Simian Sarcoma-Associated Virus Fails To Infect Chinese Hamster Cells despite the Presence of Functional Gibbon Ape Leukemia Virus Receptors

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We have sequenced the envelope genes from each of the five members of the gibbon ape leukemia virus (GALV) family of type C retroviruses. Four of the GALVs, including GALV strain SEATO (GALV-S), were originally isolated from gibbon apes, whereas the fifth member of this family, simian sarcoma-associated virus (SSAV), was isolated from a woolly monkey and shares 78% amino acid identity with GALV-S. To determine whether these viruses have identical host ranges, we evaluated the susceptibility of several cell lines to either GALV-S or SSAV infection. GALV-S and SSAV have the same host range with the exception of Chinese hamster lung E36 cells, which are susceptible to GALV-S but not SSAV. We used retroviral vectors that differ only in their envelope composition (e.g., they contain either SSAV or GALV-S envelope protein) to show that the envelope of SSAV restricts entry into E36 cells. Although unable to infect E36 cells, SSAV infects GALV-resistant murine cells expressing the E36-derived viral receptor, HaPit2. These results suggest that the receptors present on E36 cells function for SSAV. We have constructed several vectors containing GALV-S/SSAV chimeric envelope proteins to map the region of the SSAV envelope that blocks infection of E36 cells. Vectors bearing chimeric envelopes comprised of the N-terminal region of the SSAV SU protein and the C-terminal region of SSAV infect E36 cells, whereas vectors containing the N-terminal portion of the SSAV SU protein and C-terminal portion of GALV fail to infect E36 cells. This finding indicates that the region of the SSAV envelope protein responsible for restricting SSAV infection of E36 cells lies within its amino-terminal region.

The first step in retroviral infection requires binding of the viral envelope protein to a cell surface receptor (29). Binding is followed by fusion of the viral envelope with the plasma membrane, delivering the viral nucleocapsid to the cell cytosol. Blocks to either of these steps will render a cell resistant to viral infection. Although the presence of an appropriate receptor is the predominant requirement for cellular susceptibility to retroviral infection, other cellular factors accessory to the viral receptor play a role. For example, murine cells which express CD4, the receptor for human immunodeficiency virus type 1 (HIV-1), remain resistant to infection by HIV (16, 20). HIV-1 binds to all cells that express CD4, but a second factor is required for entry. Several chemokine receptors have been found to function as entry cofactors (2), which are required for HIV-1 to undergo fusion. CXCR4 is a coreceptor for T-cell-tropic HIV-1 (19), and CCR5 is a coreceptor for macrophage-tropic HIV-1 (28, 35).

The gibbon ape leukemia virus (GALV) family comprises four strains of exogenous type C retroviruses isolated from nonhuman primates in various states of disease—SEATO (S), SF, Brain (Br), and Hall’s Island (H)—as well as simian sarcoma-associated virus (SSAV) (32). All members of the GALV family use the same receptor, Pit1, a multimembrane-spanning class III phosphate transporter (18, 22), to infect human cells (27, 32). The in vitro host range of GALV is similar to that of xenotropic murine leukemia viruses (MuLVs) in that GALV can infect most mammalian (e.g., human, bat, rat, and cow) cells, while murine and hamster (CHO) cells are resistant to GALV-S infection (10, 32). Murine cells are resistant to GALV because they lack functional GALV receptors (21). The cellular components which restrict GALV infection of hamster cells have not been determined. E36 cells, derived from Chinese hamster lung tissue, differ from other hamster cells in their ability to be readily infected by GALV-S. Because of this unusual property of E36 cells, we used them to evaluate whether any host range differences among the GALV isolates could be detected. We found that SSAV differs from the other GALV strains; it is unable to infect E36 cells.

We sought to resolve the molecular mechanism underlying the block to SSAV infection of E36 cells. Our results show that SSAV cannot infect E36 cells, despite the presence of functional GALV receptors, and this appears to be due to inherent differences between the SSAV and GALV-S envelope proteins. GALV-S and SSAV chimeric envelope studies have enabled us to map the region of the SSAV envelope responsible for the block to infection of E36 cells. The N-terminal region of SSAV envelope including variable regions A and B (VRA and VRB) restricts SSAV infection of E36 cells at the entry stage.

MATERIALS AND METHODS

Cells and viruses. The following cell lines were used in this study: NIH 3T3 murine fibroblasts (ATCC CRL 1658), Mus dunni tail fibroblasts (MDTF) (ob-
tain from Olivier Danos, Institut Pasteur, France; also available as ATCC
CRL 1764), M. musculus molossinus MMK cells (ATCC CRL 6439), mink lung fibroblast cells (ATCC CL64), and E36 Chinese hamster lung cells (provided by Christine Kozak, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, Md.). 293T cells were
obtained from Cell Genesys Inc., Foster City, Calif. All cells were maintained in Dulbecco’s modified essential medium (DMEM; Whittaker Bioproducts, Inc., Walkersville, Md.) supplemented with 5% fetal bovine serum, 100 U of penicillin and 100 μg of streptomycin per ml, and 4 mM glutamine. Wild-type GALV strains Br, H, and SF were obtained from the supernatant of mink cells, and SSAV was obtained from marmoset 71-AP-1 cells.

**Production of retroviral vectors.** 293T cells were seeded at a density of 10^6 cells/10-cm-diameter dish 2 days before transfection. The following three plasmids were transfected into 293T cells by the calcium phosphate precipitation method (Promega): (i) 10 μg of pF2F-US gag-pol (from a Moloney MuLV)-based packageable genome containing the packaging signal and the β-galactosidase gene coding sequence; (ii) 2.5 μg of MoMuLV gag-pol-expressing plasmid (12); and (iii) 5 μg of pCI-neo (Promega) plasmid with GALV-S or SSAV envelope coding region. GALV-SS or SSAV enveloped retroviral vectors were harvested from supernatant of transfected 293T cells 60 to 72 h after transfection.

**Viral infections and vector transduction.** E36, Rat2, MMK, and NIH 3T3 target cells were seeded at a density of 3.0 × 10^4 cells/well in a 12-well plate 1 day prior to virus infection. Cell medium from virus producer cells (marmoset 71-AP-1 cells) was passed through a 0.45-μm-pore-size filter and adjusted to a final Polybrene concentration of 10 μg/ml. Target cells were exposed to the harvested supernatant for 24 h, trypsinized, and reseded at 1/10 density. Supernatant of cells exposed to wild-type virus was analyzed for reverse transcriptase activity by measuring the counts per minute of incorporated [3H]TTP 8 days after exposure to virus (34). For GALV-S and SSAV enveloped retroviral vectors, 1.5 × 10^4 target cells/well were seeded in a 24-well plate 1 day prior to exposure to retrovector-vector-containing supernatant. After 48 to 72 h, cells were analyzed for expression of β-galactosidase by histochemical staining with 5-bromo-4-chloro-3-indolyβ-D-galactopanoside (X-Gal) as described previously (31).

**Isolation and hybridization of unintegrated viral DNA.** E36 and Rat2 target cells were seeded at a density of 5 × 10^5 target cells 1 day prior to infection. The next day, cells were exposed to wild-type virus and incubated for 24 h. Unintegrated, low-molecular-weight DNA was isolated from the infected cells by the method of Hirt (17). The extrachromosomal Hirt DNA preparation was digested with a restriction enzyme (EcoRI or Xhol) and run on a 0.8% agarose gel. The DNA was transferred to a nitrocellulose membrane and prehybridized for 2 h at 65°C in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1 mg of salmon sperm DNA per ml and then hybridized in the same solution containing the labeled probe (2 × 10^6 cpm/ml) overnight. The nick-translated 32P-labeled BamHI-PstI DNA fragment from the MOG-GAS env plasmid (30), encoding the GALV-S envelope protein, was used as the probe. The hybridized membrane was washed in 2× SSC twice at room temperature and then twice at 65°C. The autoradiogram was developed after exposure of the hybridized membrane to Kodak X-AR-2 film with two screens at ~70°C.

**Cloning and sequencing of envelope glycoproteins.** Virus was harvested from mink lung fibroblasts infected with GALV-Br or GALV-H, and mRNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase to obtain the first-strand DNA. The cDNA was amplified by a FastTrack 2.0 kit (Invitrogen). cDNA was prepared in a reverse transcription reaction using avian myeloblastosis virus reverse transcriptase (Promega) and random hexamer primers. Several overlapping fragments were produced by PCR using primers based on the nucleotide sequence of GALV-S envelope (6). Products were subcloned into the TA vector pCRH (Invitrogen) and sequenced by dideoxy sequencing, using GALV-S-based primers. A consensus sequence was obtained, and the 5′ end was sequenced by using a sense primer derived from the pol region of GALV-S and an antisense primer derived from a previously sequenced region. 64 nucleotides downstream of the putative envelope start codon. For the 3′ end, we designed a sense primer based on a sequenced region of GALV-H and an antisense primer based on the nucleotide sequence from the U3 region of GALV-S, bases 7772 to 7749. pvG-3 (26) was used as a template to sequence the GALV-S envelope. Plasmid pGAS-2 (14) was used as a sequence template for GALV-S, and SSAV envelope was sequenced from the pb11 clone (13), by sequencing version 2.0 (U.S. Biochemicals, Cleveland, Ohio).

**Construction of GALV-S and SSAV envelope chimeras.** Chimeric SSAV envelope constructs containing both GALV-S VRA and VRB were made by exchanging the region between XhoI and XmaI sites of GALV-S with the corresponding region of GALV-H. The same strategies were used to construct chimeric GALV-S envelopes containing the SSAV VRA and VRB.

**Nucleotide sequence accession numbers.** The GenBank accession numbers for the envelope sequences of the five members of the GALV family are as follows: GALV-S, AF055060; GALV-H, AF055061; GALV-Br, AF010502; GALV-SF, AF010503; and SSAV, AF010504.**

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### RESULTS

E36 cells are resistant to infection by SSAV but not GALV-S. We have previously reported that the four GALV strains and SSAV can use Pit1 as a receptor (27, 32). To determine whether there are any differences in cell tropism between these viruses, we evaluated the susceptibility of four cell lines to individual strains of GALV and monitored the viral infection by measuring the reverse transcriptase activity from culture supernatant (Table 1). All four strains of GALV and SSAV efficiently infected Rat2 cells (positive control) and failed to infect NIH 3T3 cells (negative control). We examined the susceptibility to GALV's infection of MMK and E36 cells because of their unusual receptor properties previously reported (32, 33). We found that MMK cells were permissive for infection of all GALV strains and SSAV, while E36 cells can be efficiently infected by all four GALV strains but not SSAV.

**SSAV DNA is not detected in extrachromosomal DNA from E36 cells exposed to SSAV.** To determine the stage at which SSAV infection of E36 cells was blocked, we first analyzed DNA purified from E36 cells exposed to SSAV for the presence of unintegrated viral DNA. Twenty-four hours after exposure to SSAV, extrachromosomal, low-molecular-weight DNA was isolated by the method of Hirt (17) and examined by Southern blot analysis (Fig. 1). DNA recovered from cells exposed to SSAV or GALV-S was analyzed by restriction enzyme digestion. EcoRI cleaves SSAV DNA at a single site within the pol coding region: Xhol cleaves GALV-S DNA at a single site within the gag coding region. A nick-translated DNA fragment corresponding to the expected size of linear viral DNA was detected in DNA isolated from both E36 and Rat2 fibroblasts exposed to GALV-S by hybridization to a nick-translated 32P-labeled GALV-S probe. The additional DNA fragments hybridizing to this probe are presumably derived from defective provirus or incompletely digested DNA fragments. DNA from Rat2 cells exposed to SSAV bound to the GALV probe, indicating the presence of extrachromosomal viral DNA in Rat2 cells. The observed absence of a hybridizing DNA fragment in E36 cells exposed to SSAV under identical assay conditions suggests that the block to SSAV infection of E36 cells occurs early in the viral infection-replication process, before reverse transcription of viral RNA into double-stranded DNA.

**SSAV infection of E36 cells is restricted at the level of virus envelope-receptor interaction.** To determine the viral component which is responsible for the inability of SSAV to infect E36 cells, we used retroviral vectors differing only in their envelope glycoproteins, either from GALV-S or SSAV. These retroviral vectors contain identical MoMuLV core proteins and a MoMuLV-based packageable genome containing β-ga-

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**TABLE 1. Host range of GALV strains and SSAV**

<table>
<thead>
<tr>
<th>Strain or virus</th>
<th>NIH 3T3</th>
<th>Rat2</th>
<th>MMK</th>
<th>E36</th>
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<tr>
<td>GALV strain</td>
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<tr>
<td>SSAV</td>
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* Determined by measuring [3H]TTP incorporation in a reverse transcriptase assay performed on cell supernatant 8 days after exposure to virus as described in Materials and Methods.
lactosidase gene coding sequence. Retroviral vectors bearing GALV-S envelope efficiently infected E36 cells, whereas those bearing the SSAV envelope did not (Table 2). These results demonstrate that the SSAV envelope is the viral component which restricts SSAV infection of E36 cells.

We have previously shown that E36 cells express two distinct cell surface proteins, HaPit1 and HaPit2, which both function as receptors for GALV-S infection (33). MDTF cells, normally resistant to SSAV, are susceptible to infection by SSAV-envoloped vectors when they express HaPit2 (Table 2). This finding demonstrates that E36 cells express receptors that are functional for SSAV infection, even though SSAV cannot infect these cells.

Envelope gene sequences from four GALV strains and SSAV. We sought to elucidate the molecular basis for the observed differences in the host range of SSAV by comparing the envelope sequences of the GALV family. The GALV-Br and GALV-H envelope genes were cloned (see Materials and Methods), and these as well as previously cloned GALV-S (14), GALV-SF (26), and SSAV (13) envelope genes were sequenced. Alignment of the amino acid sequences deduced from the nucleotide sequences of the four strains of GALV and SSAV is shown in Fig. 3. We found that the predicted transmembrane (TM) portion of the envelope of GALV-S is longer than previously reported (6), extending 18 amino acids at the carboxyl terminus. Both GALV-Br and GALV-H are highly related to GALV-S, showing 91 and 93% amino acid identity, respectively, whereas SSAV and SF show 78 and 81% identity, respectively. VRA and VRB of MuLVs have been defined and shown to influence receptor specificity (1). These regions were determined for GALV-S and SSAV by alignment of their deduced envelope sequences with sequences from MuLVs (Fig. 3).

The N-terminal half of the SSAV envelope restricts the ability of SSAV to infect E36 cells. SSAV and GALV-S envelope plasmids were used as templates to construct GALV-S/SSAV chimeric envelope cDNAs to map the region of the SSAV envelope which restricts infection of E36 cells. We exchanged the regions encoding the amino-terminal portion, including VRA and VRB, between GALV-S and SSAV envelope genes and tested the susceptibility of E36 cells to infection by retroviral vectors bearing these envelope chimeras (Fig. 2). E1 chimeric envelope was constructed by replacing the portion encoding the first 219 amino acids of the SSAV envelope gene with the corresponding GALV-S region; E2 was constructed in a similar manner, where the portion encoding the first 226 amino acids of GALV-S envelope was replaced with the corresponding region of SSAV. E36 cells are resistant to vectors containing the full-length SSAV envelope. However, vectors bearing the E1 chimeric envelope infected E36 cells, and the titer of these vectors in E36 cells was similar to that of vectors bearing the full-length GALV-S envelope. Conversely, the vectors bearing the E2 chimeric envelope proteins failed to infect E36 cells. These results demonstrate that vectors bearing the N-terminal portion of the SSAV envelope cannot infect E36 cells, in correlation with the inability of SSAV to infect E36 cells (Table 2).

### DISCUSSION

E36 cells can be infected by the four strains of GALV but not by the closely related retrovirus SSAV. Blocks to viral infection can occur at any one of several stages in the infection process: (i) binding of the virus to its receptor; (ii) fusion; (iii) after entry but before reverse transcription of the viral RNA into DNA; (iv) after reverse transcription but before integration of the double-stranded DNA intermediate; or (v) after integration (including viral transcription and/or viral assem-
bly). We show here that SSAV replication in E36 cells is blocked prior to reverse transcription of the viral RNA into double-stranded DNA, suggesting that the block occurs at an early stage of infection. The vectors with SSAV envelope are unable to infect E36 cells, suggesting that this block is envelope mediated. An E36-derived receptor, HaPit2, can function as a receptor for SSAV when expressed in murine cells, even though this receptor when expressed in E36 cells fails to facilitate SSAV entry. Together these data show that the block to SSAV infection of E36 cells occurs at the stage of entry.

There are several possible explanations for the ability of SSAV to infect MDTF cells expressing the E36 Pit 2 homolog, HaPit2, but not E36 cells. The block to SSAV infection of E36 cells may be due to cell-specific posttranslational modification of the receptor. For example, the HaPit2 protein, unlike the human homolog, has a consensus sequence for N-linked glycosylation present in the second extracellular domain (33). We have previously shown that differences in glycosylation can affect ecotropic MuLV (E-MuLV) receptor function in murine and hamster cells (11, 31). The E-MuLV receptor expressed in MDTF cells functions for all E-MuLVs except MoMuLV (11). Inhibition of N-linked glycosylation in these cells or site-specific mutagenesis of one of two potential N-linked glycosylation sites in the MDTF E-MuLV receptor renders the receptor functional for MoMuLV (11). However, pretreatment of E36 hamster cells with tunicamycin, an inhibitor of N-linked glycosylation, does not allow SSAV to infect E36 cells (data not shown), suggesting that N-linked glycosylation by itself does not account for the loss of SSAV receptor function for the endogenous receptor in E36 cells. Alternatively, cellular fac-

FIG. 3. Alignment of the deduced amino acid sequences in the envelope regions of five members of GALV family. The underlined segments correspond to the putative VRA and VRB. Gaps in the alignment are indicated by dashes, with the consensus sequence indicated on the bottom line.
tors in addition to the viral receptor may influence the ability of SSAV to use the E36 GALV receptors. At least 10 coreceptors (CCR5, CXCR4, CCR3, CCR2b, STRL33, GPR15, GPR1, V28, CCR8, and US28) have been identified to be used by HIV and simian immunodeficiency virus (2, 7) and participate in the postbinding stage of entry. Furthermore, the requirement for cellular factors has been shown to be both cell type and viral strain dependent in a manner similar to what we have observed for GALV-S and SSAV. E36 cells may either express specific factors which inhibit SSAV entry or lack accessory proteins, present in murine MDTF cells, which are required for HaPit1/HaPiT2-mediated SSAV entry.

We have sequenced the SU (surface) and TM regions of the envelope gene from GALV-S, -H, -Br, and -SF, as well as SSAV, to determine what region(s) of SSAV envelope correlate with its inability to infect E36 cells. The GALV-S envelope open reading frame extends an additional 54 bp from that originally proposed by Delassus et al. (6). The protein expressed by the open reading frame predicted from the previously published sequence is highly fusogenic when expressed in mammalian cells (11a). In contrast, the full-length GALV-S envelope does not induce cell-cell fusion when expressed in murine cells or in human 293T cells. This finding is consistent with a previous report that cleavage of the corresponding C-terminal 16 residues of the E-MuLV envelope activates membrane fusion (24, 25) and suggests that the fusogenic properties that result from the removal of the terminal 16 residues of the E-MuLV TM also occurs in other members of the mammalian type C family of retroviruses.

The 10A1 MuLV, feline leukemia virus subgroup B (FeLV-B), and each of the GALV strains have been demonstrated to use Pit1 as a receptor to infect human cells (27, 32, 33). Two distinct regions, VRA and VRB (1), within the SU of MuLV envelopes are involved in receptor utilization. Interestingly, comparison of the FeLV-B (3), 10A1 (23), SSAV (13), and GALV envelopes (6) in the regions corresponding to VRA and VRB reveals considerably divergent amino acid sequences (Fig. 4), despite their common receptor utilization. VRA of FeLV-B contains 37 residues, compared to 42 residues in 10A1 and 68 in SSAV and GALV-S. VRB is much longer for both FeLV-B and 10A1 (30 residues) than for GALV-S or SSAV (12 residues). The sequences comprising VRA and VRB for FeLV-B, 10A1, and SSAV/GALV-S are not closely related, indicating that Pit1 receptor recognition does not impose substantial conservation of SU envelope sequence or that other regions are involved in receptor recognition for this group of retroviruses. Amphotropic-MuLV enveloped vectors do not utilize Pit1 as a receptor but can be modified to have 10A1 host range properties by replacing as few as three residues in their SU regions, two within VRA and one within VRB, with the corresponding 10A1 envelope residues (15). FeLV-A can be modified to utilize Pit1 as a receptor by substituting 37 residues corresponding to VRA of FeLV-B (4).

In this report, we have shown that the N-terminal half of GALV-S envelope, encompassing VRA and VRB, can be substituted for the corresponding residues within the SSAV SU, and vectors bearing these chimeric envelopes, in contrast to vectors bearing SSAV envelopes, are able to infect hamster E36 cells. There are 12 amino acid residues that differ between the 68-residue VRA sequences of GALV-S and SSAV, and 9 of the 12 residues in their VRB sequences differ. Further analysis of the SSAV and GALV-S envelopes may distinguish more specifically the regions or residues which are important for E36 receptor recognition and fusion.

**FIG. 4.** Protein sequence alignment of the GALV-S, SSAV, 10A1 MuLV, and FeLV-B envelope VRA and VRB. Gaps in the alignment are indicated by dashes, with the consensus sequence indicated on the bottom line. Residue numbers correspond to those in the mature envelope protein after removal of the signal peptide.
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