Virus Genes
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# Susceptibility of muridae cell lines to ecotropic murine leukemia virus and the cationic amino acid transporter 1 viral receptor sequences: implications for evolution of the viral receptor

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**Abstract** Ecotropic murine leukemia viruses (Eco-MLVs) infect mouse and rat, but not other mammalian cells, and gain access for infection through binding the cationic amino acid transporter 1 (CAT1). Glycosylation of the rat and hamster CAT1s inhibits Eco-MLV infection, and treatment of rat and hamster cells with a glycosylation inhibitor, tunicamycin, enhances Eco-MLV infection. Although the mouse CAT1 is also glycosylated, it does not inhibit Eco-MLV infection. Comparison of amino acid sequences between the rat and mouse CAT1s shows amino acid insertions in the rat protein near the Eco-MLV-binding motif. In addition to the insertion present in the rat CAT1, the hamster CAT1 has additional amino acid insertions. In contrast, tunicamycin treatment of mink and human cells does not elevate the infection, because their CAT1s do not have the Eco-MLV-binding motif. To define the evolutionary pathway of the Eco-MLV receptor, we analyzed CAT1 sequences and susceptibility to Eco-MLV infection of other several murinae animals, including the southern vole (*Microtus rossiaemeridionalis*), large Japanese field mouse (*Apodemus speciosus*), and Eurasian harvest mouse (*Micromys minutus*). Eco-MLV infection was enhanced by tunicamycin in these cells, and their CAT1 sequences have the insertions like the hamster CAT1. Phylogenetic analysis of mammalian CAT1s suggested that the ancestral CAT1 does not have the Eco-MLV-binding motif, like the human CAT1, and the mouse CAT1 is thought to be generated by the amino acid deletions in the third extracellular loop of CAT1.

**Keywords** Ecotropic murine leukemia virus · CAT1 · Glycosylation · Evolution

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## Introduction

A change in the cell surface receptor for a virus is one of a host defense mechanism against virus infection; for example, the C-terminally truncated CCR5 variant is known to confer resistance to human immunodeficiency virus (HIV) infection [1]. We have analyzed the viral receptors for ecotropic murine leukemia viruses (Eco-MLVs) in mouse (*Mus musculus*), rat (*Ratus norvegicus*), and *Mus dunni* cells as a model of receptor evolution that confers resistance to a virus infection [2–4].

Eco-MLVs can infect mouse and rat cells, and recognize the multi-membrane-spanning cationic amino acid transporter 1 (CAT1) as the receptor for infection [5]. Eco-MLV binds the YGE or HGE motif in the third extracellular loop of the CAT1 [6, 7]; the CAT1 has two N-linked

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glycosylation sites near the Eco-MLV-binding motif. Nucleotide sequences near the virus-binding motif are highly diversified among mouse, rat, hamster, mink, and human, suggesting that the region is under selective pressure.

Rat cells are much less susceptible to Eco-MLV infection than mouse cells, and hamster cells are completely resistant to infection. Treatment of rat and hamster cells with tunicamycin, an N-linked glycosylation inhibitor, enhances susceptibility to Eco-MLV infection [8, 9]. Furthermore, an amino acid substitution at the glycosylation site of the rat CAT1 increases susceptibility to Eco-MLV infection [2]. These results indicate that N-linked glycosylation of the rat and hamster CAT1 proteins inhibits Eco-MLV infection. Although the mouse CAT1 is also glycosylated at the same amino acid residues as the rat and hamster CAT1s, it does not affect Eco-MLV infection [2]; rat and hamster CAT1 proteins have three- and six-amino acid insertions near the viral-binding domain of the protein compared to the mouse CAT1. We have previously reported that a deletion of the amino acid insertion in the rat CAT1 confers increased susceptibility, and abrogates the glycosylation-mediated inhibition of Eco-MLV infection, indicating that the amino acid insertion in the rat CAT1 is the determinant for the glycosylation-dependent infection inhibition [3]. The longer insertion present in the hamster CAT1 compared to the rat protein may confer hamster cells a complete resistance to Eco-MLV infection. In addition, glycosylation of the *Mus dunni* CAT1 also inhibits Eco-MLV infection as a result of a one-amino acid insertion in the YGE virus-binding motif [4].

To confirm the pathway of evolution for mammalian CAT1s, we established immortalized cell lines from several murinae animals, and then determined their susceptibility to Eco-MLV infection and their CAT1 sequences. We showed that the CAT1 sequences of the southern vole, large Japanese field mouse, and Eurasian harvest mouse were shown to have amino acid insertions similar to the hamster CAT1. Phylogenetic analysis of mammalian CAT1 sequences revealed that the CAT1 ancestor is the human-type CAT1, and evolved to the mouse-type CAT1 by deletion rather than by insertion. This study reviews the evolutionary pathway of the ecotropic MLV receptor, CAT1, in relation to the viral infection.

## Materials and methods

### Animals

The southern vole (*Microtus rossiaemeridionalis*) and steppe lemming (*Lagurus lagurus*) were obtained from the closed colony maintained at the Frontier Science Research Center, University of Miyazaki. The Mongolian gerbil (*Meriones unguiculatus*) was obtained from the closed colony maintained at the Institute of Tropical Medicine, Nagasaki University. A wild large Japanese field mouse (*Apodemus speciosus*) and a Eurasian harvest mouse (*Micromys minutus*) were captured in Kiyotake, Miyazaki City, Miyazaki Prefecture, Japan, with the approval of the prefectural governor (No. 24940-2696). This animal study is approved by the Ethics Committee of Nagasaki University (No. 0812080723), and the Committee for the Ethics on Animal Experiments at the University of Miyazaki (No. 2008-505).

### Cells

Mouse NIH3T3, rat F10, and human TELCeB6 [10] cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) supplemented with 8 % fetal bovine serum (FBS). Kidney grafts of *M. minutus*, *M. rossiaemeridionalis*, and muscle grafts of *A. speciosus* were isolated and were treated with trypsin to separate cells. The cells were cultured with D-MEM containing 20 % FBS for more than one year. Cells from *M. rossiaemeridionalis* and *A. speciosus* were passed by 1/6 dilution every 3 days. Cells from *M. minutus* were passed by 1/2 dilution every 6 days.

### Expression plasmids

An expression plasmid of the ecotropic Friend MLV Env has been already described [11]. A VSV-G expression plasmid was obtained from Dr. L. Chang through the AIDS Research and Reference Reagent Program, NIAID, NIH, USA [12].

### Transduction assay

TELCeB6 cells [10] were transfected with an expression plasmid containing the Friend MLV envelope protein using the Fugene transfection reagent (Promega). Since the expression plasmid also encodes the neomycin resistance gene, the transfected cells were selected by geneticin (Invitrogen). Culture supernatants of the geneticin-resistant cell pool were inoculated into target cells in the presence of Polybrene (4 μg/ml) (Sigma–Aldrich). To construct a VSV-pseudotyped MLV vector, TELCeB6 cells were transiently transfected with a VSV-G expression plasmid and their culture supernatants were collected 2 days after transfection. The culture supernatants were then inoculated into target cells. To estimate transduction titer, the inoculated cells were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Wako) 2 days after the inoculation and then blue cells were counted.

### Isolation of CAT1 sequences

Total RNA samples were prepared from cells using Isogen (Invitrogen). cDNAs were synthesized by reverse transcriptase (TaKaRa) and a fragment containing the third extracellular loop of CAT1 and its upstream region was amplified by PCR (TaKaRa) using the cDNA as template. Nucleotide sequences of the PCR primers are AAC CTG ATT CTC TCC TAC ATC and GTG GTG GCG ATG CAG TCA AAG. The PCR products were cloned into pTarget vector (Promega) and nucleotide sequences of the insert DNAs were determined (Applied Biosystems). The primers were synthesized by Genenet Co., LTD. Accession numbers of CAT1s from A. speciosus, *M. rossiaemeridionalis,* and *M. minutus* are AB839945, AB839946, and AB839947, respectively.

### Alignments and phylogenetic analysis

Rodent CAT1 gene sequences were compared with other mammalian CAT1 gene sequences obtained from the DNA database: laboratory rat (*Rattus norvegicus*; AB066224) [2], laboratory mouse (*Mus musculus*; M26687) [5], human (*Homo sapiens*; X59155) [7], pig (*Sus scrofa*; AY371320) [13], domestic dog (*Canis lupus familiaris*; XM_854224) [14], American mink (*Neovison vison*; U49796) [15], domestic cat (*Felis catus*; XM_003980275) [16], chimpanzee (*Pan troglodytes*; XM_001139004), horse (*Equus caballus*; XM_001492839) [17], cattle (*Bos taurus*; NM_001135792) [18], giant panda (*Ailuropoda melanoleuca*; XM_002914759) [19], African elephant (*Loxodonta africana*; XM_0034 14018), bonobo (*Pan paniscus*; XM_003818324) [20], Syrian hamster (*Mesocricetus auratus*; U26454), Chinese hamster (*Cricetulus griseus*; U49797), orangutan (*Pongo abelii*; XM_002824135) [21], small-eared galago (*Otolemur garnettii*; XM_003797609), Northern white-cheeked gibbon (*Nomascus leucogenys*; XM_003270277), rabbit (*Oryctolagus cuniculus*; XM_002721425), lesser Egyptian jerboa (*Jaculus jaculus*; XM_004659984), naked mole rat (*Heterocephalus glaber*; XM_004854792), and degu (*Octodon degus*; XM_004631056).

The obtained three rodent CAT1 gene sequences were compared with other mammalian CAT1 gene sequences obtained from the DNA database. All the sequences were once translated into amino acids and then aligned using MUSCLE [22] implemented in MEGA ver 5.1 [23]. These aligned amino acid sequences were reversely translated into nucleotide sequences, and used for the analyses.

For phylogenetic analyses, we employed the CAT2 sequences of human (D29990) [24] and mouse (L03290) [25] for the outgroup. Therefore, we realigned all the sequences with these CAT2 sequences following the same procedure described above. A phylogenetic tree was constructed by Bayesian method. The dataset was divided into three partitions with codon position (1st, 2nd, and 3rd), and optimum substitution models for each partition were selected by Kakusan 4 [26] based on the Bayesian information criterion; general time reversible (GTR) [27] with gamma distribution (+G), GTR+G, and HKY85 [28] +G models were selected for the 1st, 2nd, and 3rd positions, respectively. The Bayesian analysis was conducted using MrBayes v3.2.1 [29] with 3 million generations of two independent runs of four Markov chains. We sampled one tree every 100 generations and calculated a consensus topology with discarding the first 25 % of trees. The final average standard deviation of split frequencies of the Bayesian analysis was 0.017942, and all average effective sample sizes were more than 200.

### Statistical analysis

Differences between two groups of data were determined by the Student's *t* test. The statistical significance was set at $P < 0.05$ for all the tests.

## Results

### Susceptibility of rodent cells to Eco-MLV infection

Immortalized cell lines were established from the inbred southern vole (*Microtus rossiaemeridionalis*), wild large Japanese field mouse (*Apodemus speciosus*), and wild Eurasian harvest mouse (*Micromys minutus*) to assess their susceptibility to Eco-MLV infection. Cells were inoculated with the Friend MLV Env protein-carrying MLV vector, and transduction titers were measured. Infected cells were detected in the *A. speciosus* and *M. rossiaemeridionalis* cells; we observed the transduction titers for these cells to be 1/100–1/1000 times lower than those of mouse NIH3T3 cells (*M. musculus*), similar to that found on rat F10 cells (*R. norvegicus*) (Fig. 1a). In contrast, infected cells were not detected from *M. minutus*, showing that these cells are resistant to Eco-MLV infection.

To determine whether N-linked glycosylation inhibits the Eco-MLV vector infection in these rodent cells, the cells were pretreated with tunicamycin (100 μg/ml) for 24 h, and then were inoculated with the Eco-MLV vector. In the *A. speciosus, M. minutus,* and *M. rossiaemeridionalis* cells, tunicamycin treatment enhanced Eco-MLV vector infection, as in the rat F10 cells (Fig. 1b), showing that glycosylation inhibits the Eco-MLV infection in these cells. In contrast, we have already reported that tunicamycin treatment of mouse NIH3T3 cells does not affect the Eco-MLV vector infection [2, 3]. However, infection by VSV-G-pseudotyped MLV vector was not affected by the

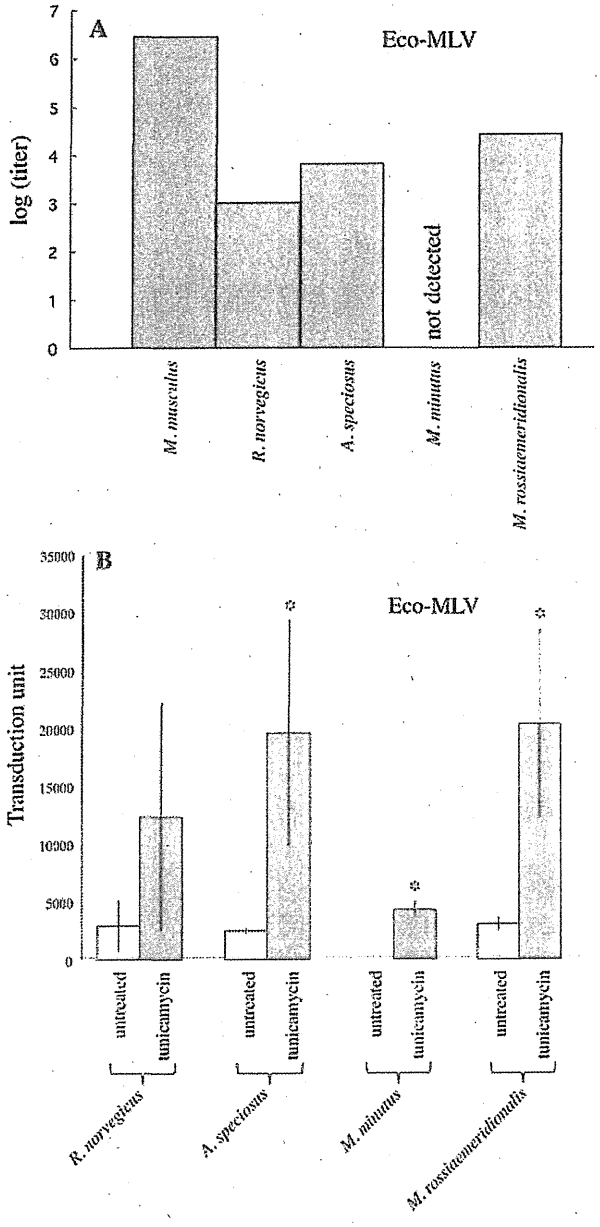


**Fig. 1** Susceptibility of rodent cells to Eco-MLV infection. Rodent cells were inoculated with the Eco-MLV (**a** and **b**). Cells were treated or untreated with tunicamycin for 24 h and then were inoculated with the viral vector. These experiments were repeated three times. The results are shown as the average titers ± SD. *Asterisks* indicate statistically significant differences compared to the titer on the untreated cells

tunicamycin treatment of these cells (data not shown), suggesting that the glycosylation-mediated inhibition of MLV infection is dependent on the virus envelope protein. These data indicate that glycosylation of CAT1 proteins of *A. speciosus, M. minutus,* and *M. rossiaemeridionalis* inhibits Eco-MLV infection, suggesting that these CAT1 sequences have the amino acid insertion similar to the rat and hamster CAT1.

## Nucleotide sequences of the CAT1 genes of rodent cells

DNA fragments containing the third extracellular loop and its upstream region of the CAT1 gene were isolated by RT-PCR from *M. rossiaemeridionalis, A. speciosus, and M. minutus* cells, and their nucleotide sequences were determined. Amino acid sequences of the third extracellular loop and its upstream region from the various mammals are shown in Fig. 2a, b, respectively. The CAT1 sequences of *M. rossiaemeridionalis, A. speciosus,* and *M. minutus* contain the amino acid insertions and the YGE or HGE Eco-MLV-binding motif in the third extracellular loop, as found in the hamster CAT1. Interestingly, the CAT1 sequences from rabbit (*Oryctolagus cuniculus*) and lesser Egyptian jerboa (*Jaculus jaculus*) have divergent types of deletions in the third extracellular loop.

When nucleotide sequences were compared among rodents including *M. musculus, R. norvegicus, M. minumus, A. speciosus, M. rossiaemeridionalis*, Chinese hamster (*C. griseus*), Syrian hamster (*M. auratus*), naked mole rat (*H. glaber*), degu (*O. degus*), rabbit (*O. cuniculus*), and lesser Egyptian jerboa (*J. jaculus*), the third extracellular loops (Fig. 3a) (76.4 ± 6.8 %) were less homologous than their upstream regions (Fig. 3b) (89.1 ± 4.8 %) ($P = 9.49 \times 10^{-23}$). Furthermore, the changes in the first and second nucleotides of codons in the CAT1 third extracellular loops (ECL3s) of rodents were more dominant than those in the upstream regions (Table 1), and nonsynonymous mutations in the ECL3s were more abundant than those in the upstream regions, compared to the mouse CAT1 ($P = 8.12 \times 10^{-11}$). In contrast, the amino acid sequences of the extracellular loops of the CAT1s among cat, dog, pig, horse, cattle, giant panda, elephant, small-eared galago, northern white-cheeked gibbon, Sumatran orangutan, bonobo, chimpanzee, and human are more similar than those among the rodents (Fig. 3c) (86.8 ± 7.2 %) ($P = 2.93 \times 10^{-14}$). These results suggest that the third extracellular region is under stronger selective pressure in rodents than in higher mammals.

Additionally, immortalized cells were established from inbred steppe lemming (*Lagurus lagurus*) and Mongolian gerbil (*Meriones unguiculatus*). CAT1 sequences could not be amplified from these cells by RT-PCR (data not shown). Consistently, when these cells were inoculated with the Eco-MLV vector, LacZ-expressing cells were not detected even in the presence of tunicamycin, although VSV-G-pseudotyped vector could transduce the cells. The *L. lagurus* and *M. unguiculatus* cells might be resistant due to the lack of susceptible CAT1 expression.

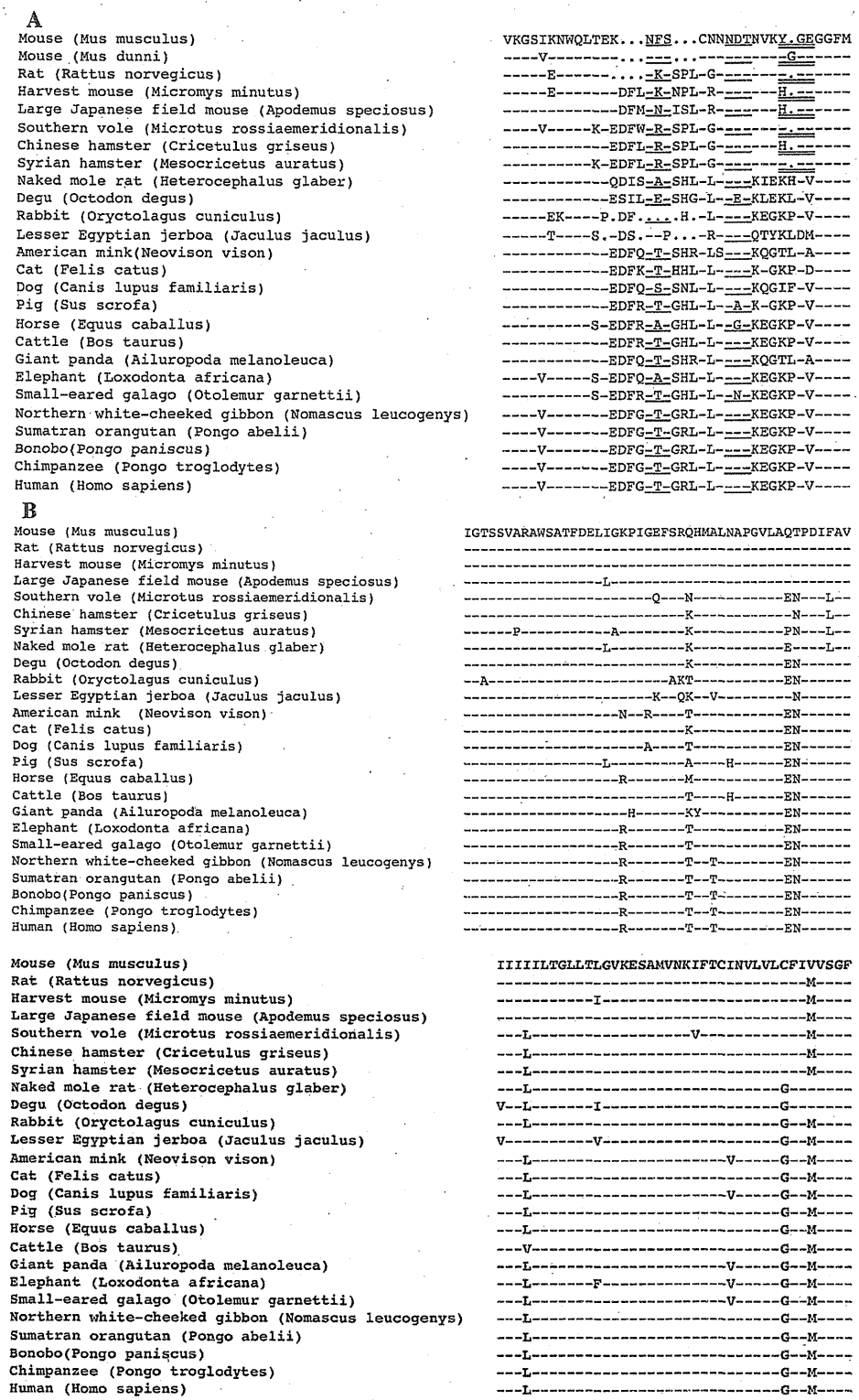


**Fig. 2** Amino acid sequences of CAT1s. The amino acid sequences of the third extracellular loops (**a**) and the upstream regions (**b**) of CAT1s are indicated. *Bars* and *dots* indicate identical amino acids and deletions, respectively. N-linked glycosylation sites are *underlined*. The Eco-MLV binding motif is *double-underlined*

**Fig. 3** Comparison of nucleotide sequences of CAT1s. Nucleotide sequences of the third extracellular loops (**a**) and the upstream regions (**b**) of CAT1s from indicated animals. Nucleotide sequences of the third extracellular loops (**a**) and their upstream regions (**b**) of rodent CAT1s are compared. Nucleotide sequences of the third extracellular loops of CAT1s from the higher animals are compared (**c**)

**A**

| | *M. musculus* | *R.norvegicus* | *M. minutus* | *A. speciosus* | *M. rossiaemeridionalis* | *C. griseus* | *M. auratus* | *H. glaber* | *O. degus* | *O. cuniculus* | *J. jaculus* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *M. musculus* | 100 | 74 | 76 | 76 | 71 | 74 | 74 | 70 | 72 | 64 | 66 |
| *R.norvegicus* | | 100 | 82 | 74 | 82 | 82 | 82 | 75 | 74 | 71 | 69 |
| *M. minutus* | | | 100 | 87 | 76 | 87 | 82 | 80 | 79 | 72 | 69 |
| *A. speciosus* | | | | 100 | 76 | 84 | 79 | 80 | 80 | 70 | 72 |
| *M. rossiaemeridionalis* | | | | | 100 | 89 | 94 | 81 | 79 | 68 | 68 |
| *C. griseus* | | | | | | 100 | 94 | 82 | 80 | 72 | 72 |
| *M. auratus* | | | | | | | 100 | 78 | 77 | 68 | 68 |
| *H. glaber* | | | | | | | | 100 | 89 | 72 | 73 |
| *O. degus* | | | | | | | | | 100 | 73 | 72 |
| *O. cuniculus* | | | | | | | | | | 100 | 74 |
| *J. jaculus* | | | | | | | | | | | 100 |

**B**

| | *M. musculus* | *R.norvegicus* | *M. minutus* | *A. speciosus* | *M. rossiaemeridionalis* | *C. griseus* | *M. auratus* | *H. glaber* | *O. degus* | *O. cuniculus* | *J. jaculus* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *M. musculus* | 100 | 98 | 96 | 98 | 88 | 92 | 90 | 88 | 86 | 85 | 88 |
| *R.norvegicus* | | 100 | 98 | 100 | 90 | 94 | 92 | 85 | 84 | 83 | 87 |
| *M. minutus* | | | 100 | 98 | 90 | 94 | 92 | 86 | 86 | 84 | 89 |
| *A. speciosus* | | | | 100 | 90 | 94 | 92 | 84 | 83 | 84 | 89 |
| *M. rossiaemeridionalis* | | | | | 100 | 96 | 96 | 87 | 87 | 83 | 85 |
| *C. griseus* | | | | | | 100 | 98 | 85 | 88 | 85 | 89 |
| *M. auratus* | | | | | | | 100 | 84 | 86 | 83 | 86 |
| *H. glaber* | | | | | | | | 100 | 92 | 87 | 85 |
| *O. degus* | | | | | | | | | 100 | 87 | 89 |
| *O. cuniculus* | | | | | | | | | | 100 | 86 |
| *J. jaculus* | | | | | | | | | | | 100 |

**C**

| | *F. catus* | *C. lupus* | *S. scrofa* | *E. caballus* | *B. taurus* | *A. melanoleuca* | *L. africana* | *O. garnettii* | *N. leucogenys* | *P. abelii* | *P. paniscus* | *P. troglodytes* | *H. sapiens* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *F. catus* | 100 | 79 | 87 | 79 | 87 | 82 | 82 | 82 | 82 | 82 | 82 | 82 | 82 |
| *C. lupus* | | 100 | 79 | 77 | 82 | 85 | 82 | 77 | 79 | 79 | 79 | 79 | 79 |
| *S. scrofa* | | | 100 | 90 | 95 | 79 | 82 | 92 | 90 | 90 | 90 | 90 | 90 |
| *E. caballus* | | | | 100 | 92 | 77 | 90 | 95 | 85 | 85 | 85 | 85 | 85 |
| *B. taurus* | | | | | 100 | 85 | 87 | 95 | 92 | 92 | 92 | 92 | 92 |
| *A. melanoleuca* | | | | | | 100 | 79 | 79 | 77 | 77 | 77 | 77 | 77 |
| *L. africana* | | | | | | | 100 | 87 | 87 | 87 | 87 | 87 | 87 |
| *O. garnettii* | | | | | | | | 100 | 90 | 90 | 90 | 90 | 90 |
| *N. leucogenys* | | | | | | | | | 100 | 100 | 100 | 100 | 100 |
| *P. abelii* | | | | | | | | | | 100 | 100 | 100 | 100 |
| *P. paniscus* | | | | | | | | | | | 100 | 100 | 100 |
| *P. troglodytes* | | | | | | | | | | | | 100 | 100 |
| *H. sapiens* | | | | | | | | | | | | | 100 |

**Table 1** Changes of the first, second, and third bases of codons in mammalian CAT1s compared to mouse CAT1s

| Species | Ratios of changes in upstream of ECL3 (%) | | | Ratios of changes in ECL3 (%) | | |
|---|---|---|---|---|---|---|
| | First | Second | Third | First | Second | Third |
| *R. norvegicus* | 0.8 | 0 | 6.9 | 4.0 | 3.4 | 5.1 |
| *M. minutus* | 0.8 | 0 | 0.5 | 5.1 | 4.5 | 6.2 |
| *A. speciosus* | 1.1 | 0 | 8.0 | 4.5 | 4.5 | 7.3 |
| *C. griseus* | 1.9 | 1.1 | 8.4 | 5.6 | 4.5 | 11.3 |
| *M. auratus* | 1.3 | 0.4 | 8.0 | 5.1 | 5.1 | 9.6 |
| *M. rossiaemeridionalis* | 3.4 | 0.4 | 9.6 | 5.6 | 5.1 | 11.3 |
| *H. glaber* | 2.7 | 0 | 9.2 | 6.8 | 6.8 | 16.4 |
| *O. Degus* | 2.7 | 0.4 | 10.7 | 7.3 | 6.8 | 13.6 |
| *O. cuniculus* | 3.8 | 1.1 | 10.0 | 7.9 | 9.0 | 18.6 |
| *J. jaculus* | 2.3 | 1.1 | 8.4 | 9.6 | 9.8 | 16.9 |

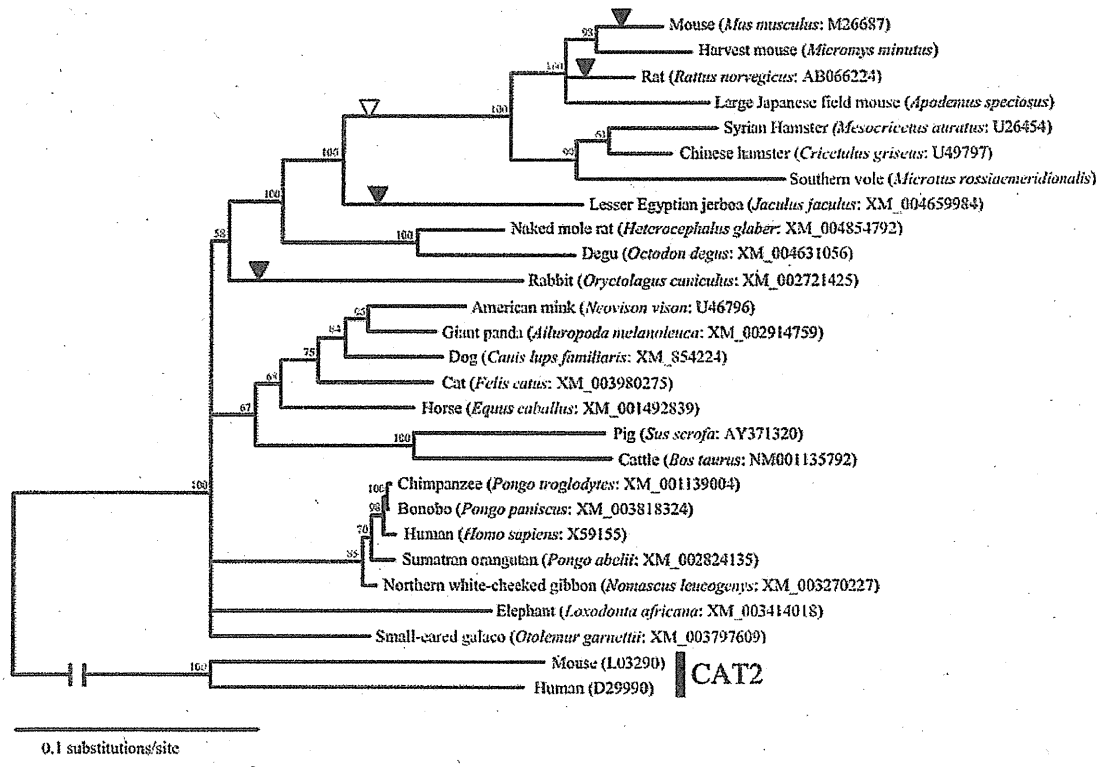


**Fig. 4** Phylogenetic tree of the mammalian CAT1s. *Closed arrow heads* indicate the time points when amino acid deletions in the third extracellular loops of CAT1s occurred. An *open arrow head* shows the time point when the Eco-MLV-binding motif was obtained

## Phylogenetic analysis of the ecotropic receptors

The phylogenetic tree of the CAT1s from the various mammals was constructed and is shown in Fig. 4. Rodents including mouse, rat, hamster, *A. speciosus, M. minutus*, and *M. rossiaemeridionalis* belong to one group. Human and primates belong to another group. The phylogenetic tree is consistent with the standard classification of mammals. By the phylogenetic tree, the Eco-MLV-binding motif could be obtained before mouse, rat, and hamster appeared (open arrow head in Fig. 4), and the amino acid deletions in the third extracellular loops of mouse, rat, rabbit, and lesser Egyptian jerboa CAT1s could occur independently (closed arrow heads in Fig. 4). This phylogenetic analysis also suggests that the ancestor CAT1 contain the amino acid insertion as present in the human CAT1s, and thereafter the mouse- and rat-type

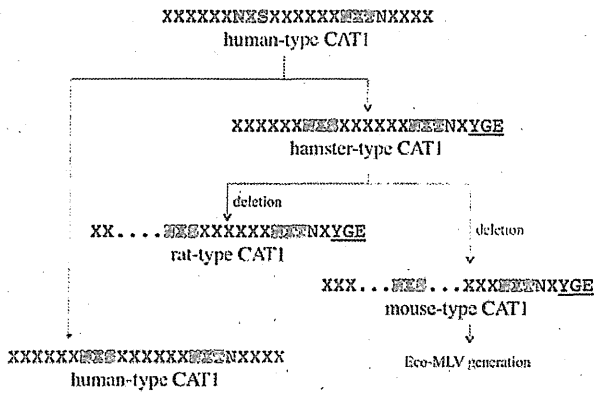


**Fig. 5** Evolutionary pathway of CAT1 predicted by this study. *Block letters* show glycosylation sites. *Dots* show amino acid deletions. The Eco-MLV-binding motifs are *underlined*

CAT1s could be generated from the hamster-type CAT1 by amino acid deletion (Fig. 5).

## Discussion

This study provides a putative evolution pathway of the mammalian CAT1s as follows (Fig. 5): the ancestor of the Eco-MLV receptor in mammals might not have the Eco-MLV-binding motif, like the human CAT1. Since Eco-MLV cannot infect the human CAT1, Eco-MLVs should not circulate at that time. The ancestral human-type CAT1 might be converted to the hamster-type CAT1, acquiring the virus-binding motif (open arrow head in Fig. 4). Since the hamster-type CAT1 is much less susceptible to Eco-MLV infection than the mouse CAT1, Eco-MLV should not spread at that time. The mouse CAT1 might be generated from the hamster-type CAT1 through amino acid deletion (closed arrow in Fig. 4). Since the mouse CAT1 is fully susceptible to Eco-MLV infection, Eco-MLV could circulate after the deletion occurred. Consistent with this speculation, it has been reported that many wild mice contain endogenous polytropic and xenotropic MLV sequences in their genomes but wild mice carrying the endogenous ecotropic MLV sequence are less numerous [30], suggesting that the ecotropic MLV appeared later than the polytropic and xenotropic viruses. Therefore, it is unlikely that Eco-MLV was derived from a virus which is related to Eco-MLV and efficiently interacts with the hamster-type CAT1 in the ancestor species of the *Mus* genus. Since the third extracellular loops of *Mus* subgenus animals, including *M. dunni*, *M. spicilegus*, and *M. minutoides*, have amino acid deletions as seen in the *M. musculus* CAT1 [31, 32], the deletion is thought to have occured before the *Mus* subgenus appeared.

The homology of the CAT1 third extracellular loop among the rodents is relatively lower than among the higher mammals, suggesting that the regions are under selective pressure in rodents. Since phylogenetic analysis of the mammalian CAT1s provided a possibility that Eco-MLV appeared after the *Mus* subgenus was generated, the selective pressure might not be the Eco-MLV infection itself. The CAT1 containing the amino acid deletion might be advantageous in the *Mus* subgenus, and eventually the deletion might permit the appearance of EcoMLVs. The observation that mouse, rat, rabbit, and lesser Egyptian jerboa CAT1s independently have amino acid deletions in the third extracellular loop suggests that the third extracellular loop of CAT1 is disadvantageous for rodents.

Since the one-amino acid insertion in the *Mus dunni* CAT1 is not found in the CAT1s of other *Mus* animals, it is thought that the one-amino acid insertion occurred to inhibit infection by Eco-MLV or other CAT1-recognizing viruses after the *Mus* subgenus appeared. CAT1-recognizing virus(s) might be widely spread among *M. dunni* population. To resolve this issue, further study is required.

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# CXCR4-Tropic, But Not CCR5-Tropic, Human Immunodeficiency Virus Infection Is Inhibited by the Lipid Raft-Associated Factors, Acyclic Retinoid Analogs, and Cholera Toxin B Subunit

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## Abstract

Development of an effective low-cost anti-acquired immunodeficiency syndrome (AIDS) drugs is needed for treatment of AIDS patients in developing countries. Host cell lipid raft microdomains, which are enriched with cholesterol, glycolipids, ceramide, and gangliosides, are important for human immunodeficiency virus type 1 (HIV-1) entry. Retinoid analogs have been shown to modulate ceramide levels in the cell membrane, while cholera toxin B subunit (CT-B) specifically binds to the ganglioside GM1. In this study, we found that the acyclic retinoid analogs geranylgeranoic acid (GGA) and NIK-333 as well as CT-B efficiently attenuate CXCR4-tropic, but not CCR5-tropic, HIV-1 vector infection. We also found that GGA and NIK-333 suppress CXCR4-tropic HIV-1 infection by attenuating CXCR4 expression. CT-B also attenuated CXCR4-tropic HIV-1 infection, but did not suppress CXCR4 expression. These results suggest a distinct role for lipid raft microdomains in CXCR4- and CCR5-tropic HIV-1 infections and illuminate novel agents for the development of AIDS therapy.

## Introduction

HIGHLY ACTIVE ANTIRETROVIRAL therapy (HAART), which suppresses human immunodeficiency virus type 1 (HIV-1) reverse transcriptase, protease, and integrase, has been found to be an effective treatment against acquired immunodeficiency syndrome (AIDS). In fact, many patients infected with HIV-1 do not progress to AIDS in developed countries due to implementation of HAART. However, HIV-1/AIDS continues to be a serious problem, as many HIV-1-infected patients in developing countries do not have access to effective anti-HIV-1 drugs due to the prohibitive cost of the therapy, and thus, the numbers of HIV-1-infected patients are increasing worldwide. In addition, HIV-1 variants resistant to current drugs have appeared.[1] To resolve these problems, novel, low-cost drugs that inhibit HIV-1 infection are critical.

Lipid raft microdomains of target cell membranes are required for HIV-1 infection.[2–6] Lipid rafts are enriched with cholesterol, glycolipids, and ceramide.[7] Extraction of cholesterol from cell membranes,[4,6] binding of cholesterol with various factors,[2,8] and inhibition of biosynthesis of cholesterol[9,10] or glycolipids[11–13] suppress HIV-1 infection, suggesting that cholesterol and glycolipids may be targets for novel anti-HIV-1 drugs. In this study, we examined the effects of lipid raft-associated factors, which were isolated from natural products, on HIV-1 vector infection.

Retinoic acid and its analogs modulate ceramide levels in cell membranes.[14–20] Retinoid analogs may inhibit HIV-1 infection by altering ceramide levels of the target cell membrane. In fact, an all-trans retinoic acid[21] and a weak nuclear retinoid receptor agonist, *N*-(4-hydroxyphenyl) retinamide (4-HPR),[22] inhibit HIV-1 infection[19,23]; however, because 4-HPR has severe toxicities, such as induction of vitamin A deficiency

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symptoms, clinical application of 4-HPR is restricted.[24] Geranylgeranoic acid (GGA), which is a natural acyclic retinoid analog present in medicinal herbs,[25] serves as a weak agonist for retinoid receptors, similar to 4-HPR.[26, 27] NIK-333, which is an artificial acyclic retinoid analog with a structure similar to GGA (Fig. 1), prevents recurrence of hepatocellular carcinoma following oral administration without any obvious side effects in clinical studies of liver cancer patients.[28,29] We analyzed the effects of the acyclic retinoid analogs GGA and NIK-333 on HIV-1 vector infection.

Cholesterol is enriched in lipid raft microdomains and requires their structural maintenance. Extraction of cholesterol from cell membranes by methyl-β-cyclodextrin (MβCD),[4, 6] inhibition of cholesterol synthesis by statin,[9,10] or binding of amphotericin B methyl ester to cholesterol[8] suppresses HIV-1 infection. Plant sterols are cholesterol analogs that reduce serum cholesterol levels by replacing cholesterol.[30] Therefore, plant sterols may function as anti-HIV-1 agents.

Because cholera toxin B subunit (CT-B) specifically binds to the ganglioside GM1, this subunit is frequently used as a lipid raft marker.[4,6] The cytopathic determinant of cholera toxin is subunit A, which has the poly(ADP) ribosylation activity of G-proteins.[31] In contrast, the B subunit has no cytopathic effect. GM1 is enriched in raft microdomains and has been reported to bind HIV-1 envelope (Env) glycoprotein.[32] Additionally, CD4-positive lymphocytes that have elevated levels of another gangliosides, GM3, are highly susceptible to HIV-1 fusion and entry.[11] Therefore, CT-B may inhibit HIV-1 infection without cytopathic effects.

In this study, we examined the effects of these raft-associated factors on HIV-1 vector infection. Our results showed that acyclic retinoid analogs and CT-B efficiently suppressed CXCR4-tropic HIV-1 vector infection, providing novel strategies for the development of CT-B or acyclic retinoid analog treatment for AIDS patients. In contrast, these factors did not affect CCR5-tropic HIV-1 vector infection, suggesting that raft microdomains are involved differently in CXCR4- and CCR5-tropic HIV-1 infections.

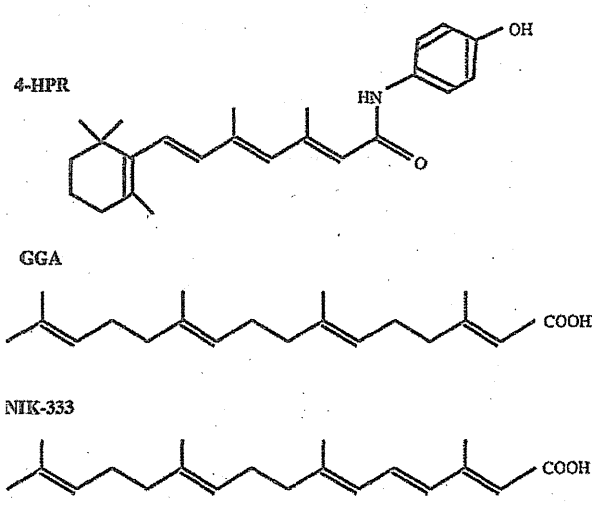


FIG. 1. Chemical structures of 4-HPR, GGA, and NIK-333.

## Materials and Methods

### Cells

COS7, 293T, NP2, TE671, and HeLa cells were cultured in Dulbecco's modified Eagle's medium (D-MEM) (Wako) supplemented with 8% fetal bovine serum (Biosource) at 37°C in 5% $CO_2$. NP2 cells expressing CD4 and CXCR4 (NP2/CD4/X4) or CD4 and CCR5 (NP2/CD4/R5) were kindly provided by Dr. H. Hoshino.[33] NP2 cells expressing CD4 and C-terminally HA-tagged CXCR4 (NP2/CD4/X4-HA) were constructed as previously reported.[34] TE671, HeLa, and 293T cells expressing CD4 (TE671/CD4, HeLa/CD4, and 293T/CD4) were constructed with a CD4-encoding murine leukemia virus (MLV) vector as previously reported.[35] MAGIC5 cells, which are derived from HeLa cells, express CD4 and CCR5 and contain the β-galactosidase (β-Gal) gene under control of the HIV-1 long terminal repeat.[36]

### Expression plasmids

CXCR4-tropic HXB2 and CCR5-tropic JRFL HIV-1 Env expression plasmids were kindly provided by Dr. Y. Yokomaku (National Hospital Organization Nagoya Medical Center). A VSV-G expression plasmid and expression plasmids required for LacZ reporter gene-containing HIV-1 vector construction were obtained from Invitrogen. An expression plasmid encoding C-terminally HA-tagged CXCR4 was constructed as already reported.[34]

### Transduction assay

To obtain HIV-1 vector particles, COS7 cells were transfected with the HIV-1 vector construction plasmids using Fugene transfection reagent (Roche). The transfected cells were washed with D-MEM medium 24 h after transfection and maintained in fresh medium for 24 h. Target cells were either left untreated or pretreated with the retinoid analogs 4-HPR (Sigma-Aldrich), GGA, or NIK-333 for 2 days or with CT-B (Sigma-Aldrich) or stigmasterol (Sigma-Aldrich) for 1 day. GGA and NIK-333 were synthesized by Kowa Company, Ltd. (Tokyo, Japan). The cells were inoculated with culture supernatants from the transfected COS7 cells and then stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) (Nacalai) 2 days after inoculation. Blue cells were counted to estimate transduction titer. Approximately $10^4$, $10^4$, and $10^6$ infected cells were detected among cells inoculated by the HXB2 Env-, JRFL Env-, and VSV-G-containing vectors, respectively. To normalize transduction titers, the VSV-G vector was diluted 100 times with medium.

### Flow cytometry

To analyze cell surface CD4 expression, suspended cells were either left untreated or treated with an anti-CD4 antibody conjugated with FITC (Sigma-Aldrich). Cell surface expression of CXCR4 or CCR5 was analyzed in suspended cells treated with rat anti-CXCR4 (A80) or anti-CCR5 (T312) monoclonal antibody.[37] As a control, cells were treated with a rat serum. The cells were then washed three times with phosphate-buffered saline (PBS) and treated with an FITC-conjugated anti-rat IgG antibody (Sigma-Aldrich). The stained cells were quantified using a flow cytometer (BD Biosciences).

### *Western immunoblotting*

NP2/CD4/X4-HA cells were treated with the retinoid analogs, and cell lysates were prepared. The cell lysates were subjected to SDS polyacrylamide gel electrophoresis (Bio-Rad) and transferred onto a PVDF membrane (Millipore). The membrane was treated with a mouse anti-HA monoclonal antibody (Covance), and then with an HRP-conjugated anti-mouse IgG antibody (Bio-Rad).

### *Vector particle binding to target cells*

Target cells were incubated with culture supernatants from the HIV-1 vector-producing cells for 1 h at 4°C. The cells were washed three times with PBS, and cell lysates were prepared. HIV-1 Gag p24 levels were measured with a p24 enzyme-linked immunosorbent assay (ELISA) (ZeptoMetrix) to estimate the numbers of HIV-1 vector particles bound to the target cells.

### *Cell fusion assay*

The 293T cells were transfected with the HXB2 Env expression plasmid, which also encodes the Tat protein. As a control, 293T cells were transfected with a Tat expression plasmid. The transfected cells were cultured with MAGIC5 cells 24 h after transfection, and cell lysates were prepared from the cells 24 h after the mixed culture. Upon cell fusion, the Tat protein induced $\beta$-Gal expression. $\beta$-Gal activity in the cell lysates was measured to estimate cell fusion capability.

### *Statistical analysis*

Differences between two groups were determined by the Student's *t*-test. The difference was considered statistically significant if the *p*-value was $<0.05$ for all tests.

## Results

### *Acyclic retinoid analogs and CT-B inhibit CXCR4-tropic HIV-1 vector infection*

To assess whether retinoid analogs inhibit HIV-1 vector infection, target cells were pretreated with 4-HPR, GGA, or NIK-333 for 2 days. The chemical structures of the analogs are shown in Fig. 1. NP2 cells expressing CD4 and CXCR4 (NP2/CD4/X4), NP2 cells expressing CD4 and CCR5 (NP2/CD4/R5),[33] and HeLa cells expressing CD4 (HeLa/CD4)[35] were used as target cells. All of the retinoid analogs inhibited infection by a CXCR4-tropic HXB2 Env-carrying HIV-1 vector (Fig. 2A). Previous reports indicated that 4-HPR inhibits HIV-1 infection,[23] and this result is consistent with our findings. In addition, cell viability was not affected by the analog treatment under these conditions. These results indicate that the acyclic retinoid analogs GGA and NIK-333 as well as 4-HPR inhibit CXCR4-tropic HIV-1 infection.

VSV-G-mediated infection is independent of lipid rafts,[4,6] so we assessed whether VSV-G-pseudotyped HIV-1 vector infection is also attenuated by the retinoid analogs. VSV-G-pseudotyped HIV-1 vector infection was not significantly affected by the retinoid analogs (Fig. 2B). Similarly, infection by HIV-1 vector pseudotyped with the Env protein of the CCR5-tropic JRFL strain was not inhibited by the retinoid analogs (Fig. 2C). These results indicate that the retinoid analogs specifically suppress CXCR4-tropic HIV-1 Env-mediated infection but not VSV-G- and CCR5-tropic HIV-1 Env-mediated infection and that the retinoid analogs inhibit CXCR4-tropic HIV-1 infection by a mechanism other than a cytopathic effect.

We next assessed whether CT-B inhibits HIV-1 vector infection. CD4-expressing TE671 (TE671/CD4), HeLa/CD4, NP2/CD4/X4, and NP2/CD4/R5 cells were pretreated with CT-B for 24 h and then inoculated with HXB2 Env- or JRFL Env-bearing HIV-1 vector in the absence of CT-B. CT-B significantly attenuated CXCR4-tropic Env-mediated infection but not VSV-G-pseudotyped HIV-1 vector infection in TE671/CD4 (Fig. 3A), HeLa/CD4 (Fig. 3B), and NP2/CD4/X4 cells (Fig. 3C). However, CT-B did not inhibit CCR5-tropic Env-mediated infection in NP2/CD4/R5 cells (Fig. 3C). If CT-B inhibited cell growth, this toxin should also suppress VSV or CCR5-tropic vector infection; however, CT-B did not affect cell growth as analyzed by microscopic observation. These results indicate that CT-B specifically suppresses CXCR4-tropic HIV-1 infection by a mechanism other than cell growth inhibition.

Additionally, we assessed whether a plant sterol, stigmasterol, inhibits HIV-1 vector infection. The target cells were pretreated with stigmasterol (80 $\mu$g/ml) for 24 h. The transduction efficiency of the HIV-1 vector was not affected by the treatment (data not shown).

### *Retinoid analogs inhibit CXCR4 cell surface expression*

As the acyclic retinoid analogs inhibited CXCR4-tropic HIV-1 vector infection, we next assessed whether these retinoid analogs suppressed cell surface expression of the HIV-1 infection receptors, CD4, CXCR4, and CCR5. 4-HPR did not affect CD4 cell surface expression in HeLa/CD4 cells (Fig. 4A). GGA and NIK-333 treatment elevated CD4 expression, though the acyclic retinoid analogs inhibited CXCR4-tropic HIV-1 vector infection. In contrast, all of these retinoid analogs reduced cell surface CXCR4 expression. Similar results were observed in NP2/CD4/X4 cells, in which CXCR4 is artificially expressed (data not shown). Furthermore, these retinoid analogs did not affect CCR5 expression (Fig. 4B). These results suggest that the retinoid analogs inhibit CXCR4-tropic HIV-1 infection by suppressing CXCR4 cell surface expression.

When NP2 cells expressing C-terminally HA-tagged CXCR4 were treated with the retinoid analogs, expression levels of the HA-tagged CXCR4 were not altered, analyzed by Western immunoblotting using an anti-HA antibody (Fig. 4C). This result suggests that the retinoid analogs inhibit the trafficking of CXCR4 to the cell surface, but do not inhibit CXCR4 expression.

CT-B also inhibited CXCR4-tropic HIV-1 vector infection but not CCR5-tropic HIV-1 vector infection; however, CT-B did not affect cell surface expression of CCR5 (Fig. 4B), CXCR4, or CD4 (Fig. 4C). These results indicate that CT-B inhibits CXCR4-tropic infection by a mechanism other than suppression of CXCR4 expression.

### *Retinoid analogs and CT-B do not affect HIV-1 particle binding to host cells*

We analyzed the effects of the retinoid analogs and CT-B on CXCR4-tropic HIV-1 vector particle binding to the target cells by p24 ELISA. The amount of p24 protein

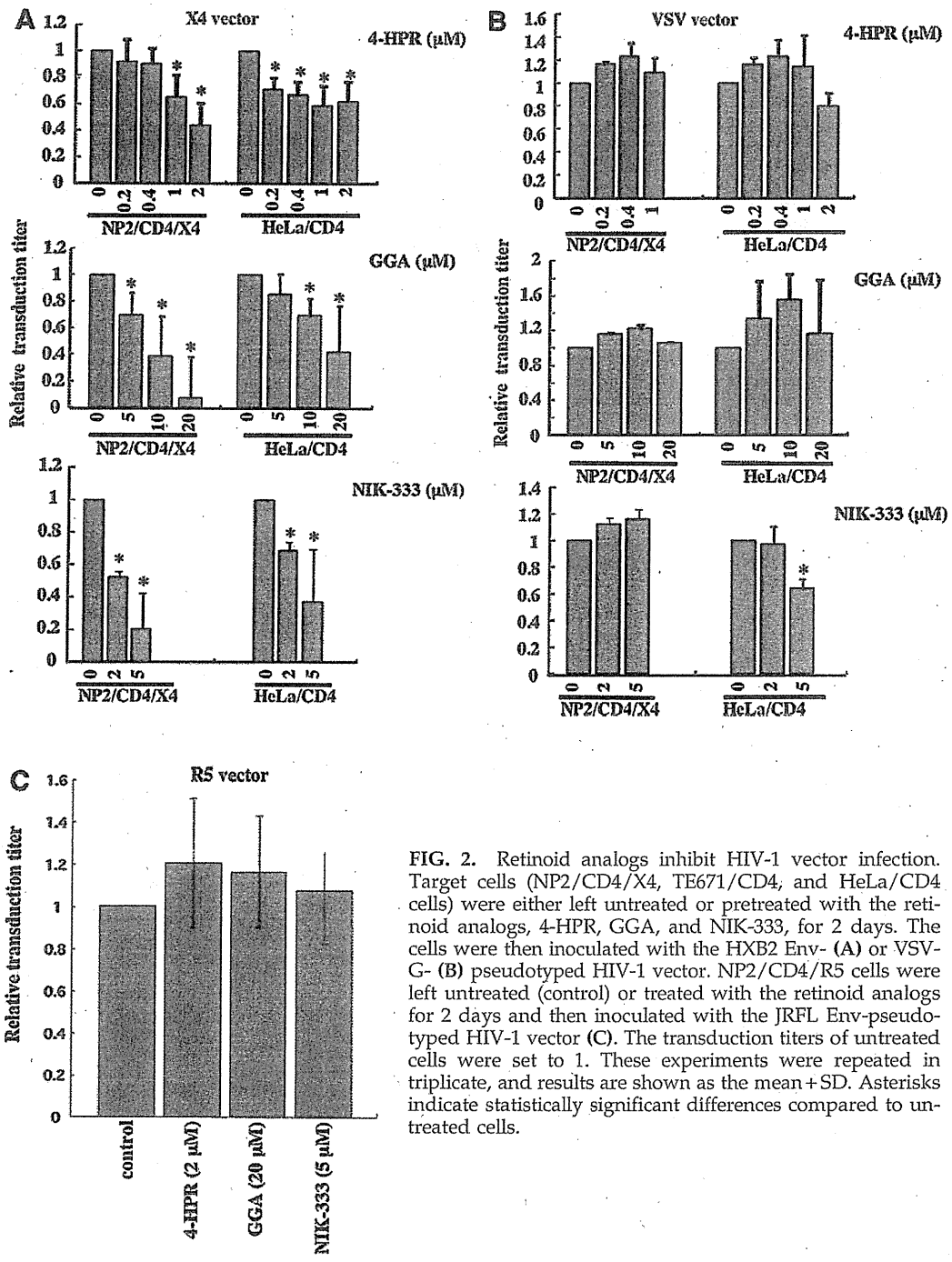


FIG. 2. Retinoid analogs inhibit HIV-1 vector infection. Target cells (NP2/CD4/X4, TE671/CD4, and HeLa/CD4 cells) were either left untreated or pretreated with the retinoid analogs, 4-HPR, GGA, and NIK-333, for 2 days. The cells were then inoculated with the HXB2 Env- (A) or VSV-G- (B) pseudotyped HIV-1 vector. NP2/CD4/R5 cells were left untreated (control) or treated with the retinoid analogs for 2 days and then inoculated with the JRFL Env-pseudotyped HIV-1 vector (C). The transduction titers of untreated cells were set to 1. These experiments were repeated in triplicate, and results are shown as the mean + SD. Asterisks indicate statistically significant differences compared to untreated cells.

bound to CD4-expressing HeLa cells was higher than that bound to CD4-negative HeLa cells, indicating that vector particle binding is CD4-dependent (Fig. 5A). None of the retinoid analogs (Fig. 5B) or CT-B (Fig. 5C) affected HIV-1 vector particle binding to the CD4-expressing target cells. These results show that the retinoid analogs and CT-B inhibit CXCR4-tropic HIV-1 infection by a mechanism other than suppression of CD4-dependent virion binding to target cells.

### *Retinoid analogs and CT-B inhibit membrane fusion activity of HIV-1 Env protein*

To assess whether the retinoid analogs or CT-B inhibit HIV-1 Env-mediated membrane fusion activity, we analyzed the effects of these agents on HIV-1 Env-induced syncytium formation. HEK293T cells transfected with the plasmid encoding the HIV-1 HXB2 Env and Tat proteins were cocultured with MAGIC5 cells for 24 h, and β-galactosidase activity was

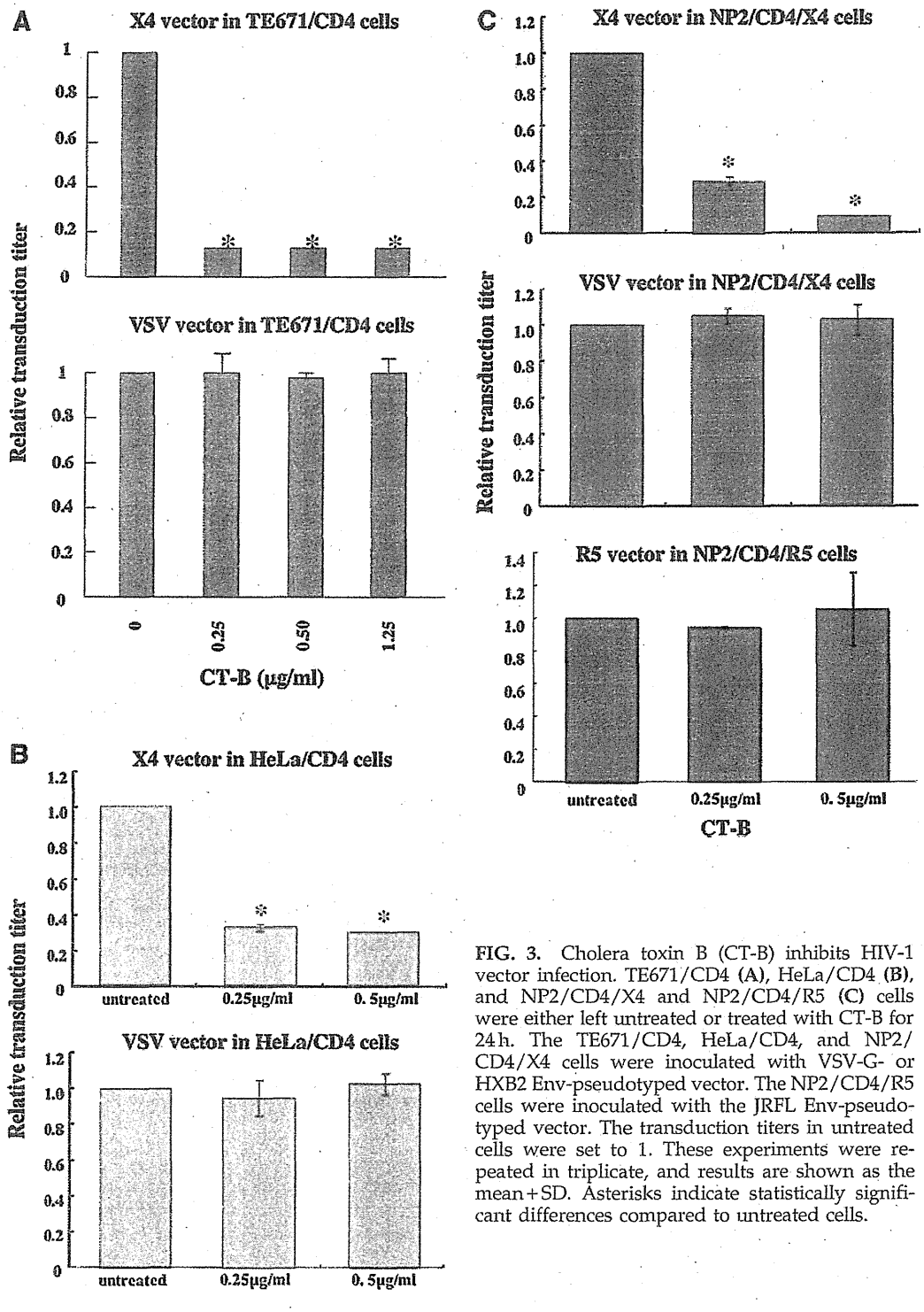


FIG. 3. Cholera toxin B (CT-B) inhibits HIV-1 vector infection. TE671/CD4 (A), HeLa/CD4 (B), and NP2/CD4/X4 and NP2/CD4/R5 (C) cells were either left untreated or treated with CT-B for 24 h. The TE671/CD4, HeLa/CD4, and NP2/CD4/X4 cells were inoculated with VSV-G- or HXB2 Env-pseudotyped vector. The NP2/CD4/R5 cells were inoculated with the JRFL Env-pseudotyped vector. The transduction titers in untreated cells were set to 1. These experiments were repeated in triplicate, and results are shown as the mean+SD. Asterisks indicate statistically significant differences compared to untreated cells.

measured in the cell lysates. The retinoid analogs (Fig. 6A) and CT-B (Fig. 6B) suppressed syncytium formation. Direct inhibition of the HIV-1 Env-mediated membrane fusion reaction by these factors would suppress both CXCR4- and CCR5-tropic HIV-1 infections; however, the factors did not affect CCR5-tropic HIV-1 infection (Fig. 2C). Taken together, these results support the hypothesis that retinoid analogs inhibit CXCR4-tropic HIV-1 infection by attenuating CXCR4 expression, although CT-B may affect the HIV-1 entry process between vector particle binding to target cells and membrane fusion.

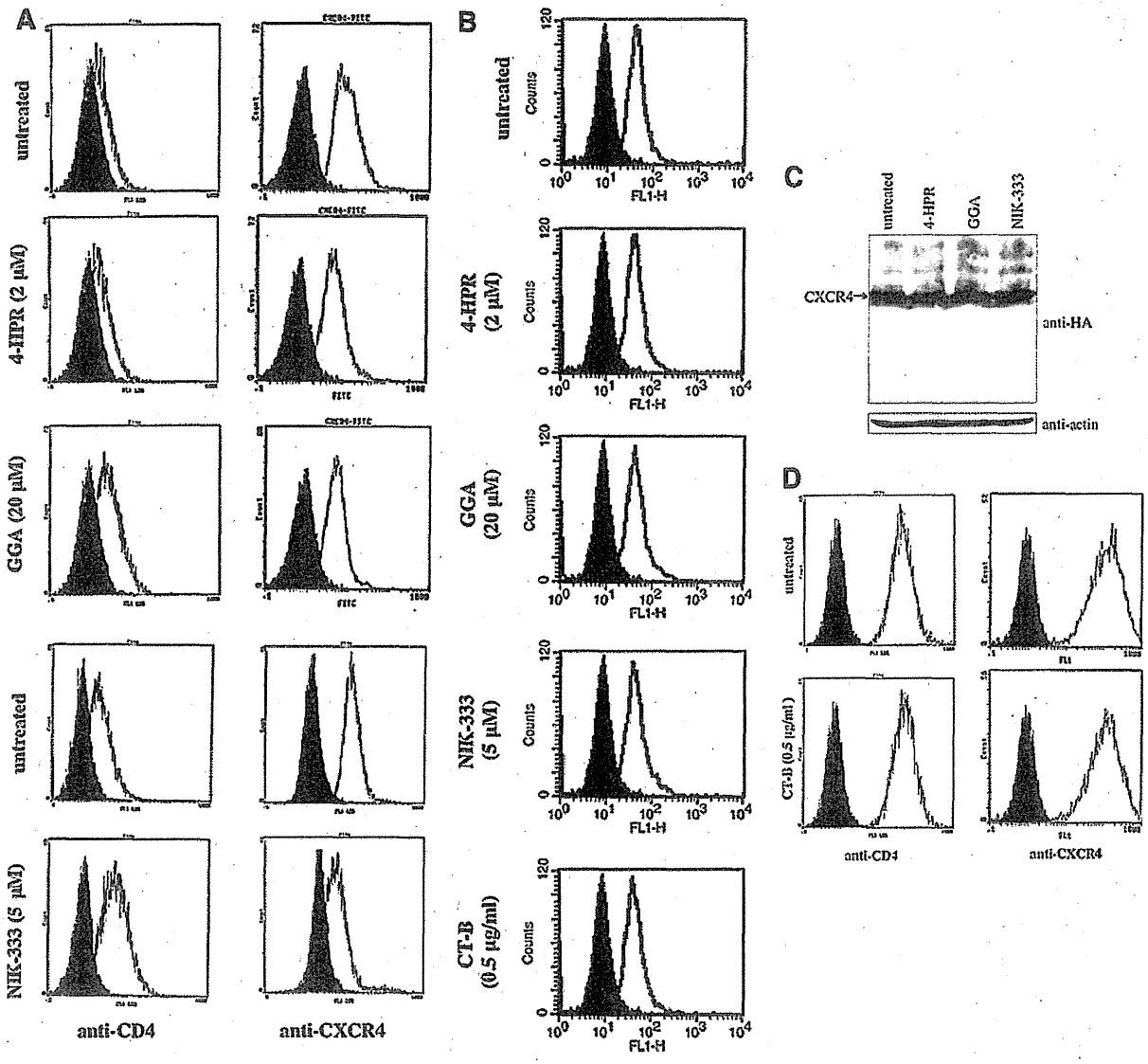


FIG. 4. Retinoid analogs inhibit cell surface expression of CXCR4. Cell surface expression of CD4 and CXCR4 in retinoid analog-treated HeLa/CD4 cells **(A)**, CCR5 in retinoid analog- or CT-B-treated NP2/CD4/R5 cells **(B)**, and CD4 and CXCR4 in CT-B-treated TE671/CD4 cells **(D)** were analyzed by flow cytometry. Closed and open areas indicate untreated cells stained with control serum or with anti-CD4, -CXCR4, or -CCR5 antibody, respectively. Representative results are shown. Expression of C-terminally HA-tagged CXCR4 was analyzed by Western immunoblotting using an anti-HA antibody **(C)**. As a control, actin expression was also analyzed.

## Discussion

HAART has dramatically reduced the mortality and morbidity of HIV-1-infected patients in developed countries. However, due to the high cost of HAART, this therapy is limited in developing countries. In addition, HIV-1 variants that are resistant to HAART have emerged. Therefore, development of novel low-cost drugs that inhibit HIV-1 replication is essential.

In this study, we found that the acyclic retinoid analogs, GGA and NIK-333, suppress CXCR4-tropic HIV-1 vector infection similarly to 4-HPR.[23] Additionally, retinoids repress expression of the HIV-1 promoter,[38–40] suggesting that retinoid analogs are possible candidates for a novel anti-HIV-1 therapy. Many reports indicate that vitamin A (retinol) supplementation reduces the mortality of HIV-1-infected patients.[41–44] NIK333, a synthetic acyclic retinoid, is orally effective against liver cancer without severe side effects.[28] GGA also suppresses HIV-1 vector infection and is present in medicinal herbs. These results suggest that oral intake of a natural acyclic retinoid analog may be novel low-cost therapy against AIDS.

We also found that CT-B efficiently suppresses CXCR4-tropic HIV-1 vector infection. Gauthier and Tremblay have shown that CT-B does not inhibit HIV-1 infection, although the concentration of CT-B (10 ng/ml) used in their study was

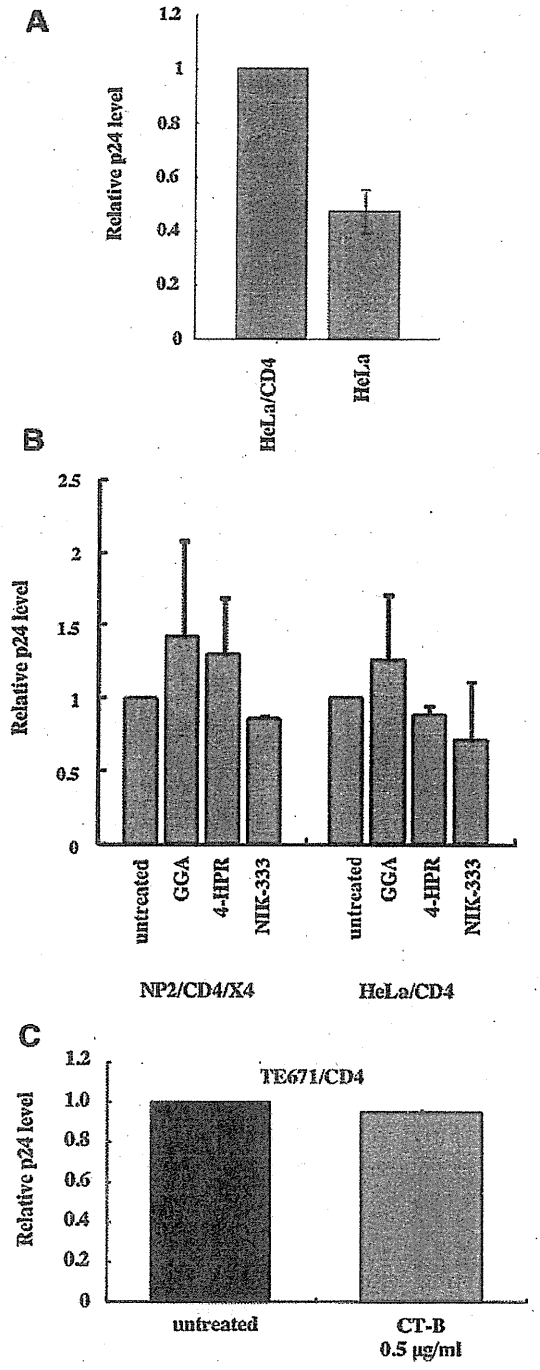


FIG. 5. Retinoid analogs and CT-B do not affect HIV-1 vector particle binding to target cells. HeLa/CD4 and HeLa cells were incubated with the HXB2 Env-containing HIV-1 vector particles at 4°C for 1 h and then washed with phosphate buffered saline (PBS) **(A)**. HIV-1 particles bound to the target cells were measured by p24 ELISA. The p24 levels in untreated HeLa/CD4 cells were set to 1. HIV-1 vector particles bound to the retinoid analog-treated NP2/CD4/X4 or HeLa/CD4 cells **(B)** or to CT-B-treated TE671/CD4 cells **(C)** were measured. The p24 levels in untreated cells were set to 1. These experiments were repeated in triplicate, and results are shown as the mean+SD.

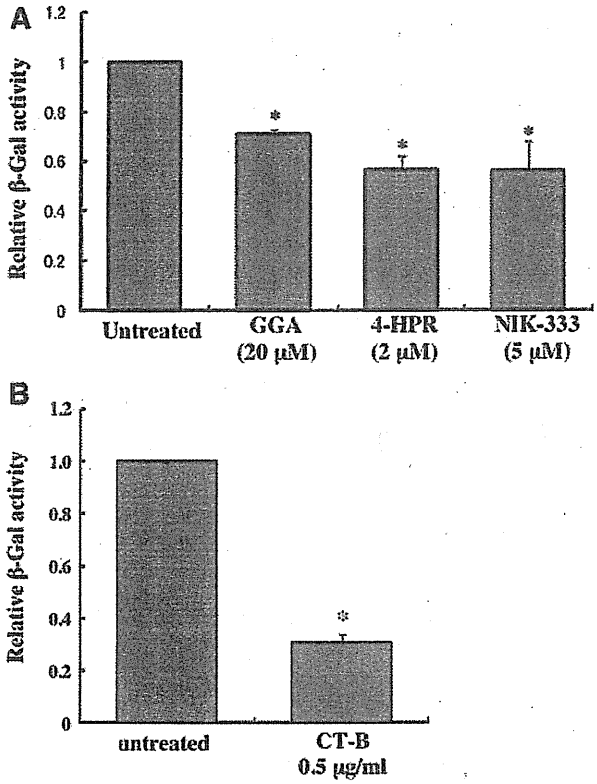


FIG. 6. Retinoid analogs and CT-B inhibit CXCR4-tropic HIV-1 Env-induced syncytium formation. Cell fusion activity of the HXB2 HIV-1 Env protein was measured in untreated and retinoid analog- **(A)** or CT-B-treated **(B)** cells (see Materials and Methods). The β-Gal activities in untreated cells were set to 1. These experiments were repeated in triplicate, and results are shown as the mean+SD. Asterisks indicate statistically significant differences compared to untreated cells.

too low to inhibit HIV-1 infection.[31] Similar to our results with CT-B, pertussis toxin B subunit also inhibits HIV-1 infection.[45–47] Although the receptor for pertussis toxin B oligomer has not yet been identified, the receptor appears to belong to a class of sialylated glycoproteins, with likely candidates being a 43-kDa protein[48] and CD11b/CD18 integrin.[49] Because the CT-B receptor GM1 is not the pertussis toxin B subunit receptor, the mechanisms by which these bacterial toxin B subunits inhibit HIV-1 infection appear to be different.

One route of HIV-1 transmission is through anal sex. As such, if gut bacteria that secrete nontoxic CT-B are present in the rectum, HIV-1 transmission through this route may be suppressed. Gut bacteria genetically engineered to express CT-B may be a useful novel low-cost strategy to prevent HIV-1 transmission through anal sex.

Use of these factors *in vivo*, however, should be approached cautiously. First, our study suggests that the acyclic retinoid analogs modulate cell surface expression of CD4 and CXCR4. Second, CT-B is used as an adjuvant for vaccination.[50,51] Therefore, these agents may induce unexpected effects *in vivo* via activation or perturbation of the human immune system. Further study is required to address this issue.

Other retinoid analogs have been reported to reduce cell surface expression of CXCR4,[52,53] similar to the retinoid analogs

used in this study. This down-regulation of CXCR4 expression is one of the mechanisms by which retinoid analogs inhibit CXCR4-tropic HIV-1 infection. HIV-1 infection is suppressed and influenza virus infection is elevated by 4-HPR through activation of endocytosis[23] without suppression of CXCR4 expression. In this study, VSV-G-mediated infection, which occurs via the endosomes, was not affected by the retinoid analogs. Further study is needed to understand the mechanism of HIV-1 infection inhibition by the retinoid analogs.

The retinoid analogs inhibited CXCR4 expression, while CT-B did not, suggesting that the mechanism of HIV-1 infection inhibition by CT-B differs from that by the retinoid analogs. Interestingly, CT-B inhibited CXCR4-tropic HIV-1 infection but not CCR5-tropic infection. Thus, CT-B may inhibit CXCR4-tropic HIV-1 entry at some point between virion binding to host cells and membrane fusion. CD4 and CCR5, but not CXCR4,[4–6] localize to lipid raft microdomains and constitutively interact.[54,55] It has been reported that CCR5-tropic HIV-1 infection is not dependent upon raft localization of CD4 and CCR5.[56] These results, together with our findings, suggest that CT-B inhibits the HIV-1 Env-induced interaction of CD4 and CXCR4 in lipid rafts and that raft microdomains are differentially involved in CXCR4- and CCR5-tropic HIV-1 infections. CT-B may have no effect on CCR5-tropic HIV-1 infection, because CD4 and CCR5 constitutively interact without binding HIV-1 Env. Recruitment of CXCR4 to CD4-containing raft microdomains by HIV-1 Env, however, has been observed in studies using CT-B as the raft marker.[5,6] Further study is required to understand the mechanism by which CT-B inhibits HIV-1 infection.

The plant sterol stigmasterol did not suppress HIV-1 vector infection. Our group previously reported that MβCD inhibits HIV-1 vector infection and that the addition of cholesterol to the MβCD-treated cells at 50 μg/ml for 30 min recovers infection, suggesting that cholesterol is incorporated into the cell membrane by the addition of cholesterol.[4] Therefore, treatment of cells with stigmasterol at 80 μg/ml for 24 h likely induces uptake of the plant sterol to the cell membrane. These results indicate that this plant sterol does not affect HIV-1 infection. Similar to mammalian cells, plant cells also have lipid raft microdomains in their membranes,[57] and these raft domains are enriched with plant sterols. Therefore, even upon replacement of cholesterol with stigmasterol in mammalian cells, the lipid raft structure is maintained, and HIV-1 infection remains unaffected.

In summary, the acyclic retinoid analogs, GGA and NIK-333, as well as CT-B, efficiently suppress HIV-1 vector infection. Another retinoid analog, 4-HPR, inhibits HIV-1 infection[23] but induces a vitamin A-deficiency syndrome. In contrast, NIK-333 induces no clinical side effects in patients with liver cancer.[28,29] This study suggests that NIK-333 can be used as a novel anti-HIV-1 agent without severe side effects. Additionally, CT-B inhibits CXCR4-tropic, but not CCR5-tropic.

HIV-1 infection, suggesting that host cell lipid raft microdomains are differentially involved in CXCR4- and CCR5-tropic HIV-1 infections.

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## Author Disclosure Statement

No competing financial interests exist.

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*Review Article*

# Retrovirus Entry by Endocytosis and Cathepsin Proteases

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Retroviruses include infectious agents inducing severe diseases in humans and animals. In addition, retroviruses are widely used as tools to transfer genes of interest to target cells. Understanding the entry mechanism of retroviruses contributes to developments of novel therapeutic approaches against retrovirus-induced diseases and efficient exploitation of retroviral vectors. Entry of enveloped viruses into host cell cytoplasm is achieved by fusion between the viral envelope and host cell membranes at either the cell surface or intracellular vesicles. Many animal retroviruses enter host cells through endosomes and require endosome acidification. Ecotropic murine leukemia virus entry requires cathepsin proteases activated by the endosome acidification. CD4-dependent human immunodeficiency virus (HIV) infection is thought to occur via endosomes, but endosome acidification is not necessary for the entry whereas entry of CD4-independent HIVs, which are thought to be prototypes of CD4-dependent viruses, is low pH dependent. There are several controversial results on the retroviral entry pathways. Because endocytosis and endosome acidification are complicatedly controlled by cellular mechanisms, the retrovirus entry pathways may be different in different cell lines.

## 1. Introduction

Retroviruses include many pathogenic agents in humans and animals. Human immunodeficiency virus (HIV) and human T-cell leukemia virus (HTLV) induce acquired immunodeficiency syndrome (AIDS) and adult T-cell leukemia (ATL), respectively. Murine leukemia viruses (MLVs) are also well-studied among retroviruses because the MLVs are used comparatively as animal models of several human diseases (leukemia, immunodeficiency, and neuropathogenic diseases) and as gene transfer tools. In addition, there are animal retroviruses that are important problems in the livestock industry, such as Visna, equine infectious anemia virus, bovine leukemia virus, and Jaagsiekte sheep retrovirus.

Retroviruses contain envelope membranes consisting of lipid bilayers derived from virus-producing cells. Genomes of simple retroviruses such as MLVs encode three essential elements, gag, pol, and env genes. Complex retroviruses including HIV additionally encode accessory genes whose products regulate the retroviral expression and suppress host antivirus factors [1]. The gag and pol genes encode viral structural proteins and enzymes, respectively. These proteins are synthesized as precursor polyproteins and then are cleaved to mature peptides by a protease encoded by the retroviral pol gene.

Retroviral envelope (Env) glycoprotein encoded by the env gene is also synthesized as a precursor protein and is cleaved to surface (SU) and transmembrane (TM) subunits by a cellular protease [2]. Retroviruses enter host cells by fusion between viral envelope and host cell membrane, following the recognition of cognate cell surface receptors. The SU protein binds to the cell surface receptor protein. The TM protein anchors the SU protein to the surface of viral particles and virus-producing cells by the complex formation of SU and TM. The TM protein mediates the membrane fusion reaction. The entry mechanisms of retroviruses are vigorously studied but are not completely understood. Elucidation of the retrovirus entry machinery