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Identification of Amino Acid Residues Critical for Infection with Ecotropic Murine Leukemia Retrovirus

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The murine cationic amino acid transporter is also the receptor for murine ecotropic leukemia retrovirus (MuLV-E). Recently, we have cloned a human gene (H13) homologous to the murine ecotropic retroviral receptor (ERR). Although the human homolog is very similar to murine ERR in sequence (87.6% amino acid identity) and structure (14 transmembrane-spanning domains), the human protein fails to function as a receptor for MuLV-E. To identify amino acid residues critical for MuLV-E infection, we took advantage of this species difference and substituted human H13 and murine ERR amino acid residues. Mouse-human chimeric receptor molecules were generated by taking advantage of using common restriction sites. These studies demonstrated that extracellular domains 3 and/or 4 contain the critical amino acid residues. Oligonucleotide-directed mutagenesis was then used to create 13 individual ERR mutants containing one or two amino acids substitutions or insertions within these two extracellular domains. Substitution of as few as one amino acid residue (Tyr) at position 235 in ERR with the corresponding H13 amino acid residue Pro abrogates the ability to function as a receptor for MuLV-E infection. Conversely, substitution of just two amino acid residues at positions 240 and 242 or 242 and 244 in H13 with the corresponding amino acid residues in ERR endows H13 with the ability to function as the receptor. This observation can be utilized to significantly improve the safety of retrovirus-mediated gene therapy in humans.

The murine ecotropic retroviral receptor (ERR) was molecularly cloned by Albritton et al. (1) and has been demonstrated to function as a cationic amino acid transporter (9, 19). Recently, we cloned a human gene (H13) homologous to the murine ERR, with 87.6% identity at the amino acid level (21). As the ERR does, the H13 protein contains 14 transmembrane-spanning domains. H13 has 629 amino acids and is 7 amino acids longer than the ERR protein (622 amino acids). Computer-guided alignments indicate that these seven amino acids are located in extracellular domain 3, which is the region most divergent between ERR and H13 sequences (5, 21). Although they are quite similar in the amino acid sequence and structure, the H13 should not function as the receptor for murine ecotropic leukemia retrovirus (MuLV-E) infection because of its tropism (20).

CD4 is the cellular receptor for human immunodeficiency virus, binding to the viral envelope glycoprotein, gp120 (16, 17). Several approaches have been taken to define the gp120 binding site of CD4, including random saturation mutagenesis coupled with selection of escape mutants (14), insertional mutagenesis (13), alanine-scanning mutagenesis (3), and homolog-scanning mutagenesis (2, 10, 11). These studies have successfully identified an amino acid stretch (residues 40 to 55) as critical for gp120 binding.

To systematically investigate which amino acids in ERR are critical for MuLV-E infection and which play a role in the dysfunction of H13 as a MuLV-E receptor, we took advantage of the species difference and performed the homolog-scanning mutagenesis between murine ERR and its human counterpart, H13. We first made chimeric molecules by using common restriction sites and determined the extracellular domains which contain the critical region. We then prepared 13 individual ERR mutants which contain mutations within these extracellular domains and identified the most critical amino acid residue Tyr at position 235. The substitution of this amino acid residue led to the abolition of the ability to function as the receptor. We also found that H13 can acquire the ability to function as the receptor by substitution of just two amino acid residues at positions 240 and 242 or 242 and 244 with the corresponding amino acid residues in ERR.

MATERIALS AND METHODS

Cell cultures and viruses. The Chinese hamster ovary (CHO-K1) and recombinant retrovirus ψCRE/BAG virion-producing (CRE BAG 2 [ATCC CRL 1858]) cell lines were maintained in Ham F-10 medium supplemented with 10% fetal bovine serum (FBS) and Dulbecco's modified Eagle's medium supplemented with 10% calf serum, respectively.

Transfections and infections. The insert of ERR cDNA was digested with *Bam*HI from pJET (1), which was kindly provided by L. M. Albritton and J. M. Cunningham (Harvard Medical School, Boston, Mass.); blunted by using the Klenow fragment of DNA polymerase I; ligated to *Eco*RI linkers; and subcloned to the *Eco*RI site of the eukaryotic expression vector, pSG5 (Stratagene, La Jolla, Calif.) (8). The *Eco*RI linker-ligated H13 cDNA *Nru*I-*Pst*I fragment (21) was subcloned to the *Eco*RI site of pSG5.

For transfection of these pSG5 constructs into CHO-K1 cells, approximately 2×10^6 cells were plated in dishes (100 by 20 mm^2). Twelve to 24 h later, 3 ml of a mixture of 20 μg of plasmid DNA and 40 μl of Lipofectin reagent (1 mg/ml; GIBCO BRL, Gaithersburg, Md.) (6) in Ham F-10 without FBS was added to the washed CHO-K1 cells. After 6 h at 37°C, 7 ml of the medium containing 10% FBS was added to the cells and incubated for 2 days. The recombinant retro-

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viruses of ψCRE/BAG virions (approximately 2 × 10^5 CFU/ml) were harvested from confluent tissue flasks, passed through 0.45-μm-pore-size filters and used to infect the transfectants in the presence of Polybrene (8 μg/ml; Sigma, St. Louis, Mo.). After 3 to 5 h, fresh medium was added and incubated for 1 day, and then each of these cells was trypsinized and divided 1:2. On the following day, 0.6 mg of G418 (Geneticin, GIBCO BRL) per ml was added to the dishes, and the plates were stained with 1% crystal violet after 10 to 14 days to quantify the presence of G418-resistant colonies.

Site-directed mutagenesis. Site-directed mutagenesis was performed by using a phagemid vector, pSELECT-1 (Promega, Madison, Wis.) (12), as recommended by the supplier. The insert of pSG5ERR was partially digested with *Bam*HI and *Eco*RI and subcloned to the *Bam*HI-*Eco*RI sites in pSELECT-1 to obtain pSELECT-1 sense and antisense ERR. Single-stranded DNA was prepared from the pSELECT-1 sense (for preparation of ERR mutants 2 and 6) and antisense (for preparation of the other mutants) ERR, and mutagenesis was carried out by following Promega's recommendations. The correctly mutated clones were selected by direct sequencing. Mutated inserts of the phagemids prepared by minipreps were excised with *Eco*RI and subcloned to the *Eco*RI site of pSG5. The presence of mutations was confirmed by sequencing each plasmid.

To prepare the H13 mutants, the insert of pSG5H13 was completely digested with *Bam*HI and partially with *Eco*RI and subcloned to the *Bam*HI-*Eco*RI sites of pSELECT-1. H13 mutants 1 to 3 and 5 were prepared by using the pSELECT-1 antisense H13 as template DNA and sense oligonucleotides 5′-AAAGAAGGGAAGTACGGTGTTGG TGG-3′, 5′-ACACAAAAGAAGTGAAGTACGGTGTTGG TGG-3′, 5′-AATGACACAAAAAACGTGAAGTACGGTG TTGGTGG-3′, and 5′-AAAGAAGGGAAGTACGGTGAGG GTGGATTCATG-3′, respectively. The inserts of pSG5H13 mutants 5 and 8 were excised with *Eco*RI and subcloned to the *Eco*RI site of pSELECT-1. H13 mutant 4 was prepared by using the pSELECT-1 antisense H13 mutant 8 and sense oligonucleotide 5′-TGAAGTACGGTGTTGGTGGATTCA TG-3′. H13 mutants 6 to 8 were prepared by using the pSELECT-1 antisense H13 mutant 5 and sense oligonucleotides 5′-ACACAAAAGAAGTGAAGTACGGTGA-3′, 5′-AATGACACAAAAAACGTGAAGTACGGTGA-3′, and 5′-AACAATGACACAAACGTGAAGTACGGTGAGGGTG GATTCATG-3′, respectively.

Sequencing. To select correctly mutated clones and confirm the presence of the mutation, sequencing was carried out by using Sequenase (USB, Cleveland, Ohio) and two ERR- and H13-specific antisense oligonucleotides (5′-GGTGGCGATGCAGTCAA-3′ for ERR mutants 1 to 7, 7A, and 7B and H13 mutants 1 to 8, and 5′-TCAGCCATGGC ATAGATA-3′ for ERR mutants 8 to 11) as primers.

RESULTS

Human H13 does not function as a receptor for MuLV-E infection. We first determined the relative abilities of murine ERR and human H13 proteins to function as receptors for MuLV-E infection. CHO-K1 cell lines were transiently transfected with a vector expressing either ERR or H13 cDNA. These transfectants were then infected with the recombinant MuLV-E, ψCRE/BAG virions, encoding the *Escherichia coli lacZ* β-galactosidase and Tn*5 neo* resistance genes (4, 15) and selected by incubation with G418. After 10 to 14 days, numbers of G418-resistant colonies were counted

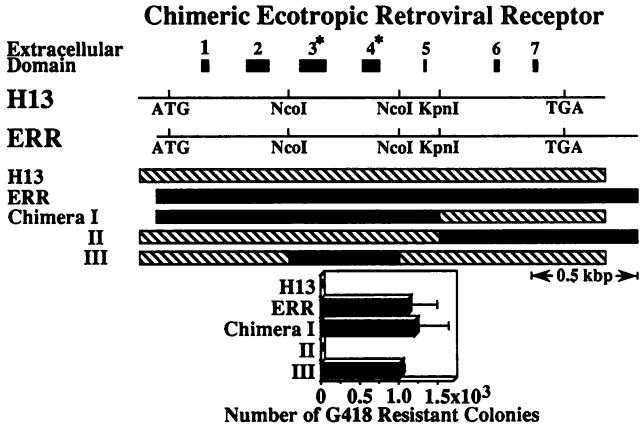

FIG. 1. Extracellular domains 3 and/or 4 (*) contain critical amino acid residues for infection by MuLV-E. Mouse-human chimeric receptor molecules (chimera I to III) were constructed by taking advantage of common restriction sites in murine ERR and human H13. Their abilities to function as a receptor for MuLV-E infection were determined by using the recombinant MuLV-E, ψCRE/BAG virions (4, 15). Black boxes at the top indicate extracellular domains of ERR and H13 proteins (1, 21), and shaded and striped bars indicate the nucleotide sequences of ERR and H13 cDNA, respectively. The results represent the average ± standard error of three transfections.

(Fig. 1). Unmodified H13 transfectants produced no resistant colonies, whereas approximately 10^3 colonies were obtained with the ERR transfectants, confirming the inability of H13 to function as a receptor for MuLV-E infection.

Extracellular domains 3 and/or 4 in ERR contain the critical region for infection. To identify amino acid residues critical for MuLV-E infection, we constructed mouse-human chimeric molecules (chimeras I and II) by taking advantage of the single common *Kpn*I restriction site which exists in both ERR and H13 cDNA and analyzed their function as a receptor for MuLV-E infection (Fig. 1). Approximately 10^3 colonies were obtained with the transfectants of chimera I, whose 5′ portion of the gene constituted the corresponding region of ERR, whereas no colonies were obtained with the transfectants of chimera II, whose 3′ portion of the gene corresponded to ERR. This indicates that the critical amino acid residues are located in the 5′ portion of the ERR cDNA. To more narrowly define the required sequences, chimera III, whose *Nco*I-*Nco*I fragment was derived from the corresponding region of ERR, was constructed and analyzed for receptor function (Fig. 1). Approximately 10^3 colonies were obtained with chimera III transfectants. This localized the region critical for infection to the *Nco*I-*Nco*I restriction sites.

Both ERR and H13 proteins are composed of seven extracellular domains (Fig. 1) (1, 21). The *Nco*I-*Nco*I fragment spans extracellular domains 3 and 4 of both proteins. Subsequent analyses focused on these two domains. Figure 2 shows the comparison of aligned sequences of extracellular domains 3 and 4 in ERR and H13 by using the Genetics Computer Group sequence analysis software package (5, 21). Extracellular domain 3 shows the highest diversity between ERR and H13 sequences, with H13 protein possessing seven additional amino acid residues within this domain. To examine the amino acid residues critical for virus binding, we employed oligonucleotide-directed mutagenesis. Eleven individual ERR mutants (ERR mutants 1 to 11), each

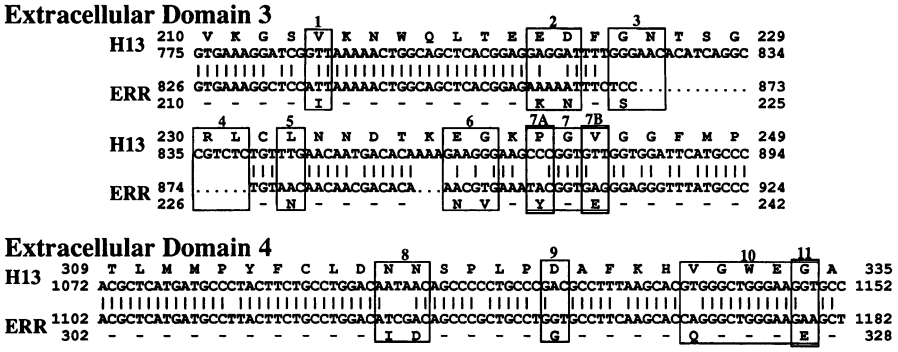

FIG. 2. Comparison of sequences of extracellular domains 3 and 4 in murine ERR and human H13. The alignment was made by using the Genetics Computer Group sequence analysis software package (5). To pinpoint the critical amino acid residues, oligonucleotide-directed mutagenesis was carried out and 13 individual ERR mutants (ERR mutants 1 to 11, 7A, and 7B) which contain one or two amino acid substitutions or insertions (as marked by boxes) were created. Dashes in ERR sequence indicate the same amino acid residues as found in the corresponding position of H13 sequence. Dots in the ERR sequence mean there are no corresponding nucleotides, according to the alignment.

containing one or two amino acid substitutions or insertions within these two extracellular domains, were created (Fig. 2 and Table 1). For each substitution we replaced amino acids of ERR with those found in equivalent positions of the H13 sequence. For each insertion we added amino acid residues of H13 into equivalent positions of the ERR sequence which aligned as shown in Fig. 2. They were transfected into CHO-K1 cells in anticipation that some mutants would abrogate the abilities to function as the receptor.

Substitution of just one amino acid residue in ERR abrogates its ability to function as the receptor. CHO-K1 cells expressing the ERR mutants proteins were tested for their

TABLE 1. Abolition of ability of ERR to function as a receptor for MuLV-E infection by mutation[a]

Domain and ERR mutant	Oligonucleotide used for mutagenesis[b]	Amino acid change (ERR → H13)	Infectivity[c]
Extracellular domain 3			
1	A AAGGCTCCGTTAAAAAC	I → V	1,140 ± 382
2	T T TACAGGAGAAATCTTCCTCCGTGAGCTG[d]	KN → ED	1,373 ± 400
3	TC --- GAGAAAAATTTCGGCAACTGTAACAACAAC	S– → GN	1,214 ± 320
4	------ AAAAATTTCTCCCGTCTCTGTAACAACAAC	-- → RL	1,190 ± 329
5	AA AATTTCTCCTGTTTCAACAACGACAC	N → L	1,441 ± 510
6	A G T TCACCGTATTTCCCTTCTGTGTCGTTGTT[d]	NV → EG	1,321 ± 400
7	TA A ACAAACGTGAAACCCGGTGTGGGAGGGTTTAT	YGE → PGV	0 ± 0
7A	TA ACAAACGTGAAACCCGGTGAGGGAGG	Y → P	38 ± 0
7B	A ATACGGTGTGGGAGGGT	E → V	1,340 ± 652
Extracellular domain 4			
8	T G TCTGCCTGGACAACAACAGCCCGCTGC	ID → NN	1,087 ± 573
9	GT GCCCGCTGCCTGACGCCTTCAAGCAC	G → D	1,583 ± 511
10	CA A GCCTTCAAGCACGTGGGCTGGGAAGGAGCTAAGTACGC[e]	QGWEE → VGWEG	1,510 ± 625
11	CA A GCCTTCAAGCACGTGGGCTGGGAAGGAGCTAAGTACGC[e]	E → G	1,423 ± 486

[a] The transfection was done three times; summarized results are shown.

[b] Letters above each oligonucleotide sequence are those in the original ERR sequence. Dashes indicate the absence of the corresponding nucleotide sequence in the original ERR sequence, according to the alignment (Fig. 2).

[c] Number ± standard error of G418-resistant colonies obtained. The numbers for ERR and H13 are 1,252 ± 689 and 0 ± 0, respectively.

[d] These two are antisense oligonucleotides and the others are sense oligonucleotides.

[e] Two mutants were recovered from the mutagenesis by using this oligonucleotide; ERR mutant 10 contains substitutions of both Gln and Glu to Val and Gly, respectively, and ERR mutant 11 contains only the Glu-to-Gly change. These two mutants were transfected separately.

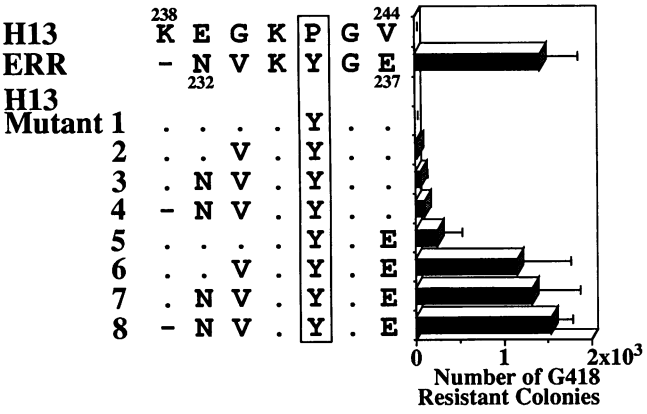

FIG. 3. Acquisition by H13 of the ability to function as a receptor for MuLV-E infection by mutation. Dashes in ERR sequence indicate the absence of the corresponding amino acid residue in ERR to the Lys at position 238 in H13, according to the alignment (Fig. 2). Dashes in H13 mutants sequences indicate deletion of the amino acid residue Lys at position 238 in H13. Dots indicate the same amino acid residues as those found in the corresponding position of the H13 sequence. The results represent the average ± standard error of three transfections.

abilities to function as the receptor (Table 1). Only ERR mutant 7, which has two amino acid substitutions (Tyr-235 residue in ERR to Pro-242 residue in H13 and Glu-237 residue in ERR to Val-244 residue in H13), failed to grow any colonies, indicating abrogated ability to function as the virus receptor. More than 10^3 colonies were obtained with all other mutants. We then constructed ERR mutants, which contained only one amino acid substitution (ERR mutant 7A, Tyr to Pro, and ERR mutant 7B, Glu to Val), and tested their abilities to function as the receptor (Table 1). ERR mutant 7A showed almost completely abrogated receptor function, while ERR mutant 7B exhibited receptor function similar to that of ERR. These results suggest that the Tyr-235 residue in ERR is essential to receptor function and that substitution of this amino acid abolishes this activity.

H13 can acquire the ability to function as the receptor by substitution of just two amino acid residues. We also examined whether the mutations introduced into human H13 near amino acid residue 242 (which is aligned in equivalent position of the critical Tyr-235 residue in ERR) affect H13's ability to function as a receptor for MuLV-E infection. Eight individual H13 mutants were constructed for this normally dysfunctional gene, with regard to receptor function (Fig. 3). All of the mutants contained Pro-to-Tyr residue substitutions at position 242. The transfectant of H13 mutant 1, which contains only one amino acid substitution at position 242, was not susceptible to infection. Transfectants with H13 mutants 2 and 3 led to slight increases in susceptibility to the infection. Transfection with H13 mutant 4 which has 3 amino acids substitutions and 1 deletion led to recovery of approximately 10% of ERR's receptor activity. H13 mutant 5, in which only two amino acid substitutions at positions 242 and 244 were made, was more active than H13 mutant 4, suggesting that the substitution at position 244 was more effective. H13 mutant 6, with three substituted residues at positions 240, 242, and 244, showed dramatically increased receptor activity, with approximately 70% of that of ERR. H13 mutant 7, with substituted residues at positions 239, 240, 242, and 244, and H13 mutant 8, with similar substitutions and a deletion at position 238, exhibited receptor activity at levels comparable to those of ERR. These results suggest that H13, the human equivalent of the murine ERR, can acquire the ability to function as the MuLV-E receptor if amino acid residues at positions 240 and 242 or 242 and 244 are substituted with those of ERR. Amino acid substitutions at positions 239, 240, 242, and 244 (H13 mutants 7 and 8) result in activities comparable to that of ERR.

DISCUSSION

In this study, we have identified the critical amino acid residue, Tyr-235, in ERR molecule for MuLV-E infection by homolog-scanning mutagenesis between murine ERR and its human counterpart, H13. We constructed a total of 13 individual mutants of ERR. Among them, only ERR mutants which contain an amino acid substitution at position 235 lost the ability to function as a receptor. All other mutants showed activity similar to that of the intact ERR.

Similarly, we found that H13 can acquire the ability to function as the receptor by substitution of just two amino acid residues at positions 240 and 242 or 242 and 244 in H13. These results suggest that the human H13 retains the protein structure necessary to function as the MuLV-E receptor and the possibility that it might function as a receptor for as yet undefined human retroviruses with similarity to MuLV-E.

Currently, the most widely used vehicles for gene therapy utilize amphotropic retrovirus (MuLV-A) vectors, because these viruses can infect cells of most mammalian species, including human cells (7, 18). The approach involves the generation of "safe" amphotropic vectors, which have the capacity to infect and integrate but cannot replicate further. This effort is aided by the use of packaging cell lines. However, the use of amphotropic viruses has inherent safety problems because of potential recombinational events that can reconstitute wild-type variants from replication-defective retroviruses, leading to possible widespread infection in the recipient. Furthermore, the broad and nonspecific infections of which replication-defective amphotropic virus are capable carry the increased and unnecessary risk of insertional mutagenesis in nontargeted cells. This might lead to cancer or other deleterious consequences in the recipient.

In contrast, ecotropic viruses can normally replicate only in cells of the species from which they originate. Thus, murine ecotropic viruses cannot infect human cells. However, transient transfection of cells with a mutated human H13 construct, such as H13 mutant 8, would permit their infection by replication-defective murine ecotropic viruses vectors carrying desired gene sequences and, very importantly, limits infection to targeted cells. Even if recombination events occur, the resulting wild-type virus would be incapable of infecting other human cells, as the normal H13 receptor does not function to permit infection by murine ecotropic viruses. The safety of ecotropic viruses for human gene therapy would also allow the use of much higher titers of virus, ensuring greater efficacy and expanding the range of cells which can be targeted. Although this approach might also work with ERR, H13 mutants might be expected to be less immunogenic, a factor which may be important for the survival of transiently expressing cells if repeated gene therapy applications prove necessary. This new approach promises to open a novel avenue for the safe and efficient delivery of therapeutic genes.

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