Analysis of Receptor Usage by Ecotropic Murine Retroviruses, Using Green Fluorescent Protein-Tagged Cationic Amino Acid Transporters

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Entry of ecotropic murine leukemia virus (MuLV) into host cells is initiated by interaction between the receptor-binding domain of the viral SU protein and the third extracellular domain (TED) of the receptor, cationic amino acid transporter 1 (CAT1). To study the molecular basis for the retrovirus-receptor interaction, mouse CAT1 (mCAT1) was expressed in human 293 cells as a fusion protein with jellyfish green fluorescent protein (GFP). Easily detected by fluorescence microscopy and immunoblot analysis with anti-GFP antibodies, the mCAT1-GFP fusion protein was expressed in an N-glycosylated form on the cell surface and in the Golgi apparatus, retaining the ecotropic receptor function. The system was applied to compare Friend MuLV (F-MuLV) and its neuropathogenic variant, PVC-211 MuLV, which exhibits a unique cellular tropism and host range, for the ability to use various CAT family members as a receptor. The results indicated that F-MuLV and PVC-211 MuLV could infect the cells expressing wild-type mCAT1 at comparable efficiencies and that rat CAT3, but not mCAT2, conferred a low but detectable level of susceptibility to F-MuLV and PVC-211 MuLV. The data also suggested that CAT proteins might be expressed in an oligomeric form. Further application of the system developed in this study may provide useful insights into the entry mechanism of ecotropic MuLV.

Binding of a viral particle to a receptor on the host cell surface is the initial step for retroviral entry. Elucidation of the molecular basis for the retrovirus-receptor interaction is thought to contribute to understanding of the viral pathogenic mechanisms and development of useful systems for retrovirus-mediated gene transduction. For a variety of retroviruses, their cognate receptors have been identified (16), and the receptor for ecotropic murine leukemia virus (MuLV) was shown to be the y+ cationic amino acid transporter 1 (CAT1) (2, 22, 39). Additional studies have indicated that the structure of the third extracellular domain (TED) of CAT1 plays an important role in determining species-specific susceptibility of host cells to ecotropic MuLV infection (1, 40). On the other hand, studies on the viral determinant for interaction with the receptor have shown that the envelope SU protein carries the receptor-binding domain (RBD) at its N-terminal region (3, 4, 13). Despite these findings, the exact mechanism for recognition and binding between the ecotropic SU RBD and the CAT1 TED has not been elucidated. We have been investigating this problem by using a unique ecotropic MuLV, PVC-211, as a model.

PVC-211 MuLV is a neuropathogenic variant of Friend MuLV (F-MuLV) that causes a rapidly progressive spongiform degeneration of the central nervous system in susceptible rats and mice (14, 18, 32). Our previous studies demonstrated that PVC-211 MuLV has an unusual tropism for capillary endothelial cells (CEC) and that the CEC tropism of the virus is important for neuropathogenicity (29, 30). We have also shown that PVC-211 MuLV can infect Chinese hamster ovary-derived CHO-K1 cells normally resistant to MuLV infection (31). From studies using chimeric viruses constructed between PVC-211 MuLV and F-MuLV, the major viral determinant for infectivity on CEC and CHO-K1 cells of PVC-211 MuLV was found to be two amino acids, Gly116 and Lys129, in the SU RBD (28, 31). These results suggested that unique SU-receptor interactions might be responsible for the unusual cellular tropism and host range of PVC-211 MuLV. Our previous observation that PVC-211 MuLV interfered with F-MuLV in a nonreciprocal manner on Rat1 fibroblasts (28) was also compatible with the possibility of a unique virus-receptor interaction by PVC-211 MuLV. Therefore, comparison of PVC-211 MuLV with other ecotropic MuLVs for the ability to use CAT1 with different TED primary structures might provide useful insights into elucidation of retroviral entry mechanism.

In this study, we developed a system to compare receptor usage of PVC-211 MuLV and F-MuLV by expressing various CAT family proteins tagged with green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* (5) in human cells. The GFP-tagged mouse CAT1 (mCAT1-GFP) was easily detected on the cell surface by fluorescence microscopy and retained its ecotropic MuLV receptor function for both PVC-211 MuLV and F-MuLV. The results also indicated, compatible with a previous study (20), that neither of the viruses used mCAT2, or chimeric mCAT1 bearing the mCAT2 TED, as a receptor. Interestingly, rat CAT3 (rCAT3) could confer a low but detectable level of susceptibility to PVC-211 MuLV and F-MuLV infection. Immunolocalization detection of CAT-GFP suggested that CAT proteins might be expressed in an oligomeric form.

**MATERIALS AND METHODS**

*Cells and viruses.* NIH 3T3 cells, human embryo kidney-derived 293 cells (11), and M-MuLV-based CRE packaging cells producing BAG retroviral vector (34)
mCAT2A, the TED-coding region of mCAT2A cDNA was amplified by PCR with two sets of primers, 5'-CCGGAGATCTGGACTGTAACATTGTTG-3' (primer A) and 5'- TTCTTCAGGCCAACCCGACACAA-3' for the 5' flanking sequence and 5'- GGTTCGCTGGCTTGCATTGCTGTG-3' and 5'-GGGCTAAGTTAGATGACGAGGGTG-3' for the 3' flanking sequence. The PCR products were then added to a new PCR mixture containing primers A and B, heated at 94°C for 5 min, annealed with each other at 37°C for 5 min, elongated at 65°C for 5 min, and subjected to a second round PCR of 25 cycles, each cycle consisting of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The products of the second-round PCR were digested with HpaI and BspHI, and the fragment obtained was used to replace the corresponding fragment of mCAT1 cDNA with pmCAT1-GFP.

Successful construction of each plasmid was confirmed by restriction enzyme digestion and nucleotide sequencing.

**Protein analysis.** To prepare protein samples, cells grown in a 60-mm-diameter plate were washed twice with ice-cold phosphate-buffered saline and harvested in 1 ml of lysis buffer (50 mM Tris HCl [pH 7.3], 150 mM NaCl, 1% Triton X-100, 20 mM EDTA) containing 10 μg of proteinase inhibitor cocktail (Calbiochem, San Diego, Calif.). Cells were centrifuged at 12,000 x g for 5 min at 4°C to remove insoluble materials, and the supernatants were collected. The protein concentration of each sample was determined with a protein assay kit (Bio-Rad Laboratories, Hercules, Calif.). Cell lysates containing 100 μg of protein were incubated with 1 μl of anti-GFP polyclonal antibody (Amersham Pharmacia Biotech AB, Uppsala, Sweden), respectively. For removal of N-linked glycosylation, immunoprecipitated samples were treated with 2 U of N-glycosidase F (Roche Diagnostics) overnight at 4°C before addition of protein A-agarose (Upstate Biotechnology, Lake Placid, N.Y.). Immunoprecipitates were washed five times with the lysis buffer, suspended in 10 μl of loading buffer containing 2× SDS-PAGE sample buffer, boiled at 100°C for 5 min. Boiled samples were fractionated by 4% to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Novex, San Diego, Calif.), transferred to a polyvinylidene difluoride (PVDF) membrane, and reacted with a mouse monoclonal antibody against GFP (Clontech) or an anti-mCAT1 rabbit serum (21) given by James Cunningham. Binding of anti-GFP and anti-mCAT1 antibodies was detected by the chemiluminescence method using peroxidase-conjugated anti-mouse immunoglobulin G (Amersham Pharmacia Biotech AB, Uppsala, Sweden), respectively. For removal of N-linked glycosylation, immunoprecipitated samples were treated with 2 U of N-glycosidase F (Roche Diagnostics) at 37°C for 1 h. The samples were then washed four times with the lysis buffer and run by SDS-PAGE, and immunoblot detection of GFP-tagged proteins was carried out as described above.

**Microscopy.** Cells were seeded on a coverglass coated with poly-L-lysine. On the next day, cells were fixed with 2% paraformaldehyde and observed under a Zeiss 410 laser scanning confocal microscope with a filter set suitable for fluorescence detection. For immunostaining, cells were fixed with 2% paraformaldehyde and stained with an mouse anti-human Golgi zone monoclonal antibody (Chemicon International, Inc., Temecula, Calif.) and a rhodamine-conjugated rabbit anti-mouse IgG antibody. Then, confocal microscopy was performed with filter sets suitable for fluorescence detection and rhodamine detection. For examination of viable cells, cells were seeded on a glass bottom dish (Matsunami Glass Ind., Ltd., Osaka, Japan) and observed under an Olympus IX70 microscope with an FLA fluorescent module.

**Retroviral transduction assay.** Cells were seeded at a density of 5 x 10^4 per well in 12-well plates and on the next day inoculated in the presence of 5 μg of Polybrene per ml with serially diluted culture supernatants containing the BAG vector (34) pseudotyped with various MuLVs. Four days after inoculation, cells were fixed with 0.5% glutaraldehyde and stained with 1 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactosidase (X-Gal) per ml in a solution containing 5 mM K3Fe(CN)6, 5 mM K4Fe(CN)6, 3 mM MgCl2, and 0.1 mM galactosidase (K4Fe(CN)6) as a chromogenic substrate. The number of blue cell foci was counted, and the transduction efficiency was calculated.

**RESULTS**

mCAT1-GFP fusion protein was expressed on the cell surface in an N-glycosylated form, conferring susceptibility to ecotropic MuLV infection. To test whether the GFP-tagged mCAT1 protein (mCAT1-GFP) functions as an ecotropic MuLV receptor, pmCAT1-GFP (Fig. 1A) was transfected into human 293 cells, and G418® cells were selected. As expected (Fig. 1B), laser confocal microscopy showed that mCAT1-GFP was expressed on the surface of 293 cells to the very end of filopodia, whereas GFP by itself exhibited homogeneous distribution in the cell (Fig. 2A). In mCAT1-GFP-expressing cells, granular accumulation of green fluorescence was observed in the cytoplasm (Fig. 2A). Immunostaining of the mCAT1-GFP-expressing cells revealed colocalization of the cytoplasmic green fluorescence with the Golgi apparatus (Fig. 2B). Expression of mCAT1-GFP was detected in viable cells.
with a conventional fluorescence microscope as well, showing cell surface and cytoplasmic granular distribution (data not shown). The mCAT1-GFP could also be detected by immunological analysis using anti-GFP antibodies, and the size of the signal (97 kDa) was compatible with the sum of 70 kDa for mCAT1 (21) and 27 kDa for GFP (Fig. 3A). We also detected an additional signal with a higher molecular mass (~200 kDa) which might represent a dimerized form of mCAT1-GFP. mCAT1-GFP was detected by an anti-mCAT1 antiserum (21) with a higher level of background signals (data not shown).

Treatment with N-glycosidase reduced the size of the signals, revealing that mCAT1-GFP molecules were expressed in an N-glycosylated form, as well as the size of the putative dimer (Fig. 3B).

To examine if mCAT1-GFP could function as an ecotropic MuLV receptor, 293 cells expressing the fusion protein were infected with the lacZ gene-bearing BAG vector produced by M-MuLV-based CRE packaging cells (34). As shown in Fig. 3C and D, the cells expressing mCAT1-GFP were as susceptible as mouse NIH 3T3 cells to M-MuLV-mediated gene

FIG. 2. (A) Cell surface expression of GFP-tagged mCAT1 detected by confocal microscopy. Human 293 cells transfected with pmCAT1-GFP or control pEGFP-N1 were observed under a laser scanning confocal microscope with a filter set suitable for differential interference contrast (DIC) (panels 1 to 3) and fluorescein detection (panels 4 to 9). Fluorescence microscopy at the midsection (panels 4 to 6) and bottom (panels 7 to 9) of the cells is demonstrated. Magnification, ×630. (B) Colocalization of GFP-tagged mCAT1 with the Golgi apparatus. mCAT1-GFP-expressing 293 cells were fixed with paraformaldehyde, stained with a mouse anti-human Golgi zone antibody and rhodamine-conjugated rabbit anti-mouse IgG antibody, and then observed under a laser scanning confocal microscope with a filter set suitable for fluorescein detection (panel 1) and rhodamine detection (panel 2). A superimposed image of panels 1 and 2 is shown in panel 3, and yellow signals indicate colocalization of mCAT1-GFP and the Golgi apparatus. Magnification, ×630.
transduction, whereas GFP alone failed to confer susceptibility to M-MuLV.

Infection of 293 cells expressing mCAT1-GFP fusion proteins with ecotropic MuLVs induced syncytium formation. To carry out interference studies, we attempted to establish mCAT1-GFP-expressing 293 cells chronically infected with F-MuLV or PVC-211 MuLV. However, when mCAT1-GFP-expressing cells were inoculated with F-MuLV, syncytium formation became noticeable 2 days after inoculation, and by day 7, massive cell fusion was observed (data not shown). Fluorescence microscopy and immunoblot analysis revealed that the cells which survived syncytium formation did not express a detectable level of mCAT1-GFP (data not shown). Similar results were obtained when the cells were infected with PVC-211 MuLV. Inoculation at a high multiplicity of infection of helper-free BAG vector produced by CRE packaging cells also induced fusion of mCAT1-GFP-expressing 293 cells (data not shown), indicating that the fusion mechanism does not require viral replication (i.e., fusion without).

mCAT2A failed to serve as ecotropic MuLV receptor, whereas rCAT3 conferred a low but detectable level of susceptibility to PVC-211 MuLV and F-MuLV. To examine whether other CAT family proteins could serve as a receptor for PVC-211 MuLV, subtype A of mCAT2 (mCAT2A) (6), a chimeric mCAT1-TED2 which carries the mCAT2 TED in the mCAT1 backbone, and rCAT3 (15) were expressed in 293 cells. As shown in Fig. 5, mCAT2A-GFP was detected in the anti-HA immunoprecipitate only in the presence of mCAT2A-HA expression (lanes 4 and 8), indicating that the fusion mechanism does not require viral replication (i.e., fusion without).
FIG. 4. Ecotropic receptor function of various CAT proteins. (A) Schematic diagrams of various CAT proteins tagged with GFP. In the diagram of the mCAT1-TED2 chimera, solid and open boxes represent segments derived from mCAT1 and mCAT2A, respectively. (B) Comparison of amino acid sequences of mCAT1-TED2 chimera, solid and open boxes represent segments derived from mCAT1 and mCAT2A, respectively. Potential N-linked glycosylation sites are underlined. Dots indicate that corresponding amino acids are missing. (C) Immunoblot detection of various CAT proteins tagged with GFP. In the diagram of the TED of CAT family proteins. The region of mCAT1 from amino acids 210 to 241 (2) and the corresponding regions of mCAT2 (6) and rCAT3 (15) are aligned. Potential N-linked glycosylation sites are underlined. Dots indicate that corresponding amino acids are missing. (C) Immunoblot detection of various GFP-tagged CAT molecules. Protein samples (100 μg) prepared from untransfected 293 cells (lane 1) or the cells transfected with the plasmid encoding mCAT1-GFP (lane 2), mCAT1-TED2-GFP (lane 3), mCAT2A-GFP (lane 4), or rCAT3-GFP (lane 5) were immunoprecipitated with an anti-GFP polyclonal antibody, fractionated by SDS-PAGE, transferred to a PVDF membrane, and reacted with an anti-GFP monoclonal antibody. Positions of molecular size markers are shown on the left in kilodaltons; arrowheads on the right indicate the signals for HA- and GFP-tagged mCAT2A.

FIG. 5. Dimerization of CAT protein. Protein samples (100 μg) prepared from 293 cells transfected with plasmid constructs (5 μg) encoding mCAT2A-GFP (lanes 1 and 5), mCAT2A-HA (lanes 2 and 6), mCAT1-GFP and mCAT2A-HA (lanes 3 and 7), or mCAT2A-GFP and mCAT2A-HA (lanes 4 and 8) were immunoprecipitated (I.P.) with an anti-HA monoclonal antibody (12CA5), fractionated by SDS-PAGE, transferred to a PVDF membrane, and reacted with an anti-HA or anti-GFP monoclonal antibody. m1 and m2 represent mCAT1 and mCAT2, respectively. Positions of molecular size markers are shown on the left in kilodaltons; arrowheads on the right indicate the signals for HA- and GFP-tagged mCAT2A.

cules associated with each other, forming homodimers. Coimmunoprecipitation of mCAT1-GFP was not detected in the presence of mCAT2A-HA expression (lanes 3 and 7), indicating the specificity of dimer formation.

DISCUSSION

In this study, we demonstrated that GFP-tagged mCAT1 was expressed on the cell surface, retaining its ecotropic receptor activity. mCAT1-GFP was colocalized with the Golgi apparatus in the cytoplasm and modified by N-linked glycosylation, suggesting that the fusion protein is synthesized and processed similarly to endogenous mCAT1 (21). In previous studies of the structure-function relationship of mCAT1, the level of exogenous mCAT1 expression was estimated by the arginine uptake assay (20, 25), which required radioactive materials and relatively complicated procedures. In another study, mCAT1 was tagged with the antigenic epitope of influenza virus HA in order to facilitate immunological detection (8). However, microscopic detection of epitope-tagged mCAT1 would require immunostaining procedures. In contrast, mCAT1-GFP used in this study could easily be detected by fluorescence microscopy in fixed, as well as viable, cells without staining. In addition, immunological detection of mCAT1-GFP with anti-GFP antibodies gave a high signal-to-noise ratio, facilitating quantitative comparison of the expression levels. Therefore, mCAT1-GFP appears to be a more versatile tool for studying virus-receptor interaction of ecotropic MuLV compared with the previously used methods.

The GFP-tagging method was able to reproduce the previous observation that mCAT2 failed to serve as an ecotropic receptor (20). In addition, it was demonstrated in this study that replacement of the mCAT1 TED with the mCAT2 TED was sufficient to abolish the ecotropic receptor function. Although the Tyr235 in the TED of mCAT1 that has been shown to be critical for the receptor function (1, 40) is conserved in mCAT2 (Fig. 4B), other residues, such as Glu37, that play additional roles in efficient receptor function (1, 40) are substituted (Fig. 4B). It is likely that these substitutions were responsible for failure of the chimera mCAT1-TED2 to serve as an ecotropic receptor. Since rCAT2 and mCAT2A have identical TED primary structures (6, 36), it is unlikely that rCAT2 contributes to the unique ability of PVC-211 MuLV to infect rat CEC or overcome interference by F-MuLV on rat fibroblasts. It was rather unexpected that rCAT3, whose TED was more distantly related to mCAT1 than mCAT2 (Fig. 4B), could confer susceptibility to PVC-211 MuLV and F-MuLV. Originally isolated from a rat brain cDNA library, rCAT3 is expressed at a high level in the brain (15). However, it is unlikely that the infectivity of PVC-211 MuLV on rCAT3-
expressing cells is significant for its neuropathogenicity, because nonneuropathogenic F-MuLV was able to use this receptor as well as, if not better than, PVC-211 MuLV. Its mouse homolog, mCAT3, which is also expressed in the brain (17), has a TED primary structure identical to that of rCAT3. Therefore, mCAT3 may also serve as an ecotropic MuLV receptor. Currently, various ecotropic MuLVs in addition to PVC-211 MuLV and F-MuLV are being tested for their ability to use rCAT3 and mCAT3 as a receptor. Attempts are also being made by transfecting infectious DNA of F-MuLV and PVC-211 MuLV into rCAT3-GFP-expressing cells to isolate an adapted virus which can use rCAT3 as a receptor more efficiently.

It was intriguing that immunoblot analysis of various CAT-GFP constructs revealed a high-molecular-weight signal whose size corresponded to a dimer of each molecule. Coimmunoprecipitation of mCAT2A-GFP with mCAT2A-HA strongly suggested that CAT molecules associate with each other to form homodimers. Since GFP alone was detected as a monomer, it appears that the CAT portion of the fusion protein is responsible for SDS-stable dimer formation. Generation of a detergent-stable dimer has previously been reported for several proteins (7, 23). As for SDS-stable dimerization of glycoporin A, intermolecular van der Waals interaction between the membrane spanning α-helices appears to be responsible (24). Since CATs are integral membrane proteins with 14 membrane-spanning regions, some of which are predicted to have an α-helical structure, it is possible that they form a dimer by a similar mechanism. It has been shown that some membrane proteins with a transporter function, such as adenosine transporter (37), glutamate transporter (9, 12), P-glycoprotein (33), and erythrocyte band 3 protein (7, 38), may exist in a dimeric form. It has also been shown that the γ1-L-type amino acid transporters function as a heterodimer with CD98 (19, 26). Therefore, dimerization of CAT molecules revealed in this study may be biologically significant. It has previously been shown that the RBD of the MuLV SU protein and mCAT1 interact with a stoichiometry of 1:1 in vitro (8). Since crystallographic studies strongly suggested trimer formation of MuLV envelope proteins (10), it is possible that mCAT1 is expressed on the cell surface as a trimer that is partially disassembled during protein sample preparation. Alternatively, it is also possible that the dimerization is an artifact caused by tagging with GFP, because in a previous study (21) a dimer signal of untagged mCAT1 was not detected by an anti-mCAT1 antiserum. To examine the biological significance of apparent oligomerization of CAT-GFP molecules, CAT molecules with various deletion mutations are being expressed in a GFP-tagged form, and the region(s) of CAT molecules required for generating the high-molecular-weight immunoblot signal is being determined.

Further studies using the fluorescent CAT molecules are necessary for elucidating the molecular basis for the unique cellular tropism and host range of PVC-211 MuLV. Our preliminary results suggested that conversion of the mCAT1 TED primary structure to that of rat or hamster CAT1 did not affect the ability to confer susceptibility to ecotropic MuLV (27). However, interference studies using these chimeric mCAT1 constructs were hampered by virus-induced syncytium formation of mCAT1-GFP-expressing 293 cells as described in this study. Similar to findings for mCAT1-expressing CHO-K1 cells (35), a high level of mCAT1-GFP expression in 293 cells might be responsible for the virus-induced fusogenicity. Additional studies are being carried out to express mCAT1-GFP in other cell lines, such as mink-derived CCL64, that may be refractory to virus-mediated fusion induction (35) and use them for interference studies. Taking advantage of the ability of mCAT1-GFP to be detected by fluorescence microscopy in viable cells, efforts are also being made in our laboratory to take motion pictures showing the effects of virus infection and various drug treatments on the localization of the receptor, which may illuminate the mechanisms of virus-receptor interaction and virus-induced membrane fusion.

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