Definition of a Domain of GLVR1 Which Is Necessary for Infection by Gibbon Ape Leukemia Virus and Which Is Highly Polymorphic between Species

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Expression of human GLVR1 in mouse cells confers susceptibility to infection by gibbon ape leukemia virus (GALV), while the normally expressed mouse Glvr-1 does not. Since human and murine GLVR1 proteins differ at 64 positions in their sequences, some of the residues differing between the two proteins are critical for infection. To identify these, a series of hybrids and in vitro-constructed mutants were tested for the ability to confer susceptibility to infection. The results indicated that human GLVR1 residues 550 to 551, located in a cluster of seven of the sites that differ between the human and mouse proteins, are the only residues differing between the two which must be in the human protein form to allow infection. Sequencing of a portion of GLVR1 from the rat (which is infectible) confirmed the importance of this cluster in that it contained the only notable differences between the rat and mouse proteins. This region, which also differs substantially between the rat and the human proteins, therefore exhibits a pronounced tendency for polymorphism.

The first stage in infection of a cell by a retrovirus involves a specific interaction between the envelope glycoprotein of the virus and a cell surface receptor. Five retrovirus receptors have been cloned. CD4, the major receptor for human immunodeficiency virus (5, 8), is involved in the recognition of antigen presented in the context of class II major histocompatibility antigen. BLVR, a receptor for bovine leukemia virus, is a protein of unknown function and has no detectable homology to other known proteins (4). tv-a, which confers susceptibility to infection by subgroup A avian viruses, has been cloned, but the open reading frame within the clone conferring susceptibility to infection has not yet been described (17). CD4 and BLVR are similar in that each contains a single transmembrane domain. This is in contrast to receptors for murine ecotropic virus and gibbon ape leukemia virus (GALV). MCAT, a receptor for ecotropic virus, is a cationic amino acid transporter (7, 15) and has 14 transmembrane domains (3). GLVR1, a receptor for GALV and feline leukemia virus B, is homologous to a phosphate permease of Neurospora crassa and is thought to contain 10 transmembrane domains (6, 14).

The availability of these receptors makes it possible to study the specifics of virus envelope-receptor interaction. To do this with MCAT, Albritton et al. (2) took advantage of the fact that expression of mouse MCAT in nonpermissive cells confers permissivity while expression of the closely related human homolog does not. By making chimeric cDNAs, it was possible to identify the amino acid residues differing between the two which controlled this effect and which were therefore critical for infection.

A similar situation exists with GALV, which infects cultured cells of many species, including human cells, but will not infect mouse NIH 3T3 cells. The differential permissivities of human and mouse cells were presumably a result of the fact that human GLVR1 and mouse Glvr-1 differ in their amino acid sequences at 64 positions. We set out to identify which of these divergent residues were involved in controlling receptor function in the hope that this would convey insight into the process of infection. We have found that a maximum of two of these divergent residues are critical in defining host range. These two are found in a domain of the protein which displays a high degree of polymorphism between species.

MATERIALS AND METHODS

Construction of expression plasmids. pOJ19, an expression plasmid for mouse Glvr-1, was made by cloning the EcoRI fragment of pMGR1 (6) (nucleotides 206 to 2721) into the EcoRI site of pcDNA1 (InVitrogen). To construct pOJ14, the KpnI-NoI fragment of pMGR1 (from the KpnI site at nucleotide 1146 to the NotI site in the vector) was used to replace the corresponding fragment of pOJ9 (an expression plasmid for human GLVR1 [6]). To construct pOJ15, the PstI fragment of pMGR1 (from nucleotide 2001 to the PstI site in the vector) was used to replace the corresponding fragment in pOJ9. To construct pOJ20, the PstI fragment of pOJ9 (from nucleotide 1926 to the PstI site in the vector [12]) was used to replace the corresponding PstI fragment of pOJ19.

To construct cDNAs with mutations in specific codons, the PstI fragment of pMGR1 or pOJ9 was cloned at the PstI site in pUC118. Mutagenesis was then carried out as described elsewhere (9). All mutants and hybrids were confirmed by sequencing junctions and mutated regions.

Isolation and testing of a portion of the rat GLVR1 cDNA. To isolate a portion of rat GLVR1, a fragment of the rat cDNA was amplified by polymerase chain reaction from rat brain cDNA by using primers corresponding to mouse Glvr-1 nucleotides 1996 to 2012 and 2633 to 2653 (the 5' primer, containing the PstI site normally encoded in human and mouse cDNAs, was 5'-CCAGTTCTCGAGATCC-3'; the 3' primer was 5'-GAGATAACTATTTGACGC-3'). The polymerase chain reaction product of 662 bp was cloned in pUC118, sequenced, and found to contain the appropriate sequence of rat GLVR1. Since the 5' primer included the first base of the codon for mouse Glvr-1 amino acid residue 524, only the 156 residues encoded downstream of the primer could

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be considered as being derived from the rat. To determine whether the rat sequence could contribute to infection by GALV (within the context of a full-size GLVR1 clone), the PstI fragment of this plasmid was used to replace the corresponding PstI fragment of pOJ19, to give pOJ52.

**Testing of expression plasmids.** NIH 3T3 cells, plated 1 day previously at 3 × 10^5/60-mm-diameter dish, were transfected with 1 μg of pSV2gpt, 3 μg of expression plasmid, and carrier DNA to a total of 20 μg of DNA per dish by CaPO₄ precipitation. pSV2gpt allows growth of cells in the presence of mycophenolic acid and xanthine (10). The next day, cells in each 60-mm-diameter dish were replated into two or three 100-mm-diameter dishes. After a further day, the cells were fed with medium containing mycophenolic acid (25 μg/ml) and xanthine (250 μg/ml). Hypoxanthine, aminopterin, and thymidine (100, 0.4, and 16 μM, respectively) were included to increase the stringency of selection. When cell death started to become evident (after about 3 days), the cells were fed with the same medium but lacking aminopterin. Colonies were counted after 2 weeks and were fed with medium containing xanthine, hypoxanthine, thymidine, and Polybrene (4 μg/ml). After 1 day, this medium was aspirated and colonies were challenged with 5 ml of GALV-pseudotyped pGV16, a replication-defective retrovirus encoding resistance to the antibiotic G418 (11). After 1 h at 37°C, pGV16 stocks were prepared as described elsewhere (12) and contained approximately 5 × 10⁶ infectious pGV16 particles/ml. Following the 1-h incubation, 5 ml of culture medium was added to each dish and the colonies were allowed to grow for 1 day. At this point, G418 was added to a concentration of 400 μg/ml. G418-resistant colonies were counted after 10 to 14 days of incubation in the presence of G418. Plasmids indicated below as permissive each rendered 30 to 50% of the pSV2gpt transfectants infectible.

**Results**

Residues controlling the phenotype of infectibility are encoded downstream of the conserved PstI site. Mouse cells, despite their expression of endogenous Glvr-1, are not normally infectible by GALV but can be rendered infectible by expression of human GLVR1 (12). It was therefore expected that some of the amino acid differences between human GLVR1 and mouse Glvr-1 controlled this difference in phenotype. To investigate this, hybrids of human GLVR1 and mouse Glvr-1 cDNAs were made by taking advantage of conserved KpnI and PstI sites (Fig. 1). These hybrids, in eukaryotic expression vectors, were transfected into mouse NIH 3T3 cells, which were then assayed for susceptibility to infection by GALV. As can be seen in Fig. 1, hybrids consisting of human sequence up to the KpnI or PstI site and of mouse sequence thereafter (pOJ14 and pOJ15) failed to confer infectibility. In contrast, pOJ20, encoding mouse sequence up to the PstI site and human sequence thereafter, was as effective as pOJ9 in rendering cells infectible. This indicated that some of the divergent residues encoded downstream of the PstI site are indispensable for receptor function and that amino acid differences between the mouse and human proteins up to human amino acid residue 502 do not affect receptor function. Two clusters of residues that are divergent between the human and mouse proteins have been noted downstream of the PstI site (6). These correspond to human residues 550 to 558 (referred to as region A) and 671 to 679 (region B).

**Human residues in region A are indispensable for infection.** To determine which of the divergent residues encoded downstream of the PstI site were involved in infection, in vitro mutagenesis was carried out on human GLVR1 and mouse Glvr-1. pOJ35, encoding mouse region B in place of human region B, conferred infectibility (Table 1). When human region A was replaced with mouse region A (pOJ34), the construct did not confer infectibility. These results suggested that the sequence coding for human region A constituted the critical difference between the two cDNAs. This was confirmed by testing of pOJ38 and pOJ37. pOJ38, the mouse cDNA encoding human region B, failed to confer infectibility, again suggesting that these residues play no essential role in infection. pOJ37, the mouse cDNA encoding human region A in place of mouse region A, rendered the mouse cells susceptible to infection. The results therefore show that, of all the amino acids which are divergent between the human and mouse proteins, only human residues 550 to 558 (region A) play a critical role in infection. To discount the possibility that mouse region A had a dominant negative effect on infection (for example, by blocking the action of another domain essential for infection), pOJ36, which encodes the residues in human GLVR1 and mouse Glvr-1, was as indicated in pOJ9; mouse, the unaltered mouse sequence encoded in pOJ9; mouse, the unaltered mouse sequence encoded in pOJ19.

**FIG. 1. Residues controlling the phenotype of infectibility are encoded downstream of the conserved PstI site.** cDNAs are shown for homologs of human, mouse, and rat GLVR1 proteins and hybrids thereof, cloned in expression vectors. Only the open reading frames involved are shown. Locations for the KpnI and PstI sites conserved in human and mouse cDNAs are indicated at the top (K and P, respectively). The primer used to amplify the rat cDNA by polymerase chain reaction introduces a PstI site at the same position.

**TABLE 1. Effect of exchanging regions A and B on permissivity**

<table>
<thead>
<tr>
<th>Clone</th>
<th>Region A</th>
<th>Region B</th>
<th>Permissivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>pOJ34</td>
<td>-KQEAESTKA</td>
<td>VFKYIILPV</td>
<td>-</td>
</tr>
<tr>
<td>pOJ35</td>
<td>KQEAESTKA</td>
<td>VPQYIILPV</td>
<td>+</td>
</tr>
<tr>
<td>Human</td>
<td>550 DTGDVSXVK</td>
<td>558 IFLYIMLV</td>
<td>679 +</td>
</tr>
<tr>
<td>Mouse</td>
<td>553 -KQEAESTKA</td>
<td>560 VFKYIILPV</td>
<td>681 +</td>
</tr>
<tr>
<td>pOJ38</td>
<td>-KQEAESTKA</td>
<td>VPQYIILPV</td>
<td>-</td>
</tr>
<tr>
<td>pOJ37</td>
<td>DTGDVSXVK</td>
<td>VFKYIILPV</td>
<td>679 +</td>
</tr>
<tr>
<td>pOJ36</td>
<td>SKVTGVGDD</td>
<td>-</td>
<td></td>
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</tbody>
</table>

* Only regions mutated in the indicated plasmids are shown. The numbers (amino acid residues) indicate the boundaries of the regions mutated. Mutant human cDNA sequences, altered to encode corresponding mouse amino acid residues, are indicated above the human sequence. Mutant mouse cDNA sequences, altered to encode corresponding human amino acid residues, are indicated below the mouse sequence (pOJ37 and pOJ38, see text).

* Only regions mutated in the indicated plasmids are shown. The numbers (amino acid residues) indicate the boundaries of the regions mutated. Mutant human cDNA sequences, altered to encode corresponding mouse amino acid residues, are indicated above the human sequence. Mutant mouse cDNA sequences, altered to encode corresponding human amino acid residues, are indicated below the mouse sequence (pOJ37 and pOJ38, see text).
region A in scrambled order, was constructed and tested. This plasmid failed to confer infectibility, indicating that residues 550 to 558 must be present in the human protein form and in a particular sequence.

**Rat GLVR1 differs from both mouse Glvr-1 and human GLVR1 in region A.** Analysis of GLVR1 sequences from species other than humans and mice should be instructive as to sequence requirements for infectibility. Rat cells are infectible by GALV. Because the rat and the mouse are so closely related, it was thought that analysis of the rat sequence would be particularly instructive in guiding later mutagenesis studies to identify residues critical for infection. A portion of rat GLVR1 cDNA was therefore isolated and sequenced. To ensure that the cloned sequence was compatible with infection, pOJ52, consisting of mouse sequence up to amino acid residue 523 and rat sequence thereafter, was tested and was found to confer infectibility (Fig. 1). Two features of the amino acid sequence of this portion of rat GLVR1 were noteworthy (Fig. 2). First, the mouse sequence diverges considerably from the rat sequence only in region A. This again highlights the importance of this region in conferring permissivity. Second, the rat sequence differs considerably from the human sequence in this region, despite the fact that both confer permissivity.

**Substitution of two amino acids in Glvr-1 allows it to function as a receptor.** Comparison of the three sequences in Fig. 2 was used to direct further mutagenesis studies to define residues critical for infection. We questioned what features of region A are conserved in both human and rat sequences but not in the mouse sequence. Such features suggest themselves as being necessary for infection. Features not conserved in human and rat sequences suggested themselves as being less likely to be important for infection. To test the possibilities raised by this comparison, residues within the mouse cDNA region A were converted to the human form. Mouse residues S-557 to A-560, corresponding to human S-555 to V-558, were not considered for mutagenesis because S-555 is the same in mouse and human sequences, S-556 is represented by a threonine in both rat and mouse sequences, K-557 is identical in the sequences of all three species, and V-558 differs in the sequences of all three. Insertion of human D-550 between mouse residues 552 and 553 (pOJ63) or changing mouse residue 553 to 556 one at a time from the mouse form to the human form in the mouse cDNA did not confer susceptibility (pOJ66, pOJ67, pOJ69, and pOJ70; Table 2). Changing mouse residues 550 to 556 (KOE) to the corresponding human residues, 550 to 553 (DTGD), rendered cells infectible (pOJ63), drawing attention to these four residues as being critical. Since the mouse sequence is one residue shorter than the human sequence in this region and lacks T-551, which is conserved in the human and rat sequences, a threonine was placed between mouse K-553 and Q-554 (pOJ64). This insertion had no effect. However, simultaneous replacement of mouse K-553 with an aspartate (pOJ68) conferred infectibility. The importance of this residue (D-550) was confirmed by removing it and showing a simultaneous loss of receptor function (pOJ71).

**DISCUSSION**

In previous work, it was demonstrated that human GLVR1 confers susceptibility to infection by GALV, while mouse Glvr-1 does not (6). This implied that some of the amino acid residues that are divergent between the two proteins are critical for infection and that their identification might provide insight into the process of infection. Analysis of a series of hybrid cDNAs and in vitro mutants allowed the determination that human residues D-550 and T-551 were the only residues that are divergent between the mouse and human sequences which were critical for infection. While these may be the only changes in the mouse cDNA necessary to confer susceptibility, it is possible that changes other than those we made within mouse residues 553 to 556 could also do so. Such a situation exists for MCAT. Albritton et al. (2) and Yoshimoto et al. (16) found different (but overlapping) changes which could render the human homolog of MCAT permissive for infection by murine leukemia virus.

The results obtained are similar to those of Tailor et al. (13). In their experiments, pOJ37 (encoding mouse Glvr-1 with human residues 550 to 558 replacing the corresponding residues) was as effective as the human cDNA in making mouse cells permissive for both other viruses in the same interference group as GALV (simian sarcoma-associated virus and feline leukemia virus subgroup B). Those authors also cloned and tested rat GLVR1 sequence identical to that presented here and also found the rat sequence to be compatible with infection by GALV.
An interesting aspect of these results is that amino acids critical for infection are contained within a domain which is highly polymorphic between species. This polymorphism suggests that the region must display only limited characteristics to allow infection, characteristics which are not present in the mouse sequence. Though it has not yet been demonstrated, it is possible that the region forms part of the virus binding site. This is because the results presented here show that the region is needed for infection and because the region is hypothesized to be exposed on the outside of the cell, constituting the fourth of five postulated extracellular domains (6). However, the lack of strong sequence conservation is incompatible with conservation of a high-affinity virus binding site. If this region does interact directly with viral envelope glycoprotein, it may be that other domains of the protein contribute to this function in order to form an overall high-affinity binding site.

Adamson et al. (1) have shown that mice carry multiple proviruses related to GALV and suggest that Glvr-1 could at one time have served as a receptor for these viruses. The fact that the closely related rat and mouse GLVR1 proteins show a cluster of differences in a region critical for infection supports this contention. Thus, if the mouse was at some time subjected to infection by a pathogenic virus using Glvr-1 as a receptor, there would have been a selection for an altered, nonpermissive form of the protein.

Despite the polymorphism in the region, the general characteristics of the amino acids within it are conserved between infectible species (humans and rats). In order, these are an acidic residue, a threonine, a variable residue, an aspartate, three neutral residues, a lysine, and a variable residue. It therefore appears that the region is not merely a linker between transmembrane domains but must retain some characteristics to allow normal function of the protein.

REFERENCES