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Contribution of Virus-Receptor Interaction to Distinct Viral Proliferation of Neuropathogenic and Nonneuropathogenic Murine Leukemia Viruses in Rat Glial Cells

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The efficiency of receptor-mediated entry of pseudotyped virus carrying the surface protein (SU) of clone A8, a neuropathogenic variant of Friend murine leukemia virus (FrMLV), to rat glial cell line F10 was 1 order of magnitude greater than that of pseudotyped virus carrying SU of nonneuropathogenic FrMLV clone 57. Introduction of the gene coding for ecotropic MLV receptor on F10 cells (F10-*ecoR*) into SIRC cells, which are naturally resistant to FrMLV infection, also revealed the difference in receptor recognition between the A8 and the 57 viruses. Our results show that the difference in receptor utilization between A8-SU and 57-SU only partially explains the 3-order-of-magnitude difference in proliferation between A8 and 57 viruses in F10 cells.

A neuropathogenic variant of Friend murine leukemia virus (FrMLV), FrC6 virus clone A8, induced spongiform degeneration in the brains of infected rats (17, 19). Studies with chimerae constructed from A8 virus and nonneuropathogenic FrMLV clone 57 (13) revealed that the *env* gene of A8 is a primary determinant for the induction of neurodegeneration and that the long terminal repeat (LTR) and/or 5′ leader sequence of A8 is also necessary for neuropathogenicity (17). Although the mechanism of induction of spongiform degeneration in brain by retrovirus infection is still not understood, it is speculated that the interaction between the *env* gene product of neuropathogenic virus and the viral receptor or other substances is critical for neuropathogenicity (20).

The A8 virus replicates efficiently in rat glial cell line F10. In contrast, the 57 virus is not very infective in F10 cells, but in NIH 3T3 cells, both 57 and A8 proliferate efficiently (17). Studies on A8 and 57 chimerae revealed that the determinants for proliferation in F10 cells are the *env* gene and the LTR and/or 5′ leader sequence of A8, but how much each gene contributes to viral production in F10 cells is not clear. Efficient viral replication in F10 cells is necessary for neuropathological manifestation in rats infected with chimerae (17). The proliferation in the central nervous system (CNS) in vivo of their chimerae and of the parental viruses A8 and 57 showed good correlation with viral production in F10 cells in vitro. Therefore, F10 cells and the A8 virus are a suitable model for analysis of the interaction between SU protein and the receptor connected with viral proliferation in CNS cells and neuropathogenicity.

Efficiency of receptor-mediated viral entry into F10 and NIH 3T3 cells. To examine receptor-virus interaction, we used an assay for receptor-mediated cell entry with pseudotyped retroviruses incorporating A8-SU (pseudo-A8) or 57-SU (pseudo-57), which transfer the β-galactosidase gene. Retrovirus vector, BAG, bearing β-galactosidase and neomycin resistance gene provided by C. Cepko, Harvard Medical School (14), was stably introduced into NIH 3T3 cells. Then, expression vectors containing the A8 *env* gene or the 57 *env* gene were stably introduced into the BAG-introduced NIH 3T3 cells by cotransfection with an expression vector for *gag-pol* of the A8 virus bearing the blasticidin deaminase (BSD) gene. Pseudo-A8 and pseudo-57 were inoculated to 2×10^4 of F10 or NIH 3T3 cells on a 12-well culture plate in the presence of 10 μg of Polybrene per ml, and the numbers of β-galactosidase-positive cells were compared. For 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) visualization of β-galactosidase activity in intact cells, the method of Dannenberg and Suga (5) was used, following fixation in 4% paraformaldehyde for 5 min and then fixation in 2% formaldehyde–0.2% glutaraldehyde for 10 min. Relative infectivity of F10 cells to NIH 3T3 cells was compared for pseudo-A8 or pseudo-57, since titers of individual virus preparations differed for each experiment. Pseudo-A8 gave F10/NIH relative infectivities of 0.17 on average, whereas pseudo-57 exhibited relative titers of 1 order of magnitude lower (Table 1). These results indicate that the viruses recognized EcoR on F10 cells as distinct from that on NIH 3T3 cells. The efficiency of receptor-mediated viral entry varied in rat cell lines derived from different tissues. In lymph node-derived cells (LYM-1) as well as in F10 cells, A8 had a higher efficiency of entry than that of 57, indicating that the efficient utilization of rat EcoR by A8 is not restricted to glial cells (Table 2). In liver-derived cells (ARLJ301-3), the efficiency of entry of 57 was similar to that of A8 (Table 2). Whole rat *ecoR* cDNAs have been isolated from hepatoma cells and intestinal cells (3, 21). The third extracellular loop domain, containing the virus-binding site of these gene products, is identical to that of the *ecoR* gene on F10 cells (described later). Therefore, posttranslational modifications of *ecoR*, such as glycosylation, numbers of EcoR molecules on cells, or cofactors other than the receptor, might account for differences in efficiency of viral entry in each cell line.

Isolation and sequence analysis of ecotropic murine leukemia virus receptor cDNA from F10 cells. We cloned the *ecoR* gene from F10 cells to compare it with the *ecoR* gene derived from NIH 3T3 cells. Total RNA (4) was extracted from F10 cells, and first-strand DNA was synthesized by a reaction with the SuperScript preamplification system for the first-strand cDNA synthesis kit (GibcoBRL) with an oligonucleotide deoxyribosylthymine primer, in accordance with the manufacturer's protocol. Second-strand DNA was synthesized by PCR with a universal primer (5′TGGAATTCATGGGCTGCAAA

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TABLE 1. Efficiency of receptor-mediated cell entry of pseudotyped virus having A8-SU or 57-SU

Pseudotyped virus, expt I trial no.	No. of β-Gal-positive cells in[a]:		F10/NIH ratio (1/10)	Pseudotyped virus, expt II trial no.	No. of β-Gal-positive cells in[a]:		F10-*ecoR*/NIH-*ecoR* ratio on SIRC (1/10)
	NIH 3T3	F10			NIH-*ecoR*/SIRC	F10-*ecoR*/SIRC[b]	
A8				A8			
1	274.3	53.3	1.9	1	159.0	33.5	2.1
2	295.0	32.8	1.1	2	405.0	50.5	1.2
3	324.6	78.4	2.4	3	360.0	31.5	0.9
4	447.6	105.4	2.4	4	1,423.5	119.0	0.8
5	612.6	65.7	1.1	5	1,632.5	115.0	0.7
6	272.5	42.5	1.6				
Mean ± SEM	371.1 ± 55.1[c]	63.0 ± 10.8[d]	1.7 ± 0.2[d]	Mean ± SEM	796.0 ± 303.5	69.9 ± 19.5	1.2 ± 0.3[e]
57				57			
1	1,079.0	20.7	0.2	1	342.5	18.0	0.5
2	199.7	1.3	0.1	2	435.5	14.0	0.3
3	144.9	2.6	0.2	3	545.0	24.5	0.4
4	197.0	1.3	0.1	4	846.0	11.0	0.1
5	429.2	6.4	0.1	5	2,848.0	54.0	0.2
6	304.5	5.1	0.2				
Mean ± SEM	392.4 ± 143.4[c]	6.2 ± 3.0	0.1 ± 0.0	Mean ± SEM	1,003.4 ± 468.9	24.3 ± 7.8	0.3 ± 0.1

[a] β-Gal, β-galactosidase. Pseudotyped viruses (2×10^4 cells) were inoculated on a 12-well culture plate in the presence of 10 μg of Polybrene per ml. At 2 days postinfection, infected cells were visualized by X-Gal staining as described in the text. Values are the means for two or three wells.

[b] NIH-*ecoR*- or F10-*ecoR*-expressing SIRC cells.

[c] This value is not significantly different from that for the other pseudotyped virus.

[d] $P < 0.01$ versus value for pseudotyped virus 57 infection.

[e] $P < 0.05$ versus value for pseudotyped virus 57 infection.

AACCTGCT) and a reverse primer (5′TGGAATTCTCATT TGCACTGGTCCAAGT), which contain the ATG and the stop codon, respectively, and an *Eco*RI recognition site. Three cDNA clones of the genes, homologous with NIH-*ecoR*, were isolated. Restriction map and partial sequence analyses revealed the structural identities of the three clones (data not shown). The cDNA of the *ecoR* homologue derived from F10 cells (F10-*ecoR*; accession no. D67087) was highly homologous with both its NIH 3T3 cell counterpart (NIH-*ecoR*), having 96% amino acid identity, and the *ecoR* isolated from rat hepatoma cells (21) (99% amino acid identity). The nucleotide sequence of F10-*ecoR* cDNA predicted a 624-amino-acid protein. The putative virus-binding site, Y-G-E (open circles, Fig. 1), of F10-*ecoR* was identical to those of NIH-*ecoR* and rat *ecoR* isolated from hepatoma cells. The greatest diversity was found in the amino acid sequence 221-ENKSSPLCGNND-232, flanking the virus-binding site in the third extracellular loop (boxed in Fig. 1). The amino acid sequence of this region of F10-*ecoR* was identical to that of rat *ecoR* isolated from hepatoma cells. The Y-G-E amino acid sequence at position 235 to 237 of the NIH-EcoR protein in the third extracellular loop has been demonstrated to be critical for ensuring proper viral receptor function (1, 22). This sequence of F10-*ecoR* is identical to that in NIH-*ecoR*. Recent studies demonstrated that the two potential sites for N-glycosylation in the third extracellular loop of EcoR (arrowheads, Fig. 1) are glycosylated (9), and these carbohydrate moieties interfere with virus binding in a combination of several cell lines and viruses (6, 12, 18). Comparison of the sequences of F10-*ecoR* and NIH-*ecoR* revealed the existence of a 3-amino-acid sequence (S-P-L) between these two potential sites for N-glycosylation of F10-EcoR, with two amino acid substitutions (F to K and N to G) (Fig. 1). Therefore, the amino acid substitutions between the two potential sites for N-glycosylation could affect the tertiary structure around the virus-binding site, resulting in a change in the capability of SU protein to bind receptor.

Comparison of receptor activity of F10-EcoR and NIH-EcoR expressed on SIRC cells. To compare the contribution of F10-*ecoR* gene and NIH-*ecoR* gene to viral proliferation in cells with the same genetic background, F10-*ecoR* cDNA or NIH-*ecoR* cDNA was introduced into rabbit cornea cell line SIRC (11). Expression vectors containing NIH-*ecoR* cDNA (pJET) were provided by J. M. Cunningham, Harvard Medical School (2). F10-*ecoR* cDNA was inserted into the pJAY3 (10) expression vector derived from pJET. These plasmids were stably introduced into SIRC cells by cotransfection with selectable plasmid pSTNeoB (8). RNA samples (20 μg each) were electrophoresed through a formaldehyde gel, transferred to a membrane, and hybridized to a ^{32}P-labeled *Pst*I-*Hin*cII fragment (0.5 kb) of NIH-*ecoR* cDNA (4, 16). Abundant expression of the introduced genes was detected in transfected SIRC cells (Fig. 2). *ecoR*-related mRNA was not found in normal SIRC cells. We examined the interaction between the viruses and the receptors expressed on SIRC cells by pseudotyped virus infec-

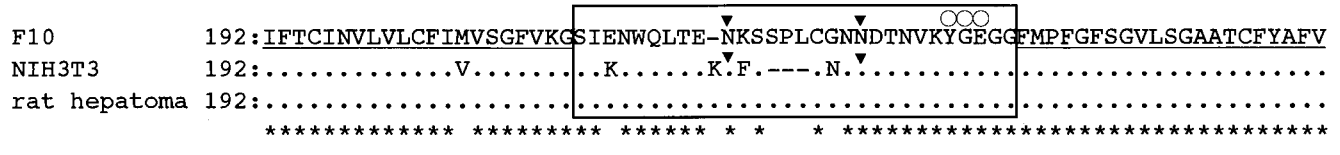

FIG. 1. Alignment of deduced amino acid sequences of F10-*ecoR* (F10), NIH-*ecoR* (NIH 3T3), and rat *ecoR* isolated from hepatoma cells (rat hepatoma) in the third extracellular loop domain (boxed). Conserved amino acids with F10-*ecoR* are represented by dots. Arrowheads indicate potential N-linked glycosylation sites. Potential membrane-spanning domains present in mouse *ecoR* (2) are underlined. Open circles indicate the amino acid sequence suggested to serve as the viral binding site (1, 22).

TABLE 2. Relative infectivity of pseudotyped virus against NIH 3T3 cells in rat-derived cells

Pseudotyped virus and trial no.	LYM-1/NIH[a] (1/10)	ARLJ301-3/NIH[a] (1/10)
A8		
1	0.7	3.6
2	1.1	2.3
3	0.7	1.7
Mean ± SEM	0.8 ± 0.2[b]	2.6 ± 0.6[c]
57		
1	0.1	1.9
2	0.1	1.1
3	0.1	0.6
Mean ± SEM	0.1 ± 0.0	1.2 ± 0.4[c]

[a] Tissue derived.
[b] $P < 0.05$ versus value for pseudotyped virus 57 infection.
[c] This value is not significantly different from that for the other pseudotyped virus.

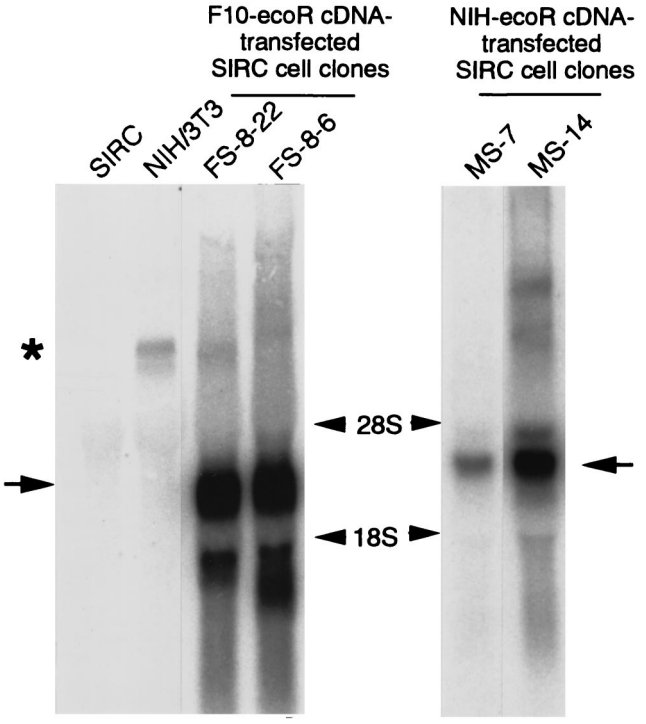

FIG. 2. Northern blot hybridization of SIRC cell clones after the introduction of F10-*ecoR* cDNA or NIH-*ecoR* cDNA. Expression vector containing 1.9 kb of F10-*ecoR* cDNA or 2.3 kb of NIH-*ecoR* cDNA was introduced. Twenty micrograms of total RNA was electrophoresed and transferred to a membrane. The quality of the RNA was confirmed by visualization of ribosomal RNA on membrane by using a UV lamp. A ^{32}P-labeled *Pst*I-*Hin*cII fragment of NIH-*ecoR* cDNA was used as a hybridization probe. Arrows indicate transfected *ecoR* mRNA. The asterisk indicates endogenous *ecoR* mRNA in NIH 3T3 cells.

tion. The relative infectivity of F10-*ecoR*-expressing SIRC cells against NIH-*ecoR*-expressing SIRC cells of pseudo-A8 was 0.12, on average, for each experiment, whereas pseudo-57 exhibited fourfold-lower relative infectivities on F10-*ecoR*-expressing SIRC cells (Table 1). To compare the viral proliferation of A8 and 57, these viruses were inoculated into 10^4 of the exogenous *ecoR*-expressing cells on a 12-well culture plate, and then viral titers at 4 days postinfection were determined by XC plaque assay on C182 cells (15). At the same time, the number of cells on the 12-well culture plates was determined by modified MTT assay with WST-1 (Dojindo) in accordance with the manufacturer's protocol (7). Virus titers were normalized by cell number to compare growth rates among different kinds of cells. In two clones of F10-*ecoR*-expressing SIRC cells, virus titers of A8 were 129- and 360-fold higher than those of virus 57 at a multiplicity of infection of 0.1 (Table 3). In NIH-*ecoR*-expressing SIRC cell clones, virus 57 proliferated with the same efficiency as A8 (Table 3).

Could a difference in receptor-mediated viral entry efficiency of 1 order of magnitude account for the final difference in neuropathogenic and nonneuropathogenic viral titers assayed at 4 days postinfection? As shown in our previous work (17), studies with chimerae from A8 and 57 demonstrated that at 4 days after infection in F10 cells, the production of Rec6, which contains the *env* gene of 57 against the background of the A8 gene, is 1 order of magnitude lower than the A8 viral titer. Furthermore, the reverse chimera to Rec6, having A8-SU on the background of virus 57, and designated Rec5, showed a 1-order-of-magnitude increase of viral production in F10 cells over virus 57. These results indicate that the interaction of the SU protein and the receptor is responsible for the increase or decrease of viral production by 1 order of magnitude. Since the LTR and/or 5′ leader sequence of A8 have been shown to be indispensable for efficient viral proliferation in F10 cells (17), the proliferation of A8 in F10 cells could be attributed to the velocity of proliferation after viral entry into cells facilitated by the LTR and/or 5′ leader sequence of A8. Therefore, the difference in receptor utilization between A8-SU and 57-SU

TABLE 3. Comparison of production of A8 and 57 in F10 cells, NIH 3T3 cells, and F10-*ecoR*- or NIH-*ecoR*-expressing SIRC cells

Cell type[a]	Receptor		Virus production (PFU/ml)[b]		A8/57 ratio
	Endogenous	Exogenous	A8	57	
F10	F10-*ecoR*	No	$1.2 \pm 0.1 \times 10^5$	$6.0 \pm 1.5 \times 10^1$	1,991
NIH 3T3	NIH-*ecoR*	No	$7.3 \pm 2.5 \times 10^5$	$2.1 \pm 0.9 \times 10^5$	3
SIRC	No	No	—[c]	—[c]	N[d]
FS-8-22	No	F10-*ecoR*	$7.0 \pm 1.0 \times 10^2$	$5.4 \pm 5.4 \times 10^0$	129
FS-8-6	No	F10-*ecoR*	$1.2 \pm 0.5 \times 10^3$	$3.3 \pm 1.7 \times 10^0$	360
MS-7	No	NIH-*ecoR*	$2.8 \pm 0.6 \times 10^3$	$1.1 \pm 0.1 \times 10^3$	3
MS-14	No	NIH-*ecoR*	$2.7 \pm 1.8 \times 10^2$	$4.2 \pm 1.2 \times 10^1$	7

[a] FS, F10-*ecoR*-expressing SIRC cells; MS, NIH-*ecoR*-expressing SIRC cells.
[b] Viruses were infected at a multiplicity of infection of 0.1. At 4 days postinfection, culture supernatants were collected and virus production was determined by XC plaque assay. Means ($n = 3$) and standard errors of the means are indicated.
[c] —, <2.5 PFU/ml.
[d] N, not available.

can only partially explain the distinct viral proliferation of A8 and 57 in F10 cells. The in vivo study with chimerae revealed that the LTR and/or 5′ leader sequence of A8 is more critical than the structural difference in the SU protein in A8 and 57 (17) for the proliferation of viruses in the brains of rats at 6 to 10 weeks postinfection. Based on the present findings and on previous studies on the proliferation of chimerae in vitro and in vivo, we conclude that A8 and 57 recognize the receptor in distinct manners; however, this difference in recognition would account only for 1 (that is, 1% at most) of a >3-order-of-magnitude difference in titer between the two viruses in the CNS.

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