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Role of a Third Extracellular Domain of an Ecotropic Receptor in Moloney Murine Leukemia Virus Infection

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The murine ecotropic retroviral receptor has been demonstrated to function as a mouse cationic amino acid transporter 1 (mCAT1), and is comprised of multiple membrane-spanning domains. Feral mouse (*Mus dunni*) cells are not susceptible to infection by the ecotropic Moloney murine leukemia virus (MoMLV), although they can be infected by other ecotropic murine leukemia viruses, including Friend MLV and Rauscher MLV. The relative inability of MoMLV to replicate in *M. dunni* cells has been attributed to two amino acids (V_{214} and G_{236}) located within the third extracellular loop of the *M. dunni* CAT1 receptor (dCAT1). Via the exchange of the third extracellular loop of the mCAT1 cDNA encoding receptor from the permissive mouse and the corresponding portion of cDNA encoding for the nonpermissive *M. dunni* receptor, we have identified the most critical amino acid residue, which is a glycine located at position 236 within the third extracellular loop of dCAT1.

We also attempted to determine the role of the third extracellular loop of the *M. dunni* CAT1 receptor with regard to the formation of the syncytium. The relationship between dCAT1 and virus-induced syncytia was suggested initially by our previous identification of two MLV isolates (S82F in Moloney and S84A in Friend MLV), both of which are uniquely cytopathic in *M. dunni* cells. In an attempt to determine the relationship existing between dCAT1 and the virally-induced syncytia, we infected 293-dCAT1 or chimeric dCAT1 cells with the S82F pseudotype virus. The S82F pseudotype virus did not induce the formation of syncytia, but did show increased susceptibility to 293 cells expressing dCAT1. The results of our study indicate that S82F-induced syncytium formation may be the result of cell-cell fusion, but not virus-cell fusion.

Keywords: retroviral receptor, pseudotype virus, syncytia, virus-cell fusion

Infection by ecotropic murine leukemia viruses is known to be restricted to mouse and rat cells which express the cationic amino acid transporter (CAT1) (Albritton *et al.*, 1989). Human cells expressing human CAT1 have been shown not to be susceptible to infection with ecotropic MLV, but can acquire susceptibility to infection via the transfection and expression of the mouse CAT1 receptor. The mCAT1 receptor is a multi-membrane spanning protein, which has previously been shown to function as a transporter of cationic amino acids across cell membranes. The receptor has 622 amino acids and can be isolated from NIH3T3 cells (Kim *et al.*, 1991; Wang *et al.*, 1991). The ecotropic murine leukemia virus recognizes the third extracellular loop of the mCAT1 receptor protein for viral entry (Albritton *et al.*, 1993; Yoshimoto *et al.*, 1993). However, *M. dunni* cells are not susceptible to Moloney MLV infection, although they can be infected by other ecotropic murine leukemia viruses (Friend MLV and Rauscher MLV). The resistance of *M. dunni* cells to Moloney MLV has been attributed to a polymorphism of the mCAT1 receptor, as *M. dunni* has been shown to harbor a different CAT1 receptor (dCAT1) (Eiden *et al.*, 1993, 1994). However, this resistance can be eliminated by tunicamycin, an inhibitor of N-linked glycosylation. Thus, the receptor expressed in *M. dunni* cells has been classified as glycosylation-dependent (Rein *et al.*, 1982; Miller and Miller, 1992; Kim and Cunningham, 1993; Tavoloni and Rudenholz, 1997). Eiden *et al.* (1994) previously showed that the dCAT1 gene of *M. dunni* cells is different from the proto-

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typical mCAT1 gene found in laboratory mice, in that the third extracellular loop harboring the virus-binding region has a substitution (I-V_{214}) as well as an inserted glycine located after the critical residue, Y235. In order to determine which amino acids in the receptor are critical with regard to Moloney murine leukemia viral infection, and which perform a role in the dysfunction of dCAT1 as an MoMLV receptor, we developed a system to compare the receptor usage of MoMLV pseudotype, via the expression of a dCAT1 variant family protein tagged with GFP in human 293 cells.

The entry of the murine leukemia virus is initiated via the binding of a surface protein within the virus to the receptor on the host cell surface (Davey *et al.*, 1997). The variants of the cell surface viral receptors, as well as differences in the receptor-binding domain of the virus, have been shown to affect pathogenicity (Park *et al.*, 1994). For example, the results of our previous studies have demonstrated that a Moloney MLV (MoMLV) variant with the S82F substitution induces the formation of large multinucleated syncytia on *M. dunni* cells two days after infection, and this syncytium formation is coupled with the accumulation of large amounts of unintegrated viral DNA (Jung and Kozak, 2003; Jung *et al.*, 2004). This suggests that a receptor-virus interaction may be the cause of the syncytium formation. However, the precise mechanism by which this receptor-virus interaction occurs has yet to be determined.

Syncytium formation is believed to arise via virus-cell fusion (fusion from without) or by cell-cell fusion (fusion from within) (White *et al.*, 1983; Jones and Risser, 1993). In order to ascertain the mechanism by which syncytium formation occurs, 293 cells expressing dCAT1 variants were infected with the S82F pseudotype virus.

Materials and Methods

Viruses and cells

M. dunni fibroblasts (MDTF cells), mouse NIH3T3 and 293 cells were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics. The pCL-eco retrovirus-packaging vector (Imgenex Co., USA) was employed in the generation of pseudotypes with Moloney ecotropic Env. The LacZ pseudotype virus was generated via the cotransfection of human 293 cells with pCLMFG-LacZ (Imgenex Co., USA), as well as expression vectors harboring a variety of ecotropic MoMLV *env* genes.

The MoMLV mutant envelope expression plasmid (pCL-eco S82F) was constructed via the substitution of the 1.3 kb *Hpa*I fragment of pCL-eco with the corresponding 1.3 kb *Hpa*I fragment harboring the single mutant S82F from the p480-Mo-S82F (Jung and Kozak, 2003).

Cloning and sequencing

The dCAT1 receptor was amplified from *M. dunni* cells via RT-PCR with forward mdCAT1: 5'-CTGTGC TACGGCGAGTTTG-3' and reverse primers 3'-mdCAT2: 5'-TCCACCAGGTCCTTCAGTTC-3' derived from the sequence of the NIH 3T3 ecotropic receptor. PCR reactions were conducted using a GeneAmp PCR system 9700 machine (PE Applied Biosystems, USA). The reactions were conducted for 35 cycles with a 30 sec DNA denaturation step at 95°C, a 30 sec annealing step, and a 1 min extension step at 72°C. The annealing temperature in the first cycle was set to 63°C, was subsequently reduced by 1°C per cycle for the next 8 cycles, and was then maintained at 55°C for the remaining 27 cycles. The 965 bp products were cloned into the pCR2.1-TOPO vector and sequenced.

Mutagenesis

The *Blp*I-*Ava*I fragment of the dCAT1 receptor was employed in order to replace the corresponding fragment of the pmCAT1-GFP plasmid, which harbors the NIH 3T3 ecotropic receptor. This plasmid was kindly provided by Dr. Jonathan Silver (NIAID, NIH, USA). The pmCAT1-GFP was then digested with *Blp*I and *Xmn*I to generate a 382 bp fragment which harbored the isoleucine residue at position 214. This fragment was then ligated into the pdCAT1-GFP vector, yielding pdCAT1(V-I_{214})-GFP. In order to delete the glycine residue at position 236, which was not present in the mCAT1-GFP protein, the pmCAT1-GFP was digested with *Xmn*I and *Ava*I, and then cloned into the pdCAT1-GFP vector from which the corresponding *Xmn*I-*Ava*I fragment had been deleted. This chimeric DNA was designated pdCAT1 (del : G_{236})-GFP. All mutants were confirmed via DNA sequencing.

Cell lines stably expressing recombinant dCAT1

DNA clones of mCAT1-GFP, dCAT1-GFP, dCAT1 (V-I_{214})-GFP, and dCAT1(del : G_{236})-GFP were introduced into cultured 293 cells using FuGENE 6 transfection reagent (Roche Applied Sci., USA). The cells were trypsinized and passed 24 h after transfection, then maintained in medium with 0.8 mg/ml geneticin (Invitrogen, USA) until colonies of drug-resistant cells became apparent. Individual colonies were picked for analysis as indicated.

Pseudotype assay

Supernatants harboring the pseudotype virus were harvested from the transfected 293 cells, then filtered and utilized to infect cells that had been plated in 6-well culture dishes, at a density of 1.5×10^5 per well. The cells were infected with 1 ml of virus in the presence

of 8 μg/ml polybrene for 3 h prior to the addition of 2 ml of fresh medium to each well. One day after infection, the cells were fixed with 0.5% glutaraldehyde, and assayed for β-galactosidase activity using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal, 2 mg/ml; ICN Biomedicals, USA) as a substrate. Infectious titers were expressed as the number of blue CFU per milliliter of viral supernatant.

Microscopy

In order to determine whether dCAT1-GFP is expressed on the surfaces of the 293 cells, the transfected 293 cells were seeded in 8-well coverglass chambers, and examined under a Zeiss 510 laser scanning confocal microscope (Zeiss, Germany). In order to assess syncytium formation in the S82F pseudotype virus-infected 293 cells, the cells were evaluated under a Nikon TS100 light microscope.

Results

Sequencing and mutagenesis

The relative inability of MoMLV to replicate on *M. dunni* cells has been attributed to two differences within the third extracellular loop of the dCAT1 receptor. The dCAT1 receptor harbors a valine residue at position 214 and an extra glycine residue at position 236, whereas the mCAT1 receptor harbors an isoleucine residue at position 214, and is not present at position 236. In order to functionally characterize the dCAT1 ecotropic receptor, we cloned and sequenced the third extracellular loop of the dCAT1 receptor from the *M. dunni* cells. The results of sequence analysis indicated that the deduced amino acid sequence was identical to that described by Eiden (Fig. 1).

The previous result suggests that the third extracellular region of the ecotropic receptor protein participates both in ecotropic virus binding and entry. In order to determine whether the presence of the additional glycine residue at position 236 in dCAT1 is attributable to the resistance of *M. dunni* cells to MoMLV infection, we constructed the dCAT1(del : G_{236})-GFP retroviral expression plasmid, and a *Xmn*I-*Ava*I fragment of mCAT1 was used to replace the corresponding fragment of the pdCAT1-GFP. The pdCAT1 (V-I_{214})-GFP harboring the isoleucine residue at position 214 was also constructed. The presence of these mutations was corroborated via sequencing (Fig. 2).

```
                        214               223               229         235   237
mCAT1             V K G S I K N W Q L T E K N F S * * C N N N D T N V K Y * G E G G
dCAT1             - - - - V - - - - - - - - - - - - - - - - - - - - - - - - G - - - -
dCAT1(del:G236)   - - - - V - - - - - - - - - - - - - - - - - - - - - - - - - - - - -
dCAT1(V-I214)     - - - - - - - - - - - - - - - - - - - - - - - - - - - - - G - - - -
```

Fig. 1. Comparison of the amino acid residues harbored within the third extracellular loop of the dCAT1 ecotropic receptors in human 293 cells. The two potential N-linked glycosylation sites are underlined. The glycine insertion located at position 236 is associated with lack of susceptibility to MoMLV.

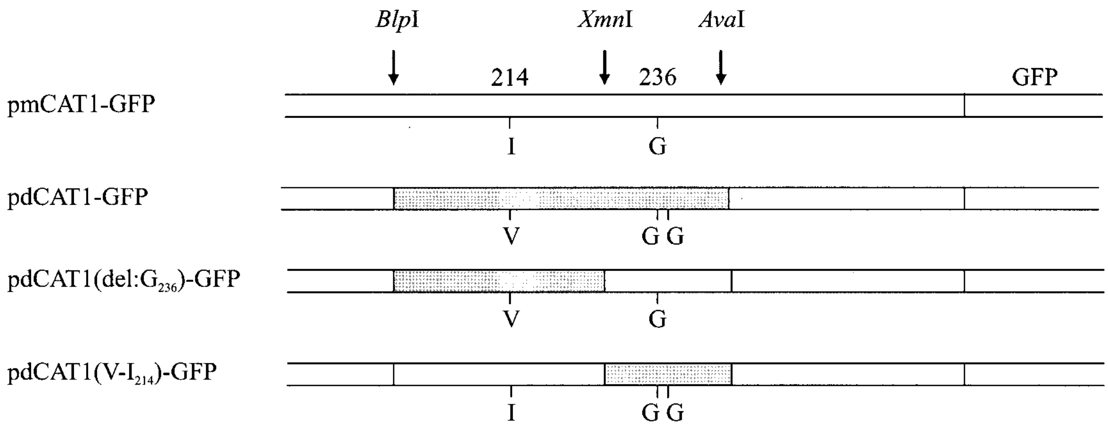

Fig. 2. The structure of recombinant dCAT1 genes. The *Blp*I-*Ava*I fragment of the mCAT1 receptor was removed and replaced by the comparable segment of dCAT1, thereby generating pdCAT1-GFP. pdCAT1(del : G_{236})-GFP was constructed via the substitution of the *Xmn*I-*Ava*I fragment of pdCAT1-GFP with the corresponding fragment of pmCAT1-GFP. In order to generate pdCAT1(V-I_{214})-GFP harboring the isoleucine residue at position 214, the *Blp*I-*Xmn*I fragment of pmCAT1-GFP was removed and ligated into pdCAT1-GFP, from which the corresponding fragment had been excised.

Cell lines stably expressing chimeric transporters

GFP-tagged mCAT1 was utilized to evaluate the intracellular localization of mCAT1, and to determine whether mCAT1 functions as an ecotropic MLV receptor. Our previous results indicated that this construct functions as an MLV receptor (Masuda *et al.*, 1999; Ou and Silver, 2003). DNA clones of mCAT1, dCAT1, dCAT1(V-I_{214}) and dCAT1(del : G_{236}) were transfected into human 293 cells. The cells were trypsinized and passed 24 h after transfection, and maintained in medium supplemented with 0.8 mg/ml of geneticin (Invitrogen, USA) until colonies of drug-resistant cells became apparent. Individual colonies were selected for analysis as indicated. Fluorescence confocal microscopy indicated that dCAT1 (del : G_{236})-GFP was expressed on the plasma membranes of the 293 cells (Fig. 3). We detected no differences between the wild-type and mutant variants in terms of the intracellular localization of the dCAT1-GFP fusion protein. Other previously published data indicated that mCAT1-GFP could also be detected via Western blot analysis, and the size of the signal was as had been expected for the chimeric mCAT1-GFP fusion protein (Masuda *et al.*, 1999).

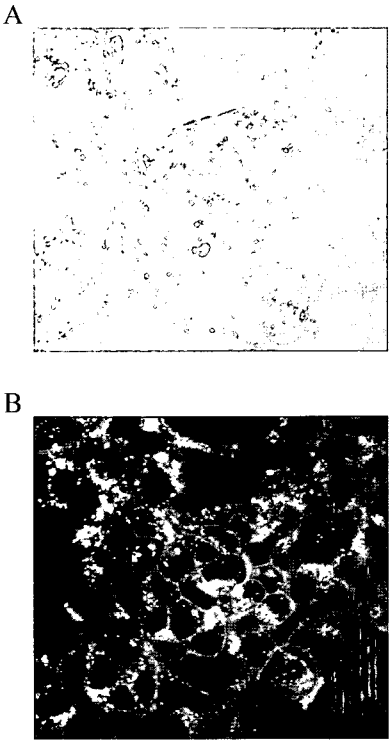

Fig. 3. Cell surface expression of GFP-tagged dCAT1 (del : G_{236}), as detected by laser scanning confocal microscopy with a filter set suitable for differential interference contrast (panel A) and fluorescein detection (panel B). Objective lens magnification, × 63.

Comparison of ecotropic MLV infectivity in cells expressing dCAT1 or chimeric dCAT1.

The importance of amino acid residues located within a putative extracellular domain of mCAT1 in infection suggests that these residues may directly interact with the MLV envelope protein, gp70. In order to determine which amino acids in dCAT1 are critical to Mo MLV infection, and which perform roles in the dysfunction of dCAT1 as a MoMLV receptor, we infected the 293 cells and transfected them with the pCLMFG-LacZ vector pseudotyped with the MoMLV envelopes. The

Table 1. Ecotropic virus titers on cell lines that express chimeric dCAT1

Target cell	Titer of LacZ pseudotype (cfu/ml)	
	LacZ (wild type)	LacZ (S82F)
NIH3T3	3.7×10^5	3.2×10^1
M. dunni	1.4×10^1	4.2×10^6
293-mCAT1	4.5×10^5	6.4×10^1
293-dCAT1	2.5×10^1	1.2×10^6
293-dCAT1(del : G_{236})	3.6×10^4	3.0×10^2
293-dCAT1(V-I_{214})	2.1×10^2	2.0×10^4

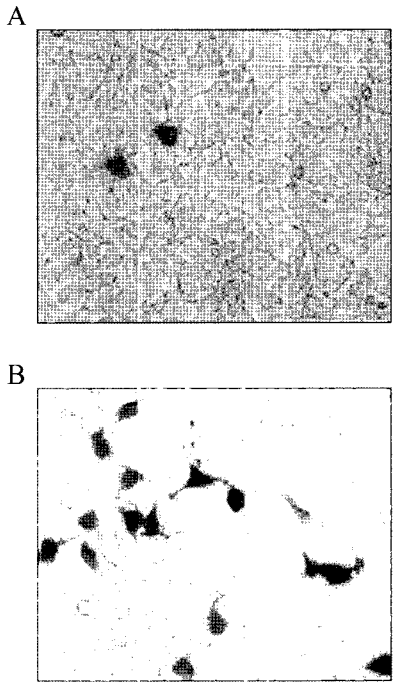

Fig. 4. Susceptibility to MoMLV pseudotype infection of 293 cells expressing recombinant dCAT1-GFP. Cells transfected with pdCAT1(V-I_{214})-GFP (panel A) or pdCAT1 (del : G_{236})-GFP (panel B) were inoculated with 1 ml of virus in the presence of 8 μg/ml of polybrene. Two days after infection, the ls were fixed and stained with X-Gal.

transfected 293 cells expressing the dCAT1 (deletion) gene were significantly more susceptible to infection with the MoMLV pseudotype than were the transfected 293 cells that expressed dCAT1(substitution) (Table 1 and Fig. 4). This indicates that the insertion at position 236 was more critical than the substitution at position 214 in terms of resistance against MoMLV infection. 293 cells expressing recombinant dCAT1 were also evaluated with regard to their sensitivity to virally-induced syncytium formation. Although the transfected 293 cells expressing dCAT1 and dCAT1 ($V\text{-}I_{214}$) efficiently infected by pseudotypes with the envelopes of syncytium-inducing MoMLV (Table 1), they did not exhibit syncytia formation (data not shown). The failure of the pseudotype virus to induce syncytia formation was attributed to failure to replicate efficiently.

Discussion

The relative inability of MoMLV to replicate on the *M. dunni* cells has been attributed to two amino acids located within the third extracellular loop of the dCAT1 receptor. The dCAT1 receptor harbors a valine residue at position 214, as compared with the isoleucine residue located at position 214 of mCAT1, and also harbors an extra glycine residue within the $YGE_{235\text{-}237}$ viral binding site, which is not present in the mCAT1 receptor. Eiden and her co-workers showed that the substitution of an isoleucine residue for this valine residue renders the dCAT1 receptor slightly functional as a MoMLV receptor (Eiden *et al.*, 1993). In order to ascertain whether the presence of the extra G residue located at position 236 accounts for the role of nonfunctional dCAT1 as a MoMLV receptor, we conducted homologue-scanning mutagenesis between mCAT1 and dCAT1. In this study, we have determined that an extra G residue inserted at position 236 is more critical than a V residue located at position 214. Similar results obtained by Eiden *et al.* indicated that the presence of the additional glycine reside most probably accounts for the loss of high-affinity monomeric gp70 binding observed in cells that express the dCAT1 receptor (Eiden *et al.*, 1993). As is shown in table 1, 293-dCAT1 (del : G_{236}) cells evidence elevated susceptibility to the MoMLV pseudotype virus, whereas the presence of the additional glycine residue is responsible for the decreased susceptibility. As expected, the 293-dCAT1 cells evidence increased susceptibility to the S82F pseudotype virus.

This observation suggests that S82F substitution induces subtle alterations in the receptor-binding pocket, which enhance interaction with the dCAT1 receptor.

The relationship between dCAT1 and virally-induced syncytia was initially suggested by our previous identification of two MLV isolates (S82F in Moloney and S84A in Friend MLV), both of which are uniquely cytopathic in *M. dunni* cells (Jung and Kozak, 2003; Jung *et al.*, 2004). Both cytopathic MLVs harbor amino acid substitutions at the same residue in the RBD (Receptor Binding Domain), which has been implicated in receptor binding. The mechanism by which retroviruses induce syncytia is currently under active investigation, but clearly involves receptor interactions. Previous mutagenesis studies regarding Friend MLV have indicated that three amino acids (S84, D86, W102) are pivotal with regard to both receptor binding and virus infection (Fass *et al.*, 1997; Davey *et al.*, 1999). Substitutions at all 3 of these sites were shown to abolish infectivity (Park *et al.*, 1994).

In order to determine the relationship between dCAT1 and virally-induced syncytia, infected 293-dCAT1 or chimeric dCAT1 cells were examined via light microscopy. The S82F pseudotype did not induce syncytia, but evidenced increased susceptibility to the dCAT1-expressing 293 cells. Our studies indicate that the replication-competent Moloney variant, S82F, is a prerequisite for the formation of syncytia. Previous results show that inoculation at a high MOI and a high concentration of mCAT1 receptor-expressing cells may be important in several steps of fusion-from-without, a process during which single virions fuse to two adjacent cells (Siess *et al.*, 1996; Chung *et al.*, 1999; Masuda *et al.*, 1999). Further studies using 293 cells that express different concentrations of dCAT1 receptors and a high titer of the MoMLV pseudotype will be required in order to elucidate the molecular basis for virally-induced syncytium formation.

In conclusion, the glycine residue located at position 236 of dCAT1 is most probably responsible for resistance against MoMLV infection, and virally-induced syncytia studies using these chimeric dCAT1 constructs will require a replication-competent Moloney variant.

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