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Envelope-Binding Domain in the Cationic Amino Acid Transporter Determines the Host Range of Ecotropic Murine Retroviruses

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Infection of rodent cells by ecotropic type C retroviruses requires the expression of a cationic amino acid transporter composed of multiple membrane-spanning domains. By exchanging portions of cDNAs encoding the permissive mouse and nonpermissive human transporters and examining their abilities to specify virus infection upon expression in human 293 cells, we have identified the amino acid residues in the extracellular loop connecting the fifth and sixth membrane-spanning segments of the mouse transporter that are required for both envelope gp70 binding and infection. These findings strongly suggest that the role of the mouse transporter in determining infection is to provide an envelope-binding site. This role is analogous to those of host membrane proteins composed of a single membrane-spanning domain that serve as binding proteins or receptors for other enveloped viruses such as human immunodeficiency virus, Epstein-Barr virus, and murine and human coronaviruses.

Infection by enveloped viruses is limited to host tissues that supply the appropriate machinery for entry and replication. Entry is initiated through binding of the virus envelope to specific receptors on the plasma membrane of the host cell. In one well-studied example, the host range of murine hepatitis virus (MHV) is determined by the expression of a type I membrane protein related to carcinoembryonic antigen that mediates binding of the MHV envelope to the intestinal brush borders of BALB/c mice (23). A monoclonal antibody that recognizes this receptor can compete with MHV for binding and thereby block infection (24). Furthermore, introduction of a cDNA encoding the receptor from BALB/c mice is sufficient for the infection of cells from nonpermissive mouse strains (5). In a similar fashion, other type I membrane proteins, such as CD4 and CR2, serve as receptors for human immunodeficiency virus (3, 13) and Epstein-Barr virus (6), respectively.

Infection by the ecotropic subclass of leukemogenic retroviruses (murine leukemia virus [MuLV]) is restricted to rodent cells that express the cationic amino acid transporter (CAT) (2, 9). Unlike the receptors composed of a single membrane-spanning domain that tether a long extracellular region containing the virus-binding site, CAT contains several small extracellular domains connected by hydrophobic membrane-spanning segments. This difference suggests that ecotropic MuLV may bind to murine CAT (MCAT) differently than other enveloped viruses bind to type I membrane proteins or that MCAT may mediate a step in infection distinct from envelope binding.

Cells that express human CAT (HCAT) (1) are not susceptible to ecotropic MuLV infection, even though HCAT is closely related to MCAT (87% identical). The resistance of human cells to infection by ecotropic MuLV can be overcome by the introduction of an expression plasmid encoding MCAT. Therefore, there must be at least one domain in MCAT that is distinct from those in HCAT and that is required for ecotropic MuLV infection but not for transport of cationic amino acids. By identifying this domain, we sought to understand the role of MCAT in mediating ecotropic MuLV infection. Our approach has been to measure the capacities of chimeric MCAT-HCAT proteins to bind the virus envelope surface protein gp70 and to confer susceptibility to ecotropic MuLV infection onto human cells.

MATERIALS AND METHODS

Cell lines and viruses. Mouse NIH 3T3 fibroblasts and nonpermissive human 293 fetal kidney cells were cultured in Dulbecco modified Eagle medium (GIBCO, Grand Island, N.Y.) supplemented with 10% donor calf serum (Hazleton, Lenexa, Kans.). Replication-defective ecotropic MuLVs include BAG (19), which encodes *Escherichia coli* β-galactosidase; DHFR (21), which encodes a mutant dihydrofolate reductase that confers methotrexate resistance; and NEO (22), which encodes neomycin phosphotransferase that confers resistance to G418 (GIBCO). OA is an ecotropic MuLV (18) that is replication competent. Cell lines producing these viruses were also grown in Dulbecco modified Eagle medium–10% calf serum.

In vitro mutagenesis. The 735-bp *Hpa*I-*Kpn*I restriction fragment of pJET (2) and the 790-bp *Sst*I-*Dra*I restriction fragment from pJLTH (1), plasmids containing the cDNAs encoding MCAT and HCAT, respectively, in the eukaryotic expression vector pJ3 (12), were excised and subcloned into M13 mp18. Phage DNA was used to prepare templates for an in vitro mutagenesis protocol (11) that utilizes oligonucleotides encoding the altered residues as primers. The clones containing mutations were identified by dideoxynucleotide sequencing (USB, Cleveland, Ohio) and then subcloned back into pJET or pJLTH and resequenced in order to confirm the presence of the mutation and the correct reading frame.

Transient BAG virus assay. Ten micrograms of pJET, pJLTH, and expression plasmids encoding chimeric transporters was applied to 5×10^5 human 293 cells by $Ca_3(PO_4)_2$ precipitation. Forty-eight and 72 h after transfection, these

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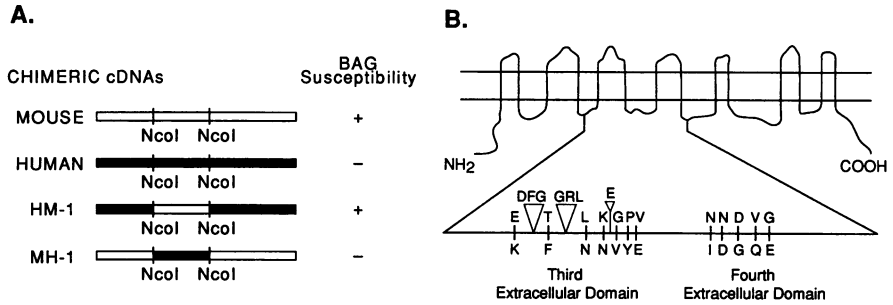

FIG. 1. Susceptibility to ecotropic MuLV infection is encoded within an internal *Nco*I-*Nco*I fragment of MCAT. (A) Capacities of chimeric cDNAs created by exchange of conserved *Nco*I-*Nco*I fragments between mouse and human transporters to confer susceptibility to ecotropic BAG infection when transiently expressed in human 293 cells. Transfection of the cDNA encoding MCAT resulted in 500 to 1,000 positively stained cells per 10^5 cells. Infection is represented by a plus when more than 100 blue cells per 10^5 cells were observed on the culture plate and by a minus when no blue cells were observed. (B) Schematic of transporter topology with respect to the membrane. The triangular inset represents the segment of mouse and human transporters encoded within the exchanged *Nco*I-*Nco*I fragments. Differences in the amino acid sequences of the mouse transporter (below the line) and the human transporter (above the line) are localized to the third and fourth extracellular domains encoded within these fragments.

cells were exposed to BAG virus in a medium containing Polybrene (8 μg/ml). Two days later, the cells were fixed in 0.5% glutaraldehyde and assayed for β-galactosidase activity with X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) as a substrate (19). Plates were examined under a light microscope for positively stained (blue) cells.

Cell lines stably expressing chimeric transporters. Ten micrograms of expression plasmids encoding chimeric transporters and 0.5 μg of pSV_{2gpt} were applied to 2.5×10^5 human 293 cells by $Ca_3(PO_4)_2$ precipitation and then selected in mycophenolic acid, as previously described (2). Resistant colonies were pooled and exposed to 10^6 CFU of NEO virus per ml for 4 h, and 2 days later they were propagated in a culture medium supplemented with G418 (GIBCO) at a dose (1 mg/ml for 25 days) sufficient to kill 293 cells transfected with the plasmid backbone. By this protocol, a polyclonal population of 293 cells that had acquired susceptibility to the NEO virus was selected. For 293 cells transfected with nonpermissive transporters, such as MH-1 and M-38, mycophenolic acid-resistant colonies were isolated as clones, expanded, and used to prepare RNA for Northern (RNA) blots. Colonies that expressed the 5.0-kb transcript derived from the transporter-encoding expression plasmid at steady-state levels comparable to or greater than levels for the MCAT transcript in mouse NIH 3T3 fibroblasts were identified. The transcript derived from the expression plasmid was clearly distinguishable from the endogenous 8.0-kb human transcript.

Virus titers. Cells were exposed to serial dilutions of virus for 4 h in a medium containing 8 μg of Polybrene per ml at 37°C. Cells infected with BAG were identified as described above and were counted. Cells exposed to DHFR virus were transferred 2 days later to a culture medium supplemented with 150 μM methotrexate for 2 weeks, and then drug-resistant colonies were fixed and stained in 2% crystal violet–90% ethanol. Cells exposed to OA virus were fixed 2 days later in cold methanol-acetone (1:1), incubated for 1 h in a 1:500 dilution of polyclonal rabbit antibody against $p30^{gag}$ (17), washed with phosphate-buffered saline (PBS), and then incubated in a 1:1,000 dilution of goat anti-rabbit antibody (Cappel, Durham, N.C.) conjugated to horseradish peroxidase. After 1 h, these cells were washed four times in PBS and incubated with 3-amino-9-ethylcarbazole (200 μg/ml) (Sigma, St. Louis, Mo.), a chromogenic substrate for horseradish peroxidase, in 50 mM sodium acetate (pH 5.0)–0.015% H_2O_2 for 10 min and then scored for red-stained cells with a light microscope.

gp70 binding. Human 293 cells (5×10^5) were incubated in 200 μl of medium containing 5% bovine serum albumin, 50 mM BES (*N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid; pH 6.8), and ^{125}I-gp70 (0 to 535 ng/ml) for 1 h at 37°C. Cells were then washed, solubilized, and counted as described previously (4). Two days after being injected with 30 ng of mRNA prepared by in vitro transcription, *Xenopus laevis* oocytes were incubated with 4 ng of ^{125}I-gp70 (2×10^4 cpm/ng) for 1 h at room temperature and then washed, solubilized, and counted as previously described (9).

RESULTS

In our initial experiment, we exchanged an *Nco*I-*Nco*I restriction fragment conserved between the cDNAs encoding both MCAT and HCAT (Fig. 1A). After the chimeric cDNAs were subcloned into the mammalian expression vector pJ3, these plasmids were transfected into nonpermissive human 293 kidney cells. Forty-eight hours later, the transfected 293 cells were exposed to BAG, a recombinant ecotropic retrovirus carrying the *E. coli lacZ* gene, and scored for infection by a histochemical assay for β-galactosidase activity with X-Gal as the substrate. The infectivity of 293 cells that express HM-1, the chimeric transporter created by placement of the murine *Nco*I-*Nco*I fragment into the HCAT-encoding plasmid, was indistinguishable from that of 293 cells transfected with MCAT (>100 BAG-positive cells per 10^5 cells). In contrast, human 293 cells that express the reciprocal construct (MH-1), created by insertion of the human *Nco*I-*Nco*I fragment into the MCAT-encoding plasmid, remained nonpermissive. Therefore, ecotropic BAG infection is determined by a portion of MCAT encoded within the *Nco*I-*Nco*I fragment.

Comparison of the deduced amino acid sequence encoded within the *Nco*I-*Nco*I fragment of MCAT with that encoded within the *Nco*I-*Nco*I fragment of HCAT revealed the divergence of 19 residues in the putative third and fourth extracellular domains (Fig. 1B). cDNAs encoding additional reciprocal MCAT-HCAT chimeras were created by in vitro mutagenesis (11) in order to examine the importance of these differences in determining susceptibility to ecotropic BAG virus (Table 1). Substitution of the 5 divergent amino acid residues found in a stretch of 7 residues near the C-terminal

TABLE 1. Susceptibilities to ecotropic BAG of human 293 cells that transiently express chimeric human-mouse transporters[a]

Chimera	Third extracellular domain	Fourth extracellular domain	BAG infection
M-22		NN D	+
M-23		V G	+
M-24	E		+
M-11	KFSCT GRL		+
M-26	L		+
M-36	KEG P V		−
M-37	KEG V		−
M-38	P V		−
M-64	P		+
M-65	V		+
Mouse	215 KNWQLTEK---NFS---CNNNDTN-VKYGEGG 239	306 PYFCLDIDSPLPGAFKHQGWEEAKY 330	+
Human	215 KNWQLTEEDFGNTSGRLCLNNDTKEGKPGVGG 246	313 PYFCLDNNSPLPDAFKHVGWEGAKY 337	−
H-21	K		−
H-12	---		−
H-17	N N-V Y		+
H-19	N-V Y E		+
H-40	Y E		+
H-62	Y		−
H-63	E		−
H-28		ID G	−
H-29		Q E	−

[a] Residues 215 to 239 compose the third extracellular domain of the mouse transporter and residues 215 to 246 compose the third extracellular domain of the human transporter. Residues composing the fourth extracellular domain of the mouse (residues 306 to 330) and human (residues 313 to 337) transporters are also given. Above each mouse sequence are the substitutions of human residues into the mouse transporter, and below each human sequence are the substitutions of mouse residues into the human transporter. The single-letter amino acid code is used throughout this table. Human 293 cells transiently transfected with cDNAs encoding these chimeric transporters were evaluated for susceptibilities to ecotropic BAG infection as described in the legend to Fig. 1.

end of the third extracellular domain of HCAT (KEGKPGV$_{238-244}$) with the equivalent residues from MCAT (N-VKYGE$_{232-237}$) was sufficient to permit infection by ecotropic BAG upon expression in 293 cells (H-19 in Table 1). In addition, expression of two chimeras related to H-19 permitted BAG infection. These chimeras came about by replacement of LNNDTKEGKP$_{233-242}$ in HCAT by NNND TN-VKY$_{227-235}$ from MCAT (H-17) and substitution of PGV$_{242-244}$ in HCAT by YGE$_{235-237}$ from MCAT (H-40). Furthermore, the reciprocal substitutions, replacement of N-VKYGE$_{232-237}$ in MCAT by KEGKPGV$_{238-244}$ from HCAT (M-36) and YGE$_{235-237}$ in MCAT by PGV$_{242-244}$ from HCAT (M-38), completely abolished the susceptibility to MuLV infection conferred by MCAT. Expression of a series of chimeric MCAT-HCAT cDNAs that together encode the remainder of the divergent amino acid residues encoded within the *Nco*I-*Nco*I fragment failed to identify additional MCAT residues that are required for BAG infection (Table 1). Also, replacement of Pro-242 in HCAT by Tyr-235, the single residue from MCAT that is shared among the permissive chimeras, H-19, H-17, and H-40, was not by itself sufficient to permit infection (H-62 in Table 1), nor did the substitution of proline for Tyr-235 prevent MCAT-mediated infection (M-64 in Table 1). Therefore, the failure of ecotropic MuLV to infect human cells is not solely a consequence of HCAT secondary structure determined by Pro-242. Indeed, the introduction of HCAT residues (KEG$_{239-241}$ and V$_{244}$) adjacent to Pro-242 was also sufficient to abolish MCAT-mediated infection (M-37).

The capacities of the chimeric transporters to permit infection were compared by determining the susceptibilities of clonal 293 cell lines that express these proteins to infection by ecotropic MuLV. These cell lines were created by cotransfection with the pJ3 expression vector carrying the cDNAs encoding chimeric transporters and pSV$_{2gpt}$ prior to their selection in a medium containing mycophenolic acid. The susceptibility of each cell line to infection was measured by end-point dilution of BAG and/or two additional ecotropic MuLVs: DHFR (22), which confers methotrexate resistance, and OA (16), which can be identified by immunoenzymatic assay for p30^{gag} (18) (Table 2). The susceptibility of the 293 cell line which expresses the HCAT chimera containing YGE$_{235-237}$ from MCAT (H-40) to BAG (2.6×10^5 IU/ml) and to DHFR (2×10^5 CFU/ml) infection is comparable to that of 293 cells that express MCAT, and this cell line is only 10-fold less susceptible to infection than mouse NIH 3T3 fibroblasts. In addition, the replacement of YGE$_{235-237}$ in MCAT by the equivalent residues, PGV$_{242-244}$, from HCAT (M-38) decreased susceptibility to ecotropic DHFR virus more than 10^5-fold: three methotrexate-resistant colonies of 293 cells expressing this chimera were identified in an experiment in which DHFR infection of 293 cells expressing MCAT resulted in 4×10^5 methotrexate-resistant colonies (data are corrected for dilution). These colonies resulted from bona fide infection, since no infected colonies were observed on 293 cells that express HCAT or MH-1. A slightly greater decrease in infectivity was observed with culture plates exposed to OA virus. This difference may reflect the spread of replication-competent OA virus during the 48 h between exposure and performance of the assay.

The importance of amino acid residues located within a putative extracellular domain of MCAT in infection suggests that these residues may interact directly with gp70, the MuLV envelope protein that determines virus host range. To examine this hypothesis, we measured binding of purified gp70 that had been labelled with ^{125}I to the cell lines which express the chimeric transporters. Each line was grown to confluence, incubated with ^{125}I-gp70 for 1 h at 37°C, washed, and solubilized, and bound ^{125}I-gp70 was determined with a gamma counter (4). A range of gp70 concentrations (0 to 535 ng/ml) that result in saturable binding to mouse NIH 3T3

TABLE 2. Ecotropic virus titers on cell lines that express chimeric transporters[a]

Chimera[b]	Third extracellular domain	Virus titer		
		BAG (IU/ml)	DHFR (CFU/ml)	OA (IU/ml)
M-38	P V	ND[c]	3	20
M-64	P	3.5×10^5	ND	ND
M-65	V	5×10^5	ND	ND
MH-1	KDFG T GRL L KEG P V	ND	0	0
Mouse	SIKNWQLTEK---NFS---CNNNDTN-VKYGEGG	4×10^5	4×10^5	8×10^6
Human	SIKNWQLTEEDFGNTSGRLCLNNDTKEGKPGVGG	0	0	0
HM-1	K--- F --- N N-V Y E	ND	4.2×10^5	8.5×10^6
H-40	Y E	2.6×10^5	2×10^5	8.2×10^6
H-62	Y	0	ND	ND
H-63	E	0	ND	ND

[a] Human 293 cell lines that express the mouse, human, or chimeric transporters were created as described in the legend to Fig. 2. The susceptibility of each cell line was determined by infection with serial 10-fold dilutions of BAG (10^6 IU/ml on NIH 3T3 fibroblasts), DHFR (10^7 CFU/ml), or OA (10^8 IU/ml) virus.
[b] Chimeric proteins are described in Table 1, footnote *a*.
[c] ND, not determined.

fibroblasts was studied (Fig. 2A). These experiments demonstrate that at the half-saturation concentration of 80 ng/ml, binding of gp70 to 293 cells that express MCAT (0.95 ng/10^5 cells) is 7.6-fold greater than that to 293 cells (0.125 ng/10^5 cells) that express HCAT and is comparable to binding by permissive mouse NIH 3T3 fibroblasts (Fig. 2B). Expression of the nonpermissive MCAT chimera that contains $PGV_{242-244}$ from HCAT (M-38) did not increase the gp70-binding capacity of 293 cells (Fig. 2B), a finding consistent with the hypothesis that the replacement of $YGE_{235-237}$ has disrupted a portion of the envelope-binding site. However, the permissive cell line that expresses H-40, the reciprocal chimera which contains $YGE_{235-237}$, also did not bind detectably more gp70 than 293 cells which express HCAT (Fig. 2B), despite demonstrating susceptibility to infection comparable to that by 293 cells expressing MCAT or HM-1 (Table 2).

It is unlikely that the lack of gp70 binding by cells expressing H-40 reflects a low level of expression, since the susceptibility of these cells was comparable to that of human cells expressing MCAT. However, we could not rule out this possibility in studies with the 293 cells. Therefore, we repeated the gp70-binding assays with frog oocytes injected with mRNA encoding the chimeric proteins. We have routinely observed a 5- to 20-fold increase in arginine uptake by oocytes injected with MCAT-encoding mRNA, thereby establishing a sensitive assay for functional protein in the plasma membrane (9). The increase in arginine uptake by oocytes injected with mRNA encoding M-38, the MCAT chimera containing $PGV_{242-244}$, is comparable to that by MCAT or HCAT (Fig. 3A). However, gp70 binding to oocytes which express MCAT is 30-fold greater than that to oocytes which express M-38 or HCAT (Fig. 3B). Therefore, the presence of $PGV_{242-244}$ in MCAT abrogated gp70 binding without influencing arginine transport, suggesting that these residues disrupt the envelope-binding site without impairing the folding or the delivery of the chimeric transporter to the cell surface. Furthermore, gp70 binding to oocytes injected with mRNA encoding H-19, the permissive chimera that

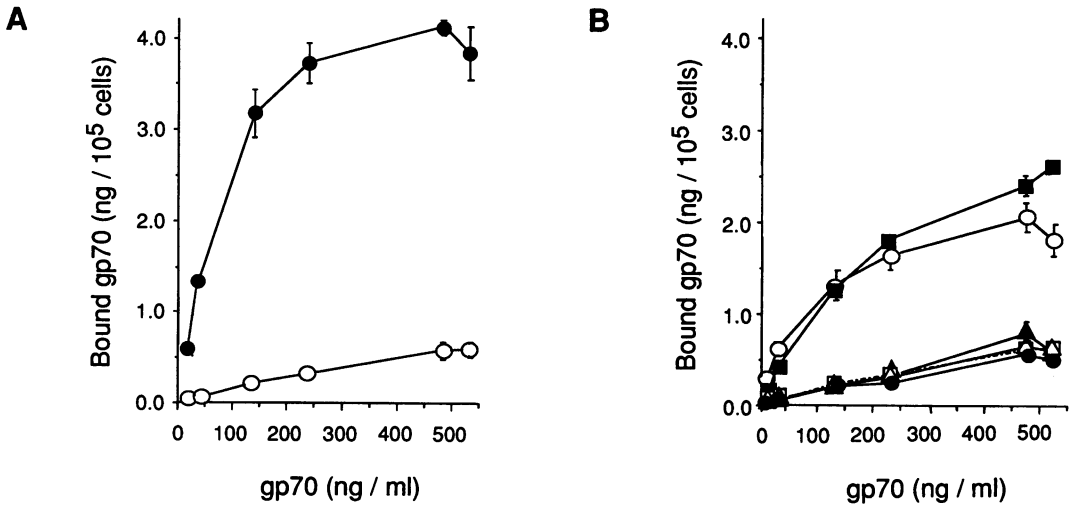

FIG. 2. Binding of purified ecotropic gp70 to human 293 cell lines expressing chimeric transporters. Each datum point for binding represents the mean ± 1 standard deviation. (A) gp70 binding to permissive murine NIH 3T3 fibroblasts (closed circles) and nonpermissive human 293 cells (open circles). (B) gp70 binding to 293 cells expressing MCAT (open circles), HM-1 (closed squares), MH-1 (closed circles), M-38 (closed triangles), and H-40 (open triangles) and to 293 cells alone (open squares). Similar data were obtained by studying two additional independent colonies that expressed MH-1 and one additional independent colony that expressed M-38.

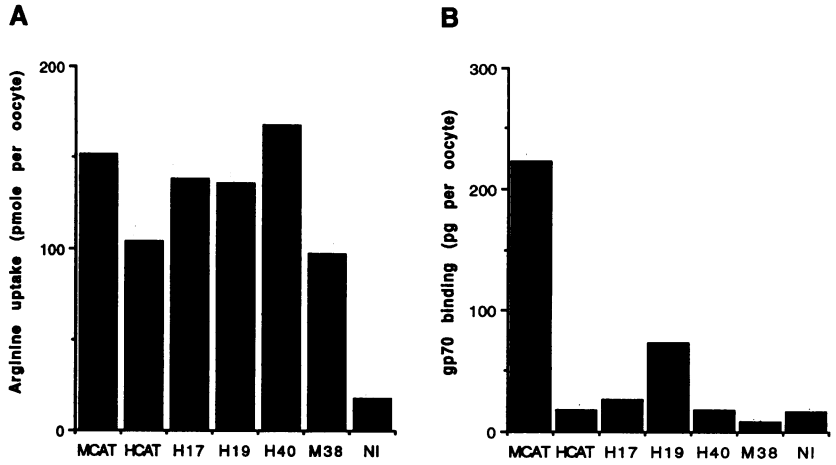

FIG. 3. Transport and envelope binding by frog oocytes that express chimeric proteins. (A) Arginine transport by frog oocytes injected with mRNA encoding MCAT, HCAT, or the chimeric proteins H-17, H-19, H-40, and M-38 described in Table 1. NI, not injected. Results for each assay are the means of data from three oocytes. (B) ^{125}I-gp70 binding to oocytes injected with mRNAs encoding the chimeric proteins described for panel A. Results for each assay are the means of data from three oocytes.

contains N-VKYGE$_{232-237}$ from MCAT, was fivefold greater than binding to oocytes which expressed HCAT. Therefore, the C-terminal portion of the third extracellular domain of MCAT is necessary for both envelope binding and ecotropic MuLV infection. In addition, no increase in gp70 binding to oocytes that express H-40, the chimeric protein that contains YGE$_{235-237}$, was observed, thereby confirming the results obtained with permissive 293 cells which express this protein.

DISCUSSION

In this study, we have identified a putative extracellular domain of MCAT that is required for infection (Fig. 1B; Table 1). Preliminary experiments demonstrate the glycosylation of two asparagine residues (residues 223 and 229) within this domain (10), consistent with a position for this domain on the extracellular face of the plasma membrane that would permit contact with the virus envelope. As yet, we have not demonstrated a direct interaction between gp70 and this domain, and it therefore remains conceivable that the critical MCAT residues identified in our studies could change the conformation of the transporter in a way that creates an envelope-binding site in another domain of MCAT or in an associated protein. However, the substitution of as few as 2 amino acid residues (PGV$_{242-244}$) from the nonpermissive human transporter abrogated both infection and gp70 binding, suggesting that these residues replace a portion of the gp70-binding site that is necessary for infection.

The behavior of H-40, the HCAT chimera containing YGE$_{235-237}$, demonstrates that it is possible to obtain efficient infection without a measurable increase in gp70 binding. This finding indicates that equilibrium binding of purified ecotropic envelope surface protein to susceptible cells is not a sensitive measure of the virus-transporter interaction which results in infection. These residues may permit sufficient binding to the virus envelope for infection, but adjacent residues, including N-V$_{233}$, apparently stabilize this interaction, as suggested by the increase in gp70 binding to oocytes which express the H-19 chimera. Alternatively, it is possible that binding of the MuLV envelope to MCAT is not the limiting step in infection, a hypothesis proposed to explain the behavior of polioviruses carrying mutations which decrease receptor binding without changing infection kinetics (8) and that of the human and mink cells transfected with the MCAT expression plasmid, pJET, that failed to demonstrate a correlation between the level of MCAT expression and susceptibility to infection (20). Other portions of MCAT which interact with the ecotropic MuLV envelope but which are conserved in HCAT cannot be identified by our experiments. However, chimeric proteins created by exchanging portions of MCAT and more divergent CATs from other species may permit the identification of these regions.

These studies suggest that MCAT may be functionally similar to the receptors for human immunodeficiency virus, Epstein-Barr virus, and murine hepatitis virus, despite their differences in membrane topology. Like these type I membrane proteins, MCAT contains a discrete extracellular domain that determines host range and that may bind to the virus envelope prior to fusion. Indeed, purified ecotropic virions coupled with antibodies to either human transferrin receptor (7) or human major histocompatibility complex class I or II antigens (16) or modified by covalent attachment of galactose (14) can infect hepatocytes that do not express MCAT. These infections are mediated by binding of the modified virus envelope to the targeted transferrin receptor or class I or II proteins or through the attachment of galactose to the asialoglycoprotein receptor expressed on susceptible cells. Therefore, envelope binding may be the sole function of MCAT in mediating infection. Also, comparison of the membrane topologies of CD4, murine carcinoembryonic antigen-like protein, and MCAT and GALV-R (15), a multiple-membrane-spanning protein that determines the host range of gibbon ape leukemia virus, identifies the presence of a suitable envelope-binding domain as the only property in common. Introduction of the MCAT domain identified in these studies into other membrane proteins may permit an additional test of the hypothesis that envelope binding is sufficient for infection.

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