/mCAT-1 cells. In contrast, tunicamycin did not induce susceptibility of mink CCL64 or human Ostk cells. In addition, we quantitatively examined susceptibility to infection of CHO15B, a CHO cell mutant that was selected for resistance to the plant lectin phytohemagglutinin (11, 13). CHO15B cells lack GlcNAc-transferase I, which is necessary for conversion of neutral high-mannose N-linked oligosaccharides into larger complex derivatives, and they predominantly contain small $Man_5(GlcNAc)_2$-oligosaccharides in their glycoproteins (11, 13). As shown in Table 3 (experiments 2A and 2B), CHO15B cells are approximately 8 to 12% as susceptible to infection as the CHO/mCAT-1 cells. Treatment of CHO cells with neuraminidase did not induce susceptibility to infection. Moreover, although the small neutral oligosaccharides made in CHO15B cells evidently cannot block the virus-binding site on hamster CAT-1, these cells bind ecotropic gp70 only weakly. Thus, in our immunofluorescence assay for gp70 binding (34), CHO15B cells did not bind gp70 to a detectable extent (results not shown). When the ^{125}I-protein A and antibody method (33) was used to label adsorbed gp70, control CHO, CHO15B, and CHO/mCAT-1(clone CERD.C19) cells bound 0 ± 7, 73 ± 14, and 839 ± 83 cpm/mg of cellular protein (n = 3), respectively, in one assay. Using this same assay, tunicamycin-treated

TABLE 3. Infection of CHO cells with ecotropic Rauscher MuLV

Expt	Cells[a]	Tunicamycin[b]	MOI[c]
1A	CHO	−	<0.001
	CHO	+	0.14 ± 0.01
	CHO/mCAT-1	−	2.50 ± 0.20
	CHO/mCAT-1	+	1.68 ± 0.11
1B	CHO	−	<0.001
	CHO	+	0.18
	Ostk/mCAT-1	−	0.96
	Ostk/mCAT-1	+	0.28
	Ostk	−	<0.001
	Ostk	+	<0.001
	CCL64	−	<0.001
	CCL64	+	<0.001
2A	CHO	−	<0.001
	CHO/mCAT-1	−	2.20 ± 0.17
	CHO15B	−	0.26 ± 0.02
2B	CHO	−	<0.001
	CHO/mCAT-1	−	1.2
	CHO15B	−	0.10

[a] The cells used were CHO, CHO/mCAT-1 (clone CERD.C19), CHO15B (11), human osteogenic sarcoma cell line Ostk, Ostk cells that express mCAT-1 (Ostk/mCAT-1) (36), and CCL64 mink lung fibroblasts. CHO15B is a mutant of CHO that lacks GlcNAc-transferase I and contains primarily small neutral oligosaccharides in its glycoproteins (11, 13).

[b] Tunicamycin treatment (9) was with 0.15 μg/ml for 18 h at 37°C.

[c] Susceptibilities of the cell clones to infection were measured by infecting with the helper-free ecotropic host-range virus that encodes human growth hormone and by analyzing the multiplicities of infection (MOI) by statistical analysis of the immunofluorescence results as described in Table 1, footnote *c*. A value of <0.001 indicates that no cells expressed human growth hormone of at least 1,000 cells that were observed.

CHO did not bind a significant quantity of gp70, although the same treatment of CHO/mCAT-1 cells (clone CERD.C19) and of mink CCL64/mCAT-1 cells (clone CEN) caused 30 to 40% increases in their binding of gp70. These results support the hypothesis that N-linked oligosaccharides partially block the gp70 binding sites in wild-type mCAT-1. In addition, they suggest that resistance of CHO cells to ecotropic MuLVs is due partly to glycosylation of the hamster receptor and partly to an inherent weakness in its viral affinity.

DISCUSSION

Several lines of evidence suggest that N-linked glycosylation of loop 3 in mCAT-1 reduces binding of ecotropic gp70 at 37°C. First, in conditions of gp70 excess, the ratio of gp70 binding to V_{max} for L-[^{3}H]arginine import or export is greater for mutant M3 than for wild-type mCAT-1 (Tables 1 and 2). However, gp70 binds to the accessible sites in wild-type and M3 mutant mCAT-1 molecules with very similar kinetics and equilibrium constants (Fig. 3). These results exclude the possibility that the mutations in the M3 mutant alter the binding constant for gp70. Rather, they seem to increase the number of sites accessible to gp70 in conditions of saturated binding. Consequently, we infer that glycosylation of wild-type mCAT-1 might occlude a proportion of the sites for virus binding without substantially perturbing the remainder of the sites. This difference in gp70 binding/V_{max} ratios for wild-type and M3 mutant mCAT-1 does not appear to be caused by an effect of glycosylation on turnover time for the transport cycle because the slow steps in the arginine import cycle are not significantly different for glycosylated and unglycosylated mCAT-1 (Fig. 4). In addition, Michaelis-Menten constants are the same for the wild-type and M3 mutant transporters in mammalian cells at 37°C (Table 1) and in *Xenopus* oocytes at 22°C. These data do not exclude the possibility that a portion of unglycosylated M3 protein on cell surfaces binds gp70 but is inactive in transport because of misfolding or because of a perturbation in its interactions with other factors. However, the transport-competent M3 molecules behave similarly to wild-type mCAT-1 transporters (Table 1 and Fig. 4). Second, the hypothesis that glycosylation reduces gp70 binding is consistent with our results (e.g., Table 3) and those of previous workers (24, 25, 37) on infection of hamster cells by ecotropic MuLVs. Inhibition of N-linked glycosylation by treatment with tunicamycin enables CHO cells to be infected approximately 7% as efficiently as tunicamycin-treated CERD.C19 cells, a CHO cell derivative that expresses mCAT-1 (Table 3, experiments 1A and 1B). Tunicamycin does not enhance infectivity of CERD.C19 because infectivity of these cells is not limited by numbers of receptors (36) and because tunicamycin causes some cytotoxicity. Moreover, CHO15B cells, which lack GlcNAc-transferase I and synthesize small neutral oligosaccharides that are predominantly $Man_5(GlcNAc)_2$- (11, 13), are infected approximately 8 to 12% as efficiently by ecotropic Rauscher MuLV as the CERD.C19 clone of CHO that expresses mCAT-1 (Table 3, experiments 2A and 2B). Together, these results support the hypothesis that a portion of glycosylated wild-type mCAT-1 (ca. 50%) may be unable to bind ecotropic gp70 in the conditions of our assays, presumably because it contains large or highly processed oligosaccharides that occlude the virus-binding site. Previous evidence that mCAT-1 consists of diverse glycoforms (17) is consistent with this interpretation. Our observation that tunicamycin causes increased binding of gp70 onto cells that express mCAT-1 also supports this conclusion. Oligosaccharides at single sites in a glycoprotein generally have diverse structures, and some sites are incompletely glycosylated (19, 27). A D residue in the middle position of the NX (S/T) consensus sequence, which occurs in loop 3 of mCAT-1 (Fig. 1), has been associated with incomplete glycosylation (29). In addition, glycosylation of one site in loop 3 of mCAT-1 could conceivably alter processing at the other site (19, 27), resulting in further divergence of glycoforms.

These interpretations are compatible with our analyses of mCAT-1 down-modulation in mink cells infected with ecotropic Rauscher MuLV (Table 2 and Fig. 2). Down-modulation of wild-type mCAT-1 transport activity is only partial (ca. 40 to 60%), as determined by studies of L-[^{3}H]arginine import or export, whereas unglycosylated M3 mutant mCAT-1 appears to be completely down-modulated. Our control studies demonstrate that ecotropic Rauscher MuLV synthesis in mink CCL64 cells has no effect on expression of the endogenous mink y^+ transporter (33) (Fig. 2). Therefore, infection down-modulates only a transporter that strongly binds the viral gp70 glycoprotein. Consequently, a partial blockage of gp70 binding sites as a result of glycosylation would cause down-modulation to be incomplete. This would be compatible with complete resistance of infected cells to superinfection by ecotropic retroviruses (36) because a portion of the residual mCAT-1 on surfaces of infected cells would be unable to bind virus because of glycosylation and the remainder could be blocked by endogenously synthesized gp70.

Effects of mCAT-1 glycosylation were previously analyzed by Kim and Cunningham (17) in a study that used antiserum to mCAT-1 as well as site-directed mutagenesis. Their results established that both potential glycosylation sites in loop 3 are glycosylated in *Xenopus* oocytes and in mammalian cells. Moreover, their results suggested that coexpression of mCAT-1 and ecotropic gp70 in oocytes resulted in a partial (ca. 50%) reduction in mCAT-1 expression on cell surfaces, probably as a consequence of inhibition in mCAT-1 processing (17). In contrast with our data, they reported an experiment suggesting that the degree of down-modulation was not noticeably affected by mCAT-1 glycosylation, but they did not indicate the significances of the datum points or whether they were based on assays of multiple oocytes. We believe this apparent discrepancy in down-modulation may have derived from their use of different mutations in mCAT-1 and of *Xenopus* oocytes at room temperature rather than mammalian cells at 37°C. Binding of gp70 to mCAT-1 is highly temperature dependent and is relatively weak at lower temperatures (12). In addition, the processing of N-linked oligosaccharides differs in *Xenopus* oocytes and in mammalian cells (unpublished results).

Further analysis of the functions of wild-type and unglycosylated mutant mCAT-1 would be facilitated by availability of species-specific antibodies that react with mCAT-1 but not with the homologous proteins of nonmurine cells. We have been unable to make such an antiserum, in part because the hydrophilic loops in mCAT-1 are short and because the sequences of these loops are similar in different species. In addition, we have not succeeded in making epitope-tagged versions of mCAT-1 that retain viral receptor function.

Glycosylation of mCAT-1 appears to have several effects that could influence pathogenesis by ecotropic MuLVs. By reducing down-modulation that occurs in infected cells, glycosylation would enable a portion of mCAT-1 to remain on cell surfaces, thereby providing a supply of arginine and lysine for protein synthesis. Arginine is also the precursor of nitric oxide, which is essential for critical aspects of metabolic regulation. Because many glycosyltransferases have tissue-specific distributions (19, 27), oligosaccharide structures on mCAT-1 and consequent pathogenic effects of infection could differ in distinct cells. Accordingly, previous studies of *M. dunni* resistance

to Moloney MuLV have demonstrated that different ecotropic MuLVs can be influenced differentially by CAT-1 glycosylation (8). Therefore, it is possible that strains of virus can have distinct pathogenic effects dependent on CAT-1 glycosylation patterns in different tissues or mouse strains.

The conservation of glycosylation sites in the hypervariable loop 3 sequences of mammals (Fig. 1) suggests that they perform an important function. Indeed, loop 3 glycosylation sites also occur in the related CAT-2 transporters that are expressed in activated T lymphocytes and the liver (15). Further studies would be required to determine whether glycosylation contributes to processing or stability of these transporters or to their metabolic regulation. The role of receptor glycosylation in protecting mammals from different retroviruses (24, 25, 33, 37) will also require additional analyses.

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REFERENCES

1. **Albritton, L. M., J. W. Kim, L. Tseng, and J. M. Cunningham.** 1993. Envelope-binding domain in the cationic amino acid transporter determines the host range of ecotropic murine retroviruses. J. Virol. **67:**2091–2096.
2. **Albritton, L. M., L. Tseng, D. Scadder, and J. M. Cunningham.** 1989. A putative murine ecotropic retrovirus receptor gene encodes a multiple membrane-spanning protein and confers susceptibility to virus infection. Cell **57:**659–666.
3. **Barchan, D., S. Kachalsky, D. Neumann, Z. Vogel, M. Ovadia, E. Kochva, and S. Fuchs.** 1992. How the mongoose can fight the snake: the binding site of the mongoose acetylcholine receptor. Proc. Natl. Acad. Sci. USA **89:**7717–7721.
4. **Bestwick, R. K., S. L. Kozak, and D. Kabat.** 1988. Overcoming interference to retroviral superinfection results in amplified expression and transmission of cloned genes. Proc. Natl. Acad. Sci. USA **85:**5404–5408.
5. **Chirgwin, J. J., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
6. **Cone, R. D., and R. C. Mulligan.** 1984. High-efficiency gene transfer into mammalian cells: generation of helper-free recombinant retrovirus with broad mammalian host range. Proc. Natl. Acad. Sci. USA **81:**6349–6353.
7. **Eiden, M. V., K. Farrell, J. Warsowe, L. C. Mahan, and C. A. Wilson.** 1993. Characterization of a naturally occurring ecotropic receptor that does not facilitate entry of all ecotropic murine retroviruses. J. Virol. **67:**4056–4061.
8. **Eiden, M. V., K. Farrell, and C. A. Wilson.** 1994. Glycosylation-dependent inactivation of the ecotropic murine leukemia virus receptor. J. Virol. **68:**626–631.
9. **Elbein, A. D.** 1991. Glycosylation inhibitors: inhibitors of N-linked oligosaccharide processing. FASEB J. **5:**3055–3063.
10. **Gazzola, G. C., V. Dall'Asta, R. Franchi-Gazzola, and M. F. White.** 1981. The cluster-tray method for rapid measurement of salute fluxes in adherent cultured cells. Anal. Biochem. **115:**368–374.
11. **Gottleib, C., J. Baenziger, and S. Kornfeld.** 1975. Deficient uridine diphosphate-N-acetylglucosamine: glycoprotein N-acetylglucosaminyltransferase activity in a clone of Chinese hamster ovary cells with altered surface glycoproteins. J. Biol. Chem. **250:**3303–3309.
12. **Handelin, B. L., and D. Kabat.** 1985. Cell surface receptors for murine leukemia viruses: two assays and their implications. Virology **140:**183–187.
13. **Harpaz, N., and H. Schacter.** 1980. Control of glycoprotein synthesis processing of asparagine-linked oligosaccharides by one or more rat liver golgi α-D-mannosidases dependent on the prior action of UDP-N-acetylglucosamine: α-D-mannoside β2-N-acetylglucosaminyltransferase I. J. Biol. Chem. **255:**4894–4902.
14. **Kabat, D.** 1989. Cell surface receptors for ecotropic murine leukemia viruses: mobile membrane components that mediate binding and slow endocytosis of the viral envelope. Virology **171:**464–474.
15. **Kavanaugh, M. P., H. Wang, Z. Zhang, W. Zhang, Y. Wu, E. Dechant, R. A. North, and D. Kabat.** 1994. Control of cationic amino acid transport and retroviral receptor functions in a membrane protein family. J. Biol. Chem. **269:**15445–15450.
16. **Kim, J. W., E. I. Closs, L. M. Albritton, and J. M. Cunningham.** 1991. Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. Nature (London) **352:**725–728.
17. **Kim, J. W., and J. M. Cunningham.** 1993. N-linked glycosylation of the receptor for murine ecotropic retroviruses is altered in virus-infected cells. J. Biol. Chem. **268:**16316–16320.
18. **Kleinerman, E. S., L. B. Lachman, R. D. Knowles, R. Snyderman, and G. J. Gianciolo.** 1987. A synthetic peptide homologous to the envelope proteins of retroviruses inhibits monocyte-mediated killing by inactivating interleukin 1. J. Immunol. **139:**2329–2337.
19. **Kornfeld, R., and S. Kornfeld.** 1985. Assembly of asparagine-linked oligosaccharides. Annu. Rev. Biochem. **54:**631–664.
20. **Lauger, P.** 1991. Electrogenic ion pumps, p. 61–91. Sinauer Associates, Sutherland, Mass.
21. **Mann, R., R. C. Mulligan, and D. Baltimore.** 1983. Construction of a retroviral packaging mutant and its use to produce helper-free defective retrovirus. Cell **33:**153–159.
22. **Masuda, M., M. P. Remington, P. M. Hoffman, and S. K. Rusetti.** 1992. Molecular characterization of a neuropathogenic and nonerythroleukemogenic variant of Friend murine leukemia virus. J. Virol. **66:**2798–2806.
23. **Miller, A. D., M.-F. Law, and I. F. Verma.** 1985. Generation of helper-free amphotropic retroviruses that transduce a dominant-acting methotrexate-resistant dihydrofolate reductase gene. Mol. Cell. Biol. **5:**431–437.
24. **Miller, D. G., and A. D. Miller.** 1992. Tunicamycin treatment of CHO cells abrogates multiple blocks to retrovirus infection, one of which is due to a secreted inhibitor. J. Virol. **66:**78–84.
25. **Miller, D. G., and A. D. Miller.** 1993. Inhibitors of retrovirus infection are secreted by several hamster cell lines and are also present in hamster sera. J. Virol. **67:**5346–5352.
26. **Park, B. H., B. Matuschke, E. Lavi, and G. Gaulton.** 1994. A point mutation in the *env* gene of a murine leukemia virus induces syncytium formation and neurologic disease. J. Virol. **68:**7516–7524.
27. **Rademacher, T. W., R. B. Parekh, and R. A. Dwek.** 1988. Glycobiology. Annu. Rev. Biochem. **57:**785–838.
28. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
29. **Sharkin-Eshlemann, S., S. L. Spitalnik, and L. Kastur.** 1996. The amino acid at the X position of an Asn-X-Ser sequon is an important determinant of N-linked core-glycosylation efficiency. J. Biol. Chem. **271:**6363–6366.
30. **Sitbon, M., B. Sola, L. Evans, J. Nishio, S. F. Hayes, K. Nathanson, C. F. Garon, and B. Chesebro.** 1986. Hemolytic anemia and erythroleukemia, two distinct pathogenic effects of Friend MuLV: mapping of the two effects to different regions of the viral genome. Cell **47:**851–859.
31. **Stoll, J., K. C. Wadhwani, and Q. R. Smith.** 1993. Identification of the cationic amino acid transporter (system y^+) of the rat blood-brain barrier. J. Neurochem. **60:**1956–1959.
32. **Szurek, P. F., P. H. Yuen, J. K. Ball, and P. K. Y. Wong.** 1990. A Val-25-to-Ile substitution in the envelope precursor polyprotein, $gPr80^{env}$, is responsible for the temperature sensitivity, inefficient processing of $gPr80^{env}$, and neurovirulence of *ts*1, a mutant of Moloney leukemia virus TB. J. Virol. **64:**467–475.
33. **Talbot, S. J., R. A. Weiss, and T. F. Schulz.** 1995. Reduced glycosylation of human cell lines increases susceptibility to CD4-independent infection of human immunodeficiency virus type 2 (LAV/2B). J. Virol. **69:**3399–3406.
34. **Wang, H., E. Dechant, M. P. Kavanaugh, R. A. North, and D. Kabat.** 1992. Effects of ecotropic murine retroviruses on the dual-function cell surface receptor/basic amino acid transporter. J. Biol. Chem. **267:**23617–23624.
35. **Wang, H., M. P. Kavanaugh, R. A. North, and D. Kabat.** 1991. Cell surface receptor for ecotropic murine retroviruses is a basic amino acid transporter. Nature (London) **352:**729–731.
36. **Wang, H., R. Paul, R. E. Burgeson, D. R. Keene, and D. Kabat.** 1991. Plasma membrane receptors for ecotropic murine retroviruses require a limiting accessory factor. J. Virol. **65:**6468–6477.
37. **Wilson, C. A., and M. V. Eiden.** 1991. Viral and cellular factors governing hamster cell infection by murine and gibbon ape leukemia viruses. J. Virol. **65:**5975–5982.
38. **Yoshimoto, T., E. Yoshimoto, and D. Mervelo.** 1993. Identification of amino acid residues critical for infection with ecotropic murine leukemia retrovirus. J. Virol. **67:**1310–1314.