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Sodium-Dependent *myo*-Inositol Transporter 1 Is a Cellular Receptor for *Mus cervicolor* M813 Murine Leukemia Virus

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Retrovirus infection is initiated by binding of the surface (SU) portion of the viral envelope glycoprotein (Env) to specific receptors on cells. This binding triggers conformational changes in the transmembrane portion of Env, leading to membrane fusion and cell entry, and is thus a major determinant of retrovirus tissue and species tropism. The M813 murine leukemia virus (MuLV) is a highly fusogenic gammaretrovirus, isolated from *Mus cervicolor*, whose host range is limited to mouse cells. To delineate the molecular mechanisms of its restricted host range and its high fusogenic potential, we initiated studies to characterize the cell surface protein that mediates M813 infection. Screening of the T31 mouse-hamster radiation hybrid panel for M813 infectivity localized the receptor gene to the distal end of mouse chromosome 16. Expression of one of the likely candidate genes (*slc5a3*) within this region in human cells conferred susceptibility to both M813 infection and M813-induced fusogenicity. *slc5a3* encodes sodium *myo*-inositol transporter 1 (SMIT1), thus adding another sodium-dependent transporter to the growing list of proteins used by MuLVs for cell entry. Characterization of SMIT1 orthologues in different species identified several amino acid variations within two extracellular loops that may restrict susceptibility to M813 infection.

Retroviral infections are initiated by binding of the viral envelope glycoprotein (Env) to a cell surface receptor protein, followed by secondary events that lead to fusion of the viral and cellular membranes, either at the cell surface or after trafficking through acidic endosomal compartments. This initial interaction is a major determinant of the tissue and species tropisms of retroviruses. Mutations in the viral *env* gene that result in altered receptor specificity are an important factor in the evolution of endogenous viruses, cross-species infection, and viral pathogenesis (4). Important examples of this have been revealed in studies of human immunodeficiency virus, but invaluable insight has also been gained in the analysis of receptor-Env interactions of other retroviruses (33, 34).

Type C murine leukemia viruses (MuLVs) belong to the genus of gammaretroviruses and can be divided into two distinct groups, classes CI and CII, based on sequence homology. Type CI viruses have been found in *Mus caroli*, *Mus cervicolor*, and *Mus dunni* and are antigenically related to type C viruses from woolly monkeys and gibbon apes (6, 20, 62). The type CII viruses are endogenous to both wild and inbred laboratory strains of *Mus musculus*, as well as most, but not all, other mouse species examined (6, 16, 18, 56). The CII viruses have been further classified into interference groups based on host range and receptor usage (32, 35, 42): classically, ecotropic (infect only murine cells), xenotropic (replicate only on nonmurine cells), and polytropic and amphotropic (infect both murine and nonmurine cells).

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Significantly, sequence analysis has confirmed that the major variations between viral genomes of the different interference groups lie in the 5′ *env* sequences, encoding amino-terminal sequences of the surface (SU) Env protein, which are important for receptor recognition and binding (reviewed in reference 11). Phylogenetic and distribution analyses suggest that a common ancestor of the xenotropic and polytropic viruses was the first to enter the *Mus* germ line (16, 18, 56). Interestingly, despite their distinct host range and nonreciprocal infection interference, polytropic and xenotropic viruses use the same cellular receptor, XPR1, for cell entry (5, 51, 63). Orthomorphic variations in XPR1 dictate whether a species is susceptible or resistant to infection by the two virus groups (23). Conceivably, the more limited host range of xenotropic viruses was expanded by mutations in the Env protein, generating polytropic viruses that were able to spread into other wild-mouse populations (23, 56). Env mutations that result in the broader recognition and/or usage of related proteins have also been previously described for 10A1 MuLV, which arose after recombination of the exogenous amphotropic virus (clone 1504) with endogenous polytropic *env* sequences (31, 32). In contrast to amphotropic viruses, which use only Pit2 as a receptor, 10A1 can also use the closely related Pit1 for cell entry (28, 58).

The unique receptor usage of the ecotropic MuLVs isolated from *M. musculus* has been speculated to have occurred by replacement of the receptor-binding domain within the amino terminus of the SU protein of a xenotropic virus by the analogous region of an unknown virus or by cellular sequences that bind to a novel receptor (48). Similar domain swapping may also account for the M813 MuLV ecotropic virus isolated from *M. cervicolor*. In contrast to other ecotropic viruses with a host

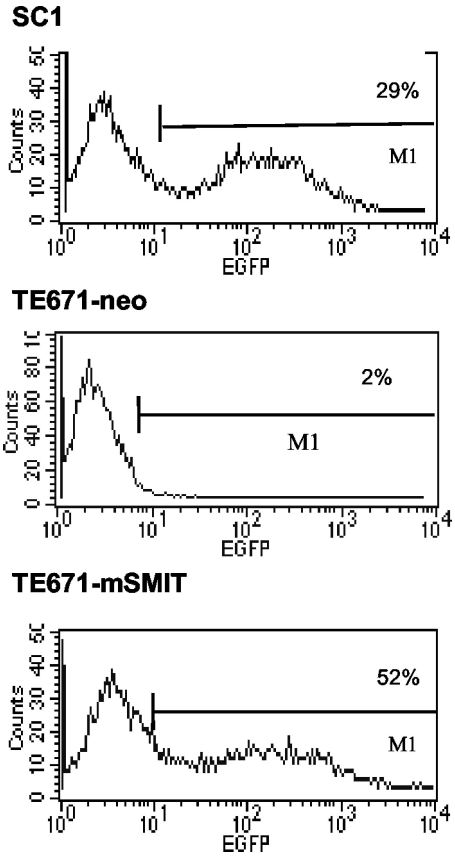

FIG. 1. Susceptibility to M813 infection is conferred to human TE671 cells by expression of mSMIT as shown by transfer of eGFP by retroviral pseudotypes. Target cells were incubated for 48 h with supernatant from SC1 fibroblasts containing M813 pseudotypes of SFα11-eGFP vectors. Infected cells were then monitored for expression of eGFP by flow cytometry. The relative fluorescence is plotted against the cell count. An analysis gate (M1) was set to measure the percentage of cells in which the fluorescent activity was above that measured in the negative control (uninfected TE671), using the 99% threshold method, thus indicating the percentage of cells that express eGFP after retroviral transfer.

range restricted to rodent cells, M813 does not use mCat1 as a cellular receptor (35). Sequence analysis of the *env* gene of M813 has found at most 37% sequence identity with other MuLVs within the amino-terminal domain of the SU protein and has identified a distinctive variable region A (VRA) domain. In contrast, sequences encoding the carboxyl end of the SU protein and the entire transmembrane domain are more highly conserved (76%). In addition to defining a unique interference group, M813 is highly fusogenic in vitro and in vivo (36). To better understand the molecular mechanisms involved in the evolution of cell recognition by viruses, this study was designed to identify the cellular protein used by M813 for cell entry. We show here that M813 uses sodium *myo*-inositol transporter 1 (SMIT1), a member of the solute/Na^+ symporter family, as a cell entry receptor.

(This work was part of the doctoral thesis of S. Hein, Department of Biology, University of Hamburg, Hamburg, Germany.)

MATERIALS AND METHODS

Cell lines, viruses, and virus assays. SC1 mouse fibroblasts were used as virus producers in all experiments (9). M813 virus was originally obtained from M813(NIH) cells, kindly provided by Ulf Rapp (39). The chimeric virus Mo-M813, in which the *env* gene encoding the SU domain of Moloney MuLV (MoMuLV) was replaced with the analogous region of a molecular clone of M813, has been previously described (35). Molecular clones of 10A1 (clone RR1) and MoMuLV (clone mov3), kindly provided by A. Rein and K. Harbers, respectively, were used to generate virus-producing SC1 fibroblasts.

Marker rescue assays, which exploit the ability of MuLV particles to transfer replication-defective retroviral vectors into target cells, were employed to monitor MuLV infection. For these studies the SFα11 retroviral vector carrying the enhanced green fluorescent protein (eGFP) was used (10). Either SC1 fibroblasts or the human rhabdomyosarcoma cell line TE671 (49) was used as target cells. Virus titers were determined by plating 3×10^4 SC1 fibroblasts into each well of a six-well plate and, after 24 h, adding serial dilutions (1:2) of supernatant of virus-producing cells. Forty-eight hours after infection, the percentage of cells expressing eGFP was determined by using a FACScalibur and Cellquest software (Becton Dickinson), and the number of fluorescent transfer units (FTU) per milliliter of virus supernatant was calculated.

Screening of the RH panel. The T31 mouse-Chinese hamster radiation hybrid (RH) panel consists of 100 cell lines, which have been characterized by using 271 sequence markers (25), and was generously provided by P. N. Goodfellow and L. C. McCarthy. A23 cells were grown in Dulbecco's modified Eagle's medium with 10% fetal bovine serum; A23-derived RH clones were grown in the same medium supplemented with hypoxanthne-aminopterin-thymidine. Ninety-nine of the 100 RH cells were plated at 5×10^4 cells per well in a six-well plate and infected the following day with M813 pseudotypes carrying Sfα11-eGFP. Two days after infection, the cells were examined for expression of eGFP under a fluorescence microscope. Wells containing more than 25 eGFP colonies were counted as positive. The infection results were analyzed by Lucy Rowe using The Jackson Laboratory Mouse Radiation Hybrid Database (41) (http://www.jax.org/resources/documents/cmdata/rhmap/RHIntro.html).

Analysis of *slc5a3* cDNAs and construction of human cell lines expressing the mSMIT protein. *slc5a3* cDNAs representative of mouse, rat, and human species were amplified from RNAs isolated from murine SC1 fibroblasts, RAT1 fibroblasts, and human rhabdomyosarcoma TE671 cells, respectively, by reverse transcriptase PCR with two sets of primers flanked by *Not*I sites (nucleotides [nt] 1 to 18 and 1048 to 1065, nt 983 to 1006, and nt 2140 to 2157 [sequence numbering as for accession no. NM_017391). The entire coding region was sequenced in both directions for all cDNAs. To generate a murine SMIT (mSMIT) expression vector, the 5′ and 3′ fragments isolated from SC1 cells were joined at a common *Sph*I site and cloned into the MPEVneo vector at its unique *Not*I site (17). The resulting plasmid (MPEV-mSMIT-neo [R895]) and control vector MPEVneo (R338) were transfected independently into Phoenix-gp packaging cells together with a plasmid encoding the vesicular stomatitis virus G protein (59). After 48 h, the supernatant was collected and used to infect TE671 cells. Cultures were selected for neomycin resistance in the presence of 800 to 1,000 μg of G418/ml (specific activity of 0.6).

Syncytium assays. To determine the fusion index, cells were seeded at a concentration of 3×10^6 to 5×10^6 cells per plate (10-cm diameter). Twenty-four hours later, the medium was replaced with virus-containing supernatant with Polybrene (8 μg/ml). Syncytium formation was visible after 1 h, and cells were fixed after 4 h and stained with Giemsa solution (Sigma). At least 500 nuclei were counted for each experiment.

Nucleotide sequence accession number. The nucleotide sequence of the cDNA isolated from RAT1 cells (see Fig. 2), derived from *Rattus norvegicus*, has been submitted to the public data banks (accession number AY231162).

RESULTS AND DISCUSSION

Mapping of the M813 receptor gene to the distal end of mouse chromosome 16. The recent success of cloning the receptor for the mouse mammary tumor virus by screening the RH panel (44) prompted us to use this approach to define the chromosomal localization of the M813 receptor. M813 pseudotypes carrying the SFα11-eGFP vector were used to infect the RH panel cell lines. Positively infected clones were identified by detection of eGFP expression. The resulting analysis placed the receptor near expressed sequenced tag

TABLE 1. Electrochemical potential-driven transporters used as cellular receptors for gammaretroviruses and type D betaretroviruses

Receptor(s)	Transporter family[a]	Transporter classification no.[b]	Virus group[c]	Reference(s)
mSMIT1	SSS	2.A.21	M813 MuLV	This paper
mCat1	APC/CAT	2.A.3.3	Ecotropic MuLV	1, 15, 61
Pit1 and Pit2[d]	PiT	2.A.20	10A1 MuLV, amphotropic MuLV, FELV-B, GALV	14, 28–30, 55, 58
XPR	DASS[e]	2.A.47	Polytropic MuLV, xenotropic MuLV	5, 23, 51, 63
FLVCR	MSF/UMF3[f]	2.A.1.28	FELV-C	37, 54
ASCT2 and ASCT1[d]	DAACS	2.A.23	RD-114, BaEV, REV, SNV, HERV-W, SRV1-5	19, 22, 40, 47, 52

[a] APC/CAT, amino acid-polyamine-organocation superfamily/cationic amino acid transporter family; PiT, inorganic phosphate transporter family; DASS, divalent anion/Na^+ symporter family; MSF/UMF3, major facilitator superfamily/unknown major facilitator-3 family; DAACS, dicarboxylate-amino acid/cation (Na^+ or H^+) symporter family.

[b] Transporter classification number of superfamily or family to which the receptor belongs, as recommended by the transport nomenclature panel of the International Union of Biochemistry and Molecular Biology (45).

[c] RD-114, feline endogenous virus; BaEV, baboon endogenous virus; REV, avian reticuloendotheliosis virus; HERV-W, human endogenous retrovirus type W; SNV, avian spleen necrosis virus; SRV, type D simian retrovirus types 1 to 5. All are gammaretroviruses except SRV, which belong to the betaretrovirus genus.

[d] Related proteins, both of which can be used as receptors for some but not all of the listed viruses.

[e] The XPR protein has been assigned to this family due to significant homology with other members of this family found by a BLAST search of the transporter database (http://www-biology.ucsd.edu/~msaier/transport/); however, functional analysis to confirm this hypothesis is lacking. Homology is also found with the yeast Syg1 protein, which is involved in the mating pheromone signal transduction pathway (5, 51, 63).

[f] FLVCR is currently classified in the UMF-3 family, but it has been shown to share homology with members of the organophosphate/P_i antiporter family and the anion/cation symporter family (54).

AA51763 (LOD score, 15.2) on the distal end of chromosome 16. Although earlier studies have mapped the receptor for M813 to chromosome 2 by using a similar approach (24, 39), the characterization of the hybrid clones used for those studies was limited to expression of a small set of isozyme markers, and thus it is likely that the receptor was incorrectly mapped.

Expression of *slc5a3* cDNA in human cells confers susceptibility to M813 infection. A search of the Celera Mouse Genome Databank in the region flanking expressed sequence tag AA51763 revealed several potential candidate genes. Of these, the *slc5a3* gene, located approximately 200 kb proximal to the identified marker, appeared to be the most promising (26). *slc5a3* encodes mSMIT1, which belongs to the family of Na^+/solute symporters (SSS family) and is a multiple membrane-spanning protein (13, 45).

To determine if mSMIT mediates cell entry of M813, the *slc5a3* cDNA was amplified from murine SC1 fibroblasts and introduced into a retroviral expression vector coexpressing the neomycin resistance gene (*neo*), which imparts resistance to G418 in eukaryotic cells. Human TE671 cells, which are resistant to infection with M813, were infected with vesicular stomatitis virus/MuLV pseudotypes carrying either the control vector or the vector expressing mSMIT and subjected to G418 selection. Drug-resistant cells were infected with the SFα11-eGFP vector pseudotyped with either M813, 10A1, or MoMuLV. In contrast to M813 and the ecotropic MoMuLV, 10A1 can infect cells of human origin. As a further control, murine SC1 fibroblasts were also infected with the same pseudotypes. As shown in Fig. 1, expression of mSMIT in the human cells imparts susceptibility to M813 infection, as measured by M813-mediated transfer of eGFP. Significantly, M813 virus titers on TE671-mSMIT1 cells were equivalent to or slightly higher than those calculated on murine SC1 fibroblasts (1.5×10^4 versus 3.2×10^4 FTU/ml, respectively [averages from two independent experiments]). Similar results were obtained when a chimeric MoMuLV/M813 retrovirus, in which the amino-terminal Env sequences are derived from M813 (35), were used for infections (data not shown). In contrast, mSMIT expression in TE671 did not mediate infectivity of the human TE671 cells by MoMuLV, although virus titers of 6.0×10^4 FTU/ml were observed on control SC1 cells. All cells were susceptible to infections with 10A1 pseudotypes with comparable efficiencies. These results clearly demonstrated that the mSMIT1 protein can mediate susceptibility to infection by M813 and that the amino-terminal half of M813 Env is necessary for this interaction. Although the RH mapping data indicate that there are no other receptors for M813 distant from mSMIT1, we cannot presently exclude the possibility that other genes encoding M813 receptors reside near *slc5a3*. Notably, no paralogues of mSMIT are found in this region.

The identification of mSMIT1 as a receptor for M813 MuLV adds another solute transporter to the growing list of retroviral receptors. Indeed, most if not all of the receptors characterized to date for gammaretroviruses (i.e., type C mammalian retroviruses) and the type D betaretroviruses (e.g., simian retroviruses) are multispanning transmembrane proteins that function as transporters for various small molecules, including amino acids and inorganic phosphate (Table 1). All utilize a carrier-mediated (versus channel-mediated) process and generally couple transport with a secondary energy source (i.e., an ion electrochemical gradient) by co- or countertransporting Na^+ and/or H^+ (reviewed in references 43 and 45). They range in size from 400 to 800 amino acid residues and contain from 6 to 14 transmembrane α-helical segments. This is in contrast to the receptors characterized for the alpharetroviruses (e.g., avian leukosis viruses), the B-type betaretroviruses (e.g., mouse mammary tumor virus), deltaretroviruses (e.g., bovine leukemia virus), and lentiviruses (e.g., human immunodeficiency virus), which are single transmembrane-spanning proteins (reviewed in reference 34), or the Jaagsiekte sheep betaretrovirus receptor, which is linked to the membrane by a glycosylphosphatidylinositol anchor (38). The fact that members of the same genera of retroviruses use cell molecules with similar structures and functions probably reflects constraints dictated by their related envelope proteins in recognizing and

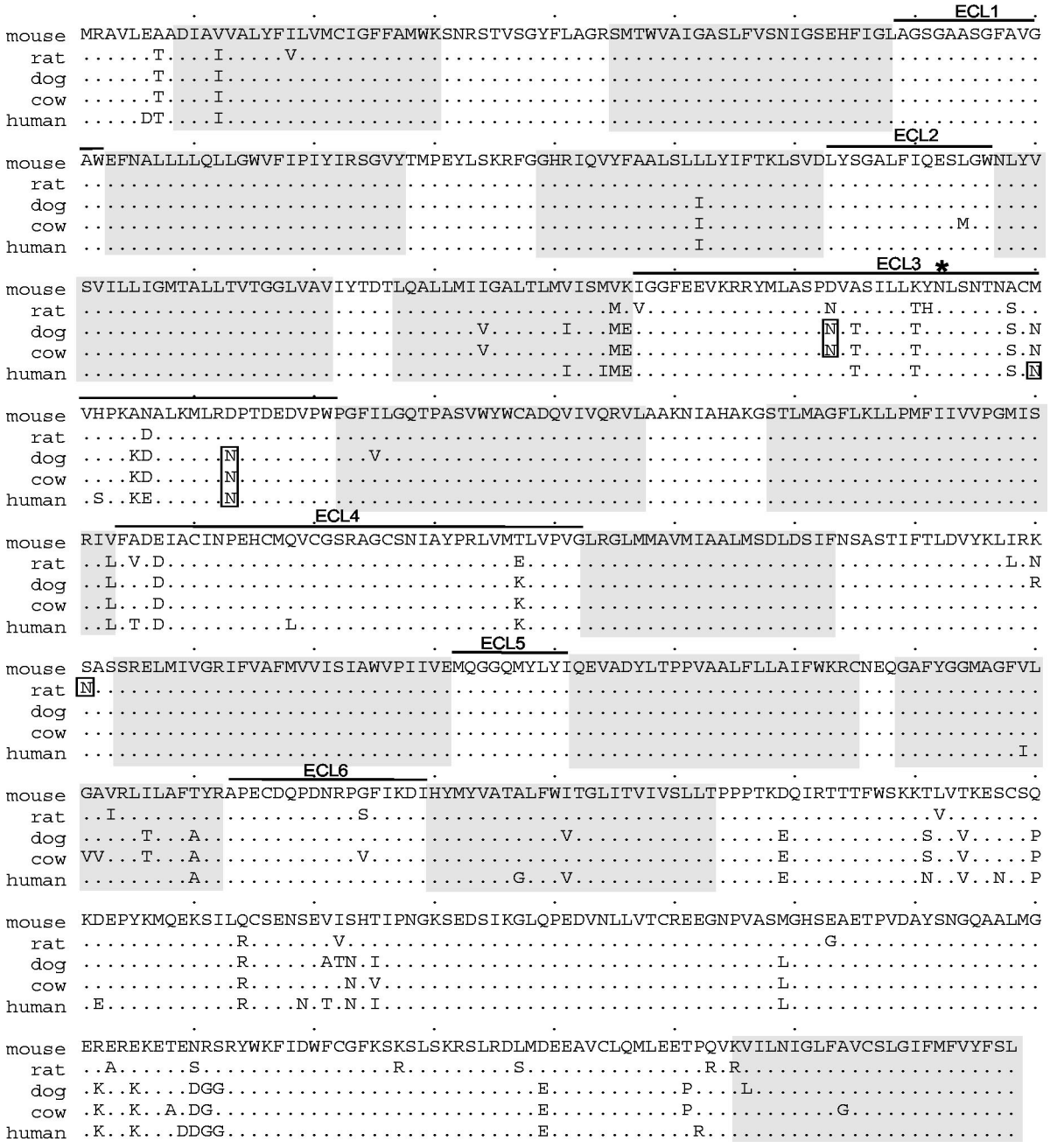

FIG. 2. Comparison of predicted SMIT1 protein sequences from various species. Sequence data were compiled from available sequences in the public data banks (*M. musculus*, NM_017391; *Canis familiaris*, M85068; *Bos taurus*, BTU41338; and *Homo sapiens*, NM_006933). cDNAs encoding SMIT1 were isolated from murine SC1 fibroblasts, rodent RAT1 fibroblasts, and human TE671 fibroblasts and sequenced. Three nucleotide deviations were found in the human sequence compared to the published sequence, at nt 92 (G → C), nt 148 (A → G), and nt 1696 (C → A), which would create codons for a Ser residue at position 31 (instead of Cys), Ala at position 50 (instead of Thr), and Lys at position 566 (instead of Gln), all consistent with those found in other species. One nucleotide deviation was found in the mouse sequence at nt 1958 (A → G), resulting in an Arg codon instead of a Gln at position 653. The predicted transmembrane α-helices are shaded, and predicted ECLs are overscored. The highly conserved Asp-linked glycosylation site, found in all mammalian homologues of the SLC5 subfamily of the SSS family, is indicated by an asterisk. Novel potential Asp-linked glycosylation sites present in mSMIT orthologues are boxed.

binding a membrane protein that triggers conformational changes necessary for fusion and cellular uptake.

Comparison of predicted amino acid sequences of SMIT1 from different species reveals several polymorphisms in two ECLs. Previous studies have shown that M813 infectivity is restricted to cells of mouse origin; rat cells were slightly permissive for infection, but no infectivity was observed in cells derived from other species, including human, dog, and rabbit (6, 35). To determine if receptor polymorphisms could possibly explain the resistance to M813 infection, available sequence data were compiled and compared. As no complete sequence data were available for the rat orthologue, the cDNA was

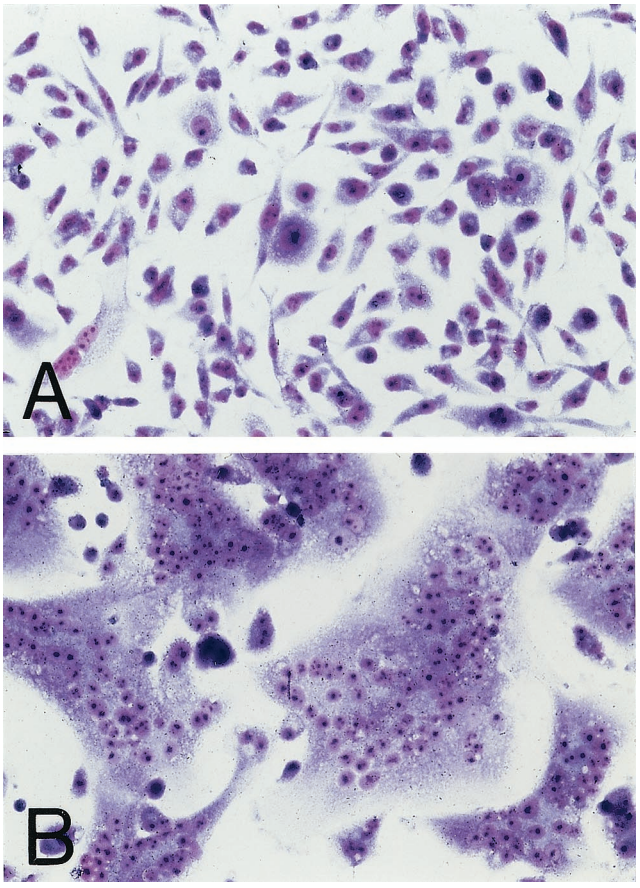

FIG. 3. Expression of mSMIT1 in human TE671 cells imparts susceptibility to M813-induced syncytium formation. Control TE671-neo cells (A) or TE671-mSMIT cells (B) were incubated with M813 for 4 h and Giemsa stained. Magnification, ×152.

amplified from Fischer rat fibroblasts (RAT1) and sequenced. The predicted amino acid sequence of the SMIT1 proteins expressed in rat, dog, cow, and humans are shown in Fig. 2. In addition, the proposed transmembrane and extracellular domains are indicated. Fourteen transmembrane domains in α-helical conformation have been predicted by experimental and computational analyses of homologues of the SSS family (57). The N terminus is extracellular, whereas the hydrophobic C terminus forms the 14th transmembrane span, preceded by a large, highly charged C-terminal domain located in the cytoplasm. A highly conserved Asp-linked glycosylation site is located in the third extracellular loop (ECL).

The SMIT1 protein is highly conserved in the N terminus, consistent with the hypothesis that this region contains the functional domains (13). Interestingly, however, clusters of mutations are observed in the third and fourth ECLs between the different species (Fig. 2). Single or multiple nonconserved substitutions found in these domains may be pivotal in inhibiting receptor function, as demonstrated for Pit1 and Pit2 (12, 21, 53) and Cat1 orthologues (2, 64). Alternatively, several novel NX(S/T) sites for potential Asp-linked gylcosylation are present in the nonfunctional receptors in this region (Fig. 2). Glycosylation has been shown to inhibit the receptor function of a number of retroviral receptors, including mCat1 and ASCT1/ASCT2 (8, 19, 22, 60). Further analysis is necessary to determine which amino acid variations, compared to mSMIT, prevent the human and dog proteins to function as cell receptors for M813. Other mechanisms that may be responsible for inhibiting infections, such as subthreshold levels of receptor expression or receptor masking due to expression of inhibitors, cannot be presently ruled out (50).

M813 infection of human TE671 cells expressing mSMIT1 induces massive syncytium induction. A striking characteristic of M813 is its high fusogenicity both in vitro and in vivo (36). Mice infected with M813 develop a peripheral T-cell lymphoma, which is associated with large multinucleated cells (36). In vitro, syncytium formation rapidly occurs after exposure to M813 but requires both the presence of a functional receptor and that the cells be preinfected with another MuLV. To test the role of mSMIT in fusion formation, human TE671 cells expressing mSMIT or the control vector were tested for fusogenicity directly after exposure to M813. As reported earlier, no syncytium formation was observed when TE671-neo cells were exposed to M813, regardless of whether they were previously infected with 10A1 MuLV or not (Fig. 3A). In striking contrast, giant syncytia were observed in TE671-SMIT cells within 3 h after exposure to M813 (Fig. 3B). M813-induced syncytium formation did not require preinfection with MuLV. Indeed, no significant increase in the levels of syncytium formation was observed in TE671-SMIT1 cells expressing 10A1 MuLV (Table 2). Thus, we hypothesize that high expression levels of mSMIT in TE671-SMIT cells negate the requirement for other Env-receptor interactions that may facilitate fusion. Further studies are necessary to determine what levels of mSMIT are required for infection and fusion induction. Previous work has shown that high expression levels of the mCat1 or PiT2 receptors also induce syncytium formation in cells productively infected with ecotropic or amphotropic MuLVs, respectively (46).

It is important to note that the fusion observed with M813 occurs from without, i.e., when a virion simultaneously fuses with two cells, in contrast to that which has been observed when an MuLV receptor is overexpressed, which occurs from within, i.e., when an infected cell expressing Env on its surface fuses with an adjacent cell. This underlines the high fusogenic capacity of the M813 virus. Interestingly, recent work has shown that the HERV-W Env also induces syncytium forma-

TABLE 2. mSMIT expression in human TE671 cells confers susceptibility to M813-induced fusogenicity with or without prior infection with 10A1 MuLV

Cell type	Fusion index after M813 inoculation	
	No preinfection	10A1 preinfection[b]
SC1	3.5	75
TE671-neo	3	3.5
TE671-mSMIT	84	87

[a] The fusion index was determined 4 h after cells were inoculated with M813 and was defined as $(N - S)/T$, where N is the number of nuclei in the syncytia, S is the number of syncytia, and T is the total number of nuclei counted (3). Values are means from at least two independent experiments.

[b] At least 2 weeks before the assay, cells were infected with cell supernatant containing 10A1 virus at an approximate multiplicity of infection of 1. Cells were maintained at low density to ensure proliferation and thus complete infection of the culture.

tion upon interaction with its receptor (ASCT2 or ASCT1) (7, 19). Indeed, it has been speculated that these endogenous retroviruses, specifically expressed in placenta cells, have evolved to facilitate syncytiotrophoblast differentiation by fusing the underlying cytotrophoblast cell layer (19, 27). Identifying mSMIT as the receptor for M813 will enable further studies to determine the critical Env-receptor interactions that regulate fusion.

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