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Characterization of a Naturally Occurring Ecotropic Receptor That Does Not Facilitate Entry of All Ecotropic Murine Retroviruses

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A fibroblast cell line (MDTF) derived from the feral mouse *Mus dunni* is resistant to infection by Moloney murine leukemia virus (Mo-MuLV), an ecotropic murine leukemia virus (E-MuLV) (M. R. Lander and S. K. Chattopadadhyay, J. Virol. 52:695–698, 1984). MDTF cells can be infected by other E-MuLVs such as Friend MuLV and Rauscher MuLV, which have been demonstrated to use the same receptor as Mo-MuLV in NIH 3T3 cells (A. Rein and A. Schultz, Virology 136:144–152, 1984). We have now shown that the block to Mo-MuLV infection of MDTF cells occurs at the level of the envelope-receptor interaction. We have cloned the ecotropic receptor cDNA from MDTF cells (dRec) and compared its sequence with that of the NIH 3T3 cell receptor (mRec). Although the deduced dRec and mRec proteins differ at only four amino acid residues, we demonstrate that these changes account for the resistance of MDTF cells to Mo-MuLV infection. Our findings suggest that retroviruses in the same receptor class can exhibit different host ranges due to single amino acid differences in their cellular receptor.

Two methods exist for defining groups of viruses that use the same cellular receptor. One method, the infection interference assay, is based on the observation that infection of a cell by a retrovirus renders that cell resistant to superinfection by other retroviruses that use the same receptor to gain entry into cells. Resistance is presumably mediated by the envelope proteins interacting with the viral receptor, thereby preventing subsequent virus binding and infection. The second method of receptor group classification depends on the identification of a molecular clone of a cellular receptor for a retrovirus. Expression of a specific virus receptor in a cell lacking a functional receptor can render the cell susceptible to all retroviruses requiring that receptor for efficient viral entry. For example, the cDNA encoding a receptor for ecotropic murine leukemia viruses (E-MuLVs) has been isolated from NIH 3T3 cells (2). Human cells do not express functional receptors for E-MuLVs but acquire susceptibility to infection following transfection and expression of the NIH 3T3 ecotropic receptor cDNA (2).

Interference assays carried out with NIH 3T3 cells have determined that E-MuLVs belong to the same receptor class (13). Even though E-MuLVs have been demonstrated to use a common cellular receptor, members of the E-MuLV group of viruses differ with respect to the cells they can infect. For example, *Mus dunni*-derived tail fibroblast (MDTF) cells are not susceptible to infection by the ecotropic virus Moloney murine leukemia virus (Mo-MuLV), although they serve as efficient host cells for the propagation of other E-MuLVs (5, 8; our published data). To determine whether the differences in susceptibility to E-MuLV infection observed between NIH 3T3 and MDTF cells are attributable to differences in the nature of their ecotropic receptors, we cloned and functionally characterized the MDTF ecotropic receptor.

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MATERIALS AND METHODS

Cells and viruses. The cells used in this study include *M. dunni* fibroblasts (MDTF cells), kindly provided by Janet Hartley, National Institute of Allergy and Infectious Diseases, Bethesda, Md. (8); NIH 3T3 murine fibroblasts (ATCC CRL 1658); the human osteosarcoma cell line, HOS (ATCC CRL 1543); PA317 cells (ATCC CRL 9078); CRE/BAG, provided by C. Cepko, Harvard Medical School, Boston, Mass. (6, 12); and the GP9122 cell line (10). MDTF/mRec and HOS/mRec cells were established by infection of MDTF or HOS cells with the PA317 packaged pLNSmRec genome (17). All cell lines were maintained in Dulbecco's modified Eagle's medium (Whittaker Bioproducts, Inc., Wakersville, Md.), supplemented with 5% fetal bovine serum, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 40 mM glutamine.

Retroviral vectors. Friend MuLV (F-MuLV)/BAG virions were produced by transfecting GP9122/BAG cells with a F-MuLV envelope expression vector. The BAG genome, a Mo-MuLV-based packageable genome which contains both the bacterial β-galactosidase gene and G418 resistance gene (12), was introduced by exposing GP9122 cells to BAG virions produced in PA317 cells followed by selection in G418 medium. The F-MuLV envelope expression plasmid (pMOV-Frenv) was constructed by replacing the *Sph*I-*Cla*I fragment of the MOV-MOVenv plasmid (18) with the corresponding *Sph*I-*Cla*I DNA fragment of the F-MuLV envelope gene from the p57 plasmid (15). The pMOV-Frenv and pREP8 (a plasmid conferring resistance to histidinol) (Invitrogen, San Diego, Calif.) plasmids were cotransfected into GP9122/BAG cells by calcium phosphate-mediated gene transfer, and the transfected GP9122/BAG cells were then selected in 2.5 mM histidinol (4).

Target cells were infected by retroviral vectors as previously described (18). Briefly, cells were seeded at densities of approximately 5×10^4 cells per well in a 12-well dish. At 24 h later, cell medium from postconfluent monolayers of

either F-MuLV/BAG or CRE/BAG producer cells was filtered through a 0.45-μm-pore-size filter and adjusted to 6 μg of Polybrene per ml. After the target cells were exposed to the filtered medium for 18 h, the cells were rinsed and fresh medium was added. At 48 to 72 h after exposure to retroviral vectors, the cells were assayed for β-galactosidase expression by histochemical staining (17).

Northern blot analysis. Polyadenylated RNAs were prepared as previously described (11). We used 5 μg of poly(A)$^+$ mRNA per sample for Northern (RNA) blot analysis. Ethidium bromide photographs of the gel verified that equivalent amounts of mRNA were present in each lane. The blots were hybridized to an antisense riboprobe (Promega, Madison, Wis.) derived from the NIH 3T3 receptor cDNA for 18 h in 50% formamide–5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7])–2× Denhardt's solution–0.1% sodium dodecyl sulfate (SDS)–0.25 mg of yeast tRNA per ml at 65°C. The blots were washed three times for 45 min each at room temperature in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS, once for 2 h at 65°C in 0.4× SSC–0.1% SDS, and finally twice for 30 min each in 0.1× SSC at 65°C. The filters were exposed with intensifying screens to X-ray film at −70°C for 24 h.

Cloning, sequencing, and mutagenesis of the MDTF ecotropic receptor (dRec) cDNA. The dRec cDNA was cloned by using the polymerase chain reaction (PCR). A mixture of random hexadeoxyribonucleotides was used to prime cDNA synthesis from MDTF polyadenylated RNA in an in vitro reverse transcription reaction. The dRec cDNA was then selectively amplified by PCR with sense and antisense oligomeric primers derived from the mRec cDNA sequence (2). The PCR-amplified dRec product was cloned into the TA vector (Invitrogen). Direct dideoxy sequencing of several amplified templates was carried out to ensure that changes in the dRec nucleotide sequence were authentic and not attributable to misincorporation of a base during the amplification process. Sequencing was performed with T7, SP6, or synthetic oligonucleotide primers (9, 14). To construct the dRec retroviral expression plasmid, a *Hpa*I-*Sph*I (nucleotides 735 to 1792) fragment of the MDTF ecotropic receptor cDNA was used to replace the corresponding fragment of the pLNSmRec plasmid. This chimeric cDNA was designated pLNSdRec. The dRecV-I_{214} cDNA is similar to the dRec cDNA except that the valine codon at position 214 has been converted to an isoleucine codon. The dRecV-I_{214} cDNA was synthesized from the dRec cDNA plasmid by a PCR mutation amplification strategy with a 5′ PCR primer encoding amino acids 208 to 220 and including a mutation at codon 214 such that the valine codon in the dRec cDNA was converted to an isoleucine codon in the amplified dRecV-I_{214} cDNA plasmid. The entire segment of amplified DNA product was subjected to sequence analysis to confirm the presence of the appropriate mutation and to determine that no unscheduled base changes had occurred during the amplification process. The pLNSdRec and pLNSdRecV-I_{214} vectors were packaged in PA317 cells. HOS cells were exposed to the PA317 filtered cell supernatant in 3 μg of Polybrene per ml. HOS cells expressing either the dRec or dRecV-I_{214} cDNAs were selected with 300 μg of G418 per ml. The G418-resistant colonies were pooled and designated HOS/dRec and HOS/dRecV-I_{214}, respectively.

R-MuLV and Mo-MuLV gp70 iodination and binding. Purified Mo-MuLV gp70 was provided by Stephen W. Pyle and Larry O. Arthur, National Cancer Institute-Frederick Cancer Research Facility, Frederick, Md. Purified RaMLV gp70 was provided by V. S. Kalyanaramen, Advanced BioScience Laboratories, Rockville, Md. Mo-MuLV and R-MuLV gp70s were radioiodinated to a specific activity of 0.8×10^4 to 1.2×10^4 cpm/ng as previously described (19). A competitive binding assay was performed in which the ability of ^{125}I-labeled Mo-MuLV gp70 to bind to NIH 3T3 cells was measured in the presence of increasing concentrations of unlabeled Mo-MuLV or R-MuLV gp70, and a 50% inhibitory concentration of approximately 4.6 nM was determined. The binding of ^{125}I-labeled Mo-MuLV and R-MuLV gp70 to NIH 3T3 cells was fully displaceable to levels of nonspecific binding observed with receptor-negative mink cells (3). Therefore, to conserve gp70, mink cells were used as a determinant of nonspecific binding in all further assays. An approximate affinity for gp70 binding to its receptor of 5 nM is in agreement with previous findings (3), and in all subsequent binding assays gp70 concentrations up to and exceeding this value by twofold were used.

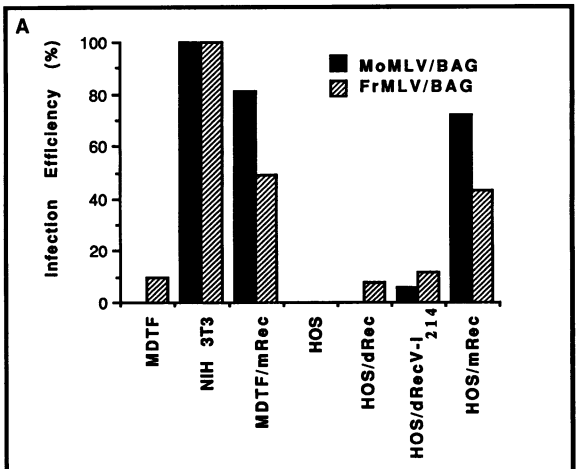
RESULTS

MoMLV infection of MTDF cells is restricted at the level of envelope-receptor interaction. MDTF cells were exposed to retroviral vectors bearing envelopes derived from different E-MuLVs. Mo-MuLV/BAG vectors, produced from the CRE packaging cell line, contain Mo-MuLV envelope glycoproteins. We developed a cell line which produces F-MuLV/BAG vectors bearing the envelope glycoproteins of the ecotropic F-MuLV. Both of these retroviral vectors contain the same Mo-MuLV-derived core proteins and genome, and both vectors efficiently infect NIH 3T3 cells. Only vectors bearing the F-MuLV envelope efficiently infect MDTF cells (Fig. 1). Therefore, the Mo-MuLV envelope glycoproteins account for the reduced Mo-MuLV infection of MDTF cells.

A retroviral vector containing the NIH 3T3-derived ecotropic receptor (mRec) cDNA was introduced and stably expressed in MDTF cells (designated MDTF/mRec cells) to determine whether expression of the NIH 3T3-derived ecotropic receptor could render MDTF cells susceptible to infection by Mo-MuLV. Expression of a functional mRec cDNA in MDTF cells conferred susceptibility to infection by retroviral vectors bearing either Mo-MuLV or F-MuLV envelopes (Fig. 1) as well as wild-type replication-competent Mo-MuLV (data not shown). These results suggest that changes in the amino acid composition of the MDTF ecotropic receptor compared with the mRec receptor protein, rather than cell-specific modification of a MDTF protein identical to mRec, account for differences in MDTF susceptibility to F-MuLV and Mo-MuLV.

Comparison of the mRec and dRec cDNAs. When polyadenylated RNA isolated from MDTF cells was evaluated by Northern blot, transcripts with homology to the mRec cDNA were detected (Fig. 2). These transcripts were similar in size and relative abundance to the 7.0-kb mRec mRNA observed in NIH 3T3 cells. The lower-molecular-size mRec mRNA (6.1 kb) present in the RNA isolated from NIH 3T3 cells but not MDTF cells possibly represents an alternately polyadenylated ecotropic receptor transcript.

Oligomeric primers complementary to specific regions of the mRec receptor cDNA were used to amplify, by PCR, the corresponding regions of the MDTF ecotropic receptor (dRec) cDNA. The complete coding region of the dRec cDNA was sequenced, and the predicted amino acid sequence was deduced. Four amino acid differences distinguish mRec from dRec (Fig. 3).



B Actual Values for Infection Efficiency

TARGET CELL LINE	RETROVIRAL VECTOR MoMLV/BAG	FrMLV/BAG
MDTF	0.1	10
NIH 3T3	100	100
MDTF/mRec	81	49
HOS	0.0	0.0
HOS/dRec	0.1	8.0
HOS/dRecV-I_{214}	6.0	12
HOS/mRec	72	43

FIG. 1. (A) Relative susceptibilities of different target cell lines to infection by Mo-MuLV/BAG and F-MuLV/BAG retroviral vectors. MDTF, NIH 3T3, MDTF/mRec, and HOS cells and HOS cells expressing the mRec, dRec, or dRecI-V_{214} cDNAs (HOS/mRec, HOS/dRec, and HOS/dRecI-V_{214} cells, respectively) were exposed to vectors containing the Mo-MuLV-based BAG genome, Mo-MuLV core proteins, and viral envelopes of either Mo-MuLV (Mo-MuLV/BAG) or F-MuLV (F-MuLV/BAG) strain of E-MuLV. The BAG retroviral genome contains the bacterial β-galactosidase gene (11). Cells exposed to either F-MuLV/BAG or Mo-MuLV/BAG virions were histochemically stained 72 h postexposure to detect β-galactosidase gene expression as a measure of retroviral vector infection. (B) Infection efficiencies of MDTF, MDTF/mRec, HOS, HOS/dRec, HOS/dRecI-V_{214}, and HOS/mRec cells by Mo-MuLV/BAG or F-MuLV/BAG vectors expressed as 100 × the number of blue foci (BFU) obtained 72 h after exposure to either Mo-MuLV/BAG or F-MuLV/BAG divided by the number of BFU obtained with NIH 3T3 cells under identical conditions. Approximately 5×10^4 BFU/ml were obtained on NIH 3T3 cells with F-MuLV/BAG, compared with 5×10^5 BFU/ml obtained with Mo-MuLV/BAG.

The functional importance of these amino acid differences between the dRec and mRec receptors was tested by constructing a dRec cDNA that contains dRec nucleotides 735 to 1792 (corresponding to amino acid residues 178 to 532) substituting for the corresponding region of the mRec cDNA (Fig. 3). HOS cells are resistant to infection by E-MuLVs (Fig. 1). HOS cells which stably expressed either the dRec cDNA (designated HOS/dRec) or mRec were compared for their relative susceptibility to infection by ecotropic retroviral vectors. HOS/mRec cells are similar to NIH 3T3 cells in that they are efficiently infected by both Mo-MuLV/BAG (exhibiting an infection efficiency of 72% compared with the

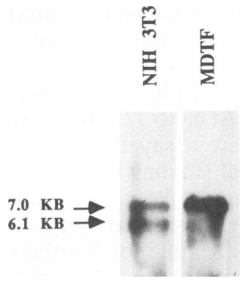


FIG. 2. Northern blot analysis of RNA isolated from NIH 3T3 and MDTF cells. Polyadenylated RNA (5 μg) extracted from NIH 3T3 cells (left lane) and MDTF cells (right lane) hybridized to an mRec cDNA probe.

100% infection efficiency obtained on NIH 3T3 cells) and F-MuLV/BAG (infection efficiency of 43% relative to NIH 3T3 [Fig. 1]). In contrast, HOS cells expressing dRec are phenotypically indistinguishable from MDTF cells in their susceptibility to infection by E-MuLVs. Both MDTF and HOS/dRec cells are susceptible to infection by F-MuLV (an 8% Fr/BAG infection efficiency on HOS/dRec cells compared with a 10% F-MuLV/BAG infection efficiency on MDTF cells) but not to infection by Mo-MuLV (Fig. 1).

The dRec protein differs from the mRec protein at amino acid residue 214, where they contain a valine codon and an isoleucine codon, respectively (Fig. 3). To determine the role of this amino acid difference, we changed the valine codon in dRec to an isoleucine codon. Although HOS cells expressing this form of the dRec cDNA and MDTF cells were infected with approximately equivalent efficiency by F-MuLV/BAG cells, they were 60-fold more efficiently infected by Mo-MuLV/BAG cells than by either MDTF or HOS/dRec cells (Fig. 1). Therefore, substitution of an isoleucine for a valine residue at position 214 of the dRec protein substantially restores Mo-MuLV receptor function to the dRec ecotropic receptor.

Comparison of E-MuLV monomeric gp70 binding in cells expressing dRec or mRec. We have determined that MDTF and NIH 3T3 cells, in addition to exhibiting marked differences in their susceptibility to infection by E-MuLVs, exhibit differences in their ability to bind E-MuLV envelope glycoprotein. The relative E-MuLV gp70-binding capacity of MDTF and NIH 3T3 was compared by using radioiodinated envelope glycoprotein purified from R-MuLV (an E-MuLV capable of infecting MDTF cells [8]) and from Mo-MuLV (MDTF cells are resistant to the ecotropic virus Mo-MuLV [8]). Specific high-affinity binding of ^{125}I-labeled R-MuLV or Mo-MuLV gp70 to NIH 3T3 cells could be readily detected (Fig. 4A). No comparable high-affinity binding could be detected on MDTF cells with either radiolabeled Mo-MuLV gp70 (Fig. 4A) or R-MuLV gp70 (Fig. 4B). The observation that MDTF cells, although susceptible to R-MuLV infection, fail to exhibit high-affinity R-MuLV gp70 binding comparable to that of NIH 3T3 cells suggests that the ecotropic receptors on these two types of cells are different (e.g., they have markedly different affinities for various gp70s). Further support of the inherent functional differences between the MDTF or dRec and NIH 3T3 or mRec receptor proteins was provided in experiments in which it was determined that MDTF cells expressing the mRec cDNA (MDTF/mRec cells) exhibit comparable high-affinity binding to both R-MuLV (Fig. 5A) and Mo-MuLV gp70 (data not shown).

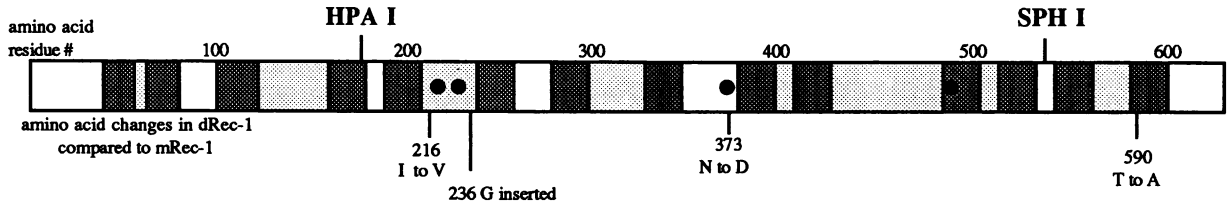


FIG. 3. Comparison of the deduced amino acid residue sequence of the NIH 3T3-derived ecotropic receptor (mRec) and the MDTF ecotropic receptor (dRec). Four amino acid differences were observed between the mRec and dRec proteins. Two of the changes are present in the third extracellular region of the receptor at amino acids 214 and 236. Symbols: ▩, hydrophobic regions; ▩, potential extracellular regions; ●, potential site of N-linked glycosylation.

Therefore the absence of E-MuLV gp70 binding observed on MDTF cells is attributable exclusively to dRec protein function and is not dependent on the contribution of other factors present in MDTF cells such as a cell surface protease.

Taken together, these findings predict that HOS cells expressing dRec would exhibit binding properties similar to those of MDTF cells. The abilities of HOS, HOS/mRec, and HOS/dRec cells to bind iodinated monomeric gp70 were compared. Radioiodinated R-MuLV gp70 was found to bind HOS cells expressing mRec cells at a level comparable to the binding observed on NIH 3T3 cells (Fig. 5B). In contrast, HOS cells expressing the dRec cDNA failed to bind R-MuLV gp70 (Fig. 5B) under similar conditions. These findings suggest that HOS cells expressing the dRec protein exhibit E-MuLV gp70-binding properties similar to those of MDTF cells. Furthermore, substitution of an isoleucine residue for the valine residue at position 214 of the dRec receptor did not restore high-affinity gp70 binding in HOS cells expressing this form of the dRec receptor, although these cells were rendered susceptible to Mo-MuLV (Fig. 5B). Therefore, one or both of the remaining amino acid residue differences that distinguish the mRec and the dRec proteins must account for the failure of the dRec receptor to bind gp70 with high affinity (Fig. 3). The observed lack of high-affinity R-MuLV gp70 binding exhibited by cells expressing the dRec protein does not discount low-affinity binding by these cells of a degree sufficient to facilitate whole R-MuLV particle entry at virus titers normally used in infection assays.

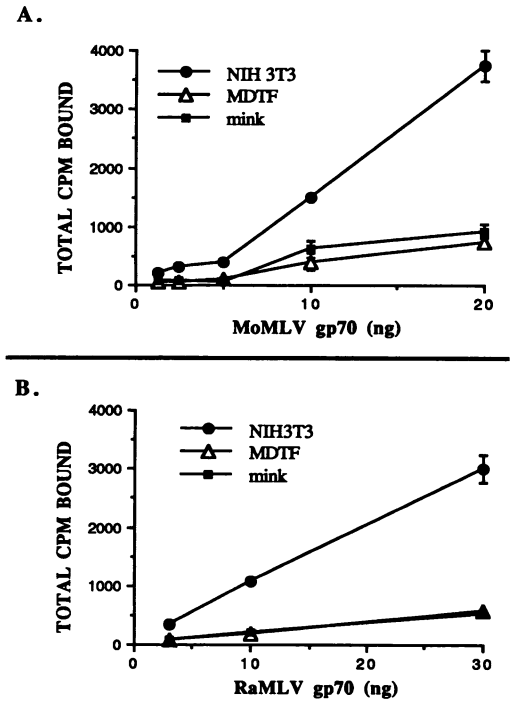


FIG. 4. Specific binding of radioiodinated E-MuLV envelope gp70 to cells. NIH 3T3, mink, or MDTF cells were incubated at room temperature in 30 μl of assay buffer containing 1.25 to 20 ng of purified ^{125}I-labeled Mo-MuLV gp70 (A) or 1.25 to 30 ng of purified ^{125}I-labeled R-MuLV gp70 (B). The cells were washed, and the radioactivity associated with the cells was assayed as previously described (19). Mink cells were included as a control for nonspecific gp70 binding since these cells lack detectable E-MuLV receptors (3). Binding assays whose results are presented in each of the panels were performed simultaneously and in triplicate.

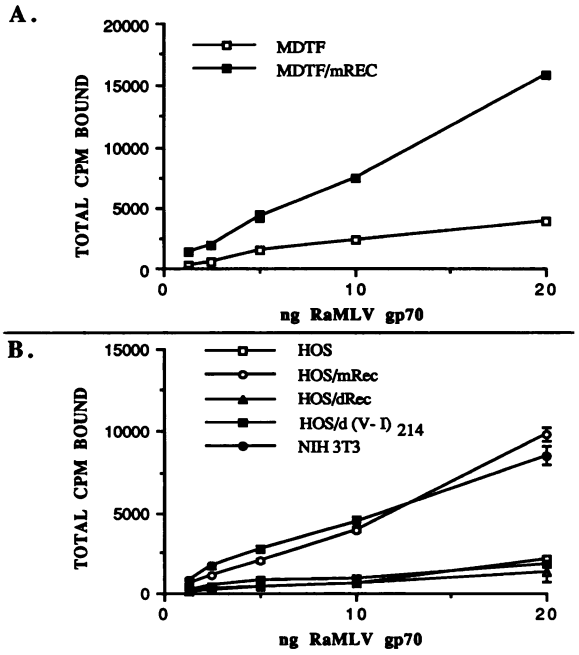


FIG. 5. Specific binding of 1.25 to 20 ng of ^{125}I-labeled R-MuLV gp70 in 30 μl of assay buffer measured on MDTF and MDTF/mRec cells (A) and ecotropic virus-resistant HOS cells and HOS cells expressing transfected mRec, dRec, and dRecV-I cDNAs and NIH 3T3 cells (B). Binding assays whose results are presented in each of the panels were performed simultaneously and in triplicate.

FIG. 6. Alignment of the deduced protein sequences of the third extracellular region of the NIH 3T3 ecotropic receptor, mRec (2); the MDTF ecotropic receptor, dRec; and the human T-cell homolog, hRec (20).

DISCUSSION

NIH 3T3 cells express an ecotropic receptor, mRec, which functions as a receptor for all E-MuLVs (e.g., F-MuLV, R-MuLV, and Mo-MuLV). This receptor has been cloned, sequenced, and preliminarily characterized as a multiple-membrane-spanning glycoprotein (2). More recently, the ecotropic receptor has been demonstrated to function as a transporter of cationic amino acids (7, 16). Human cells express an mRec homolog, hRec, but this protein does not serve as a receptor for any of the E-MuLVs (21). The ecotropic receptor present on MDTF cells is an intermediate between the nonfunctional hRec and the fully functional mRec in that it can serve as a receptor for most E-MuLVs, e.g., R-MuLV and F-MuLV, but not for Mo-MuLV. Alignment of the deduced protein sequences of mRec, dRec, and hRec (20) revealed that most nonconservative substitutions present in hRec, as well as two of the four substitutions present in dRec, occur in the third extracellular region (positions 210 to 239) (Fig. 6).

The mRec protein has an isoleucine residue at position 214, whereas both the dRec and hRec proteins contain a valine at this position. This valine substitution restricts the use of the dRec receptor by Mo-MuLV but not F-MuLV (Table 1). Substitution of an isoleucine residue for this valine residue renders the dRec protein functional as a Mo-MuLV receptor without compromising the efficiency of F-MuLV infection (Table 1). The dRec receptor also contains an extra glycine residue at position 236, not present in the mRec protein, and an aspartic acid residue at position 373 of dRec compared with an asparagine residue at position 373 of the mRec protein. The presence of the additional glycine residue most probably accounts for the loss of high-affinity monomeric gp70 binding observed in cells expressing the dRec receptor, since this change, in contrast to the change at position 373, occurs in an extracellular region of the Rec protein previously demonstrated to play a role in E-MuLV entry (1, 21). Our collective findings support the conclusion that the third extracellular region of the ecotropic receptor protein participates in ecotropic virus binding and entry.

TABLE 1. Summary of binding and infection results

Cell line	High-affinity gp70 binding	Susceptible to CRE/BAG	Susceptible to F-MuLV/BAG
MDTF	No	No	Yes
HOS/dRec[a]	No	No	Yes
HOS/dRecV-I_{214}[b]	No	Yes	Yes
HOS/mRec[c]	Yes	Yes	Yes

[a] The dRec ecotropic receptor, isolated from MDTF cells, contains a V residue at position 214 and an extra G residue inserted at position 236.

[b] The dRecV-I_{214} receptor is similar to dRec; however, the V residue at position 214 has been changed to an I residue.

[c] The mRec ecotropic receptor, isolated from NIH 3T3 cells, contains an I residue at position 214 and lacks the additional G residue at position 236.

The elucidation of the primary structure of this naturally occurring MDTF ecotropic receptor, dRec, has allowed the mapping of specific amino acid residues within the mRec receptor protein required for both high-affinity E-MuLV monomeric gp70 binding and Mo-MuLV entry. Different amino acid changes in the MDTF receptor appear to account for the lack of high-affinity gp70 binding and for the failure of this receptor to facilitate Mo-MuLV entry. Therefore, failure to detect high-affinity Mo-MuLV gp70 binding on a particular cell does not always predict cellular resistance to Mo-MuLV infection.

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