Complementation of a Binding-Defective Retrovirus by a Host Cell Receptor Mutant
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The entry of ecotropic murine leukemia virus (MLV) into cells requires the interaction of the envelope protein (Env) with its receptor, mouse cationic amino acid transporter 1 (mATRC1). An aspartic acid-to-lysine change at position 84 (D84K) of ecotropic Moloney MLV Env abolishes virus binding and infection. We recently identified lysine 234 (rK234) in mATRC1 as a residue that influences virus binding and infection. Here we show that D84K virus infection increased 3,000-fold on cells expressing receptor with an rK234A change and 100,000-fold on cells expressing an rK234D change. The stronger complementation of D84K virus infection by rK234D than by the rK234A receptor suggests that although the major reason for loss of infection of D84K and D84R virus is due to steric hindrance and charge repulsion, the loss of an interaction of D84 with receptor appears to contribute as well. Taken together, these results indicate that D84 is very close to rK234 of mATRC1 in the bound complex and there is likely an interaction between them. The definitive localization of the receptor binding site on SU should facilitate the design of chimeric envelope proteins that target infection to new receptors by replacing the receptor binding site with an exogenous ligand sequence.

Infection by retroviruses is mediated by interacting between the viral envelope protein (Env) and host cell receptor. The Env contains two subunits: the surface subunit (SU), responsible for receptor recognition and binding, and the transmembrane subunit (TM), promoting viral and cellular membrane fusion. Among several residues that influence receptor binding (3, 13), aspartate 84 (D84) on ecotropic Moloney murine leukemia virus (MoMLV) appears to have the greatest importance. Replacement of D84 with lysine (D84K) completely abolishes virus binding and infection (13). Other substitutions, e.g., replacement by valine (D84V), gave a 250-fold decrease in infection (13). Similar results were obtained in ecotropic Friend 57 MLV (FrMLV) when the homologous aspartic acid 86 was changed (5). Notably, the single substitution that did not affect infection of NIH 3T3 cells was a semiconservative change to asparagine (5).

We sought to determine if D84 directly interacts with the virus receptor or if the D84K change affects infection by acting at a distance from the actual site of Env-receptor contact. Since receptor binding appears to trigger the conformation changes that activate Env for membrane fusion, this information is of importance in understanding the triggering mechanism. It may also be an important consideration in developing new approaches to the design of chimeric Env that redirect or target infection to a new receptor, particularly if targeted infection is to approach the efficiency of natural MoMLV infection.

The ecotropic MLVs utilize a common receptor, the mouse cationic amino acid transporter 1 (mATRC1, also called MCAT-1) (2, 11, 19). Although all mammals have a homologous ATRC1 transporter, only ATRC1s from mice and rats are capable of functioning as virus receptors. Several residues within its third extracellular domain have been shown to influence virus binding: asparagine 232 (rN232), valine 233 (rV233), tyrosine 235 (rY235), and glutamic acid 237 (rE237) (1, 21). Substitution of two or more of these residues greatly reduced binding of purified SU and infection, whereas replacement of any single one does not affect binding or infection, indicating that none of them is essential (1). Addition of all of these critical residues to the human ATRC1 gave 30% purified SU binding compared to that with the wild-type (WT) receptor (1).

We recently showed that lysine 234 (rK234) in the receptor also influences binding. Although infection via mutant receptors carrying a lysine 234-to-alanine (rK234A), a lysine-to-glutamate (rK234E), or a lysine-to-aspartate (rK234D) change was comparable to that via WT receptor, virus binding was reduced (16). In addition, 293 cells expressing double mutant rY235A plus rK234D receptors showed greatly reduced infection and loss of binding, whereas infection was not affected by double mutant rE237A plus rK234D (16).

Structural data on the SU-receptor complex would reveal a precise molecular description of binding. The structure of the receptor binding domain (RBD; residues 1 to 236) of SU has been solved (6); however, limitations in solving the structure of complexes consisting of large soluble proteins bound to multiple membrane pass proteins like mATRC1 have prevented solution of the binary complex. Based on genetic data, Davey et al. proposed that a hydrophobic pocket at the top of the RBD is the receptor binding site (5). This putative site includes
the critical aspartate. In Davey’s model, the critical receptor residues rY235 and rE237 bind in this pocket directly to the FrMLV equivalent of D84 and a nearby tryptophan residue, W102 (5).

Two obvious questions are (i) why does a D84K change reduce binding and (ii) which, if any, residue(s) in the receptor interacts with the critical D84. Here we report that D84K virus infection increased 3,000-fold on cells expressing receptor with a rK234A change and 100,000-fold on cells expressing a rK234D change compared to WT receptor. This complementation suggests that steric hindrance and charge repulsion between the lysine at position 84 of the D84K Env mutant and the lysine in position 234 of the receptor accounts for the largest part of the loss of infection of D84K virus and by inference that D84 is close to rK234 in the SU-receptor complex. The stronger complementation of D84K virus infection by rK234D than by rK234A receptor supports that a potential interaction between these two residues influences binding.

RESULTS

In this study, we used genetic approaches to investigate the reason D84K virus is noninfectious and to identify potential binding partners in the receptor. First, we asked why D84K virus lost binding and infection activity. Since D84V virus infection was only 250-fold lower than that with WT virus, we hypothesized that replacement of lysine at position 84 sterically hinders binding to receptor or prevents binding by charge-charge repulsion with a positively charged receptor residue. To test this hypothesis, arginine (D84R) and alanine (D84A) were introduced into position 84 of ecotropic MoMLV Env.Titers of viruses carrying the individual D84K, D84R, and D84A mutations were determined on mouse NIH 3T3 cells expressing endogenous mATRC1 and human 293 cells stably expressing mATRC1 (293/mATRC1). All of the mutant Env were as efficiently incorporated into virions as WT Env and were processed normally (Fig. 1).

In agreement with previous reports (5, 13), D84K substitution completely abolished virus infection on both cell types (Fig. 1). Although slightly greater infection was observed with the novel D84R substitution, this virus was almost noninfectious on NIH 3T3 cells and showed 10,000-fold lower infection of 293/mATRC1 cells when compared to WT virus infection. In contrast, D84A virus was almost as infectious as WT virus on NIH 3T3 cells and was comparable to WT virus infection on 293/mATRC1. With respect to the difference of D84A virus titer between NIH 3T3 and 293/mATRC1 cells, the receptor levels on 293/mATRC1 cells are several times that on NIH 3T3 cells (4). Although cell-type-specific differences might also be involved, Davey and coworkers previously reported that overexpressing mATRC1 in NIH 3T3 cells gave a substantial increase of infection by virus carrying a D86A change in the SU of FrMLV (homologous to D84A in MoMLV) (5).

Virus binding. Virus binding assays were performed exactly as previously described (16). Briefly, virus stock was concentrated and shedded SU was removed using a Centrifloc Plus-80 device (100-kDa molecular mass cutoff). A total of 10⁶ cells were incubated with concentrated virus stock for 1 h at 4°C, after which unbound virus was removed by two washes with ice-cold PBA (4% fetal bovine serum, 0.02% sodium azide in phosphate-buffered saline), and virus-cell complexes were incubated sequentially at 4°C with goat anti-gp70 antisera (1:100) and with fluorescein isothiocyanate-conjugated donkey anti-goat anti-antiserum (1:200; Jackson Laboratories). The fluorescence intensity was analyzed by flow cytometry (Epics Profile Analyzer; Coulter Cytometry). Parent 293 cells incubated with WT virus and 293/mATRC1 incubated with virus-free medium served as negative controls. In some cases, viruses and cells were incubated for 20 min at 23 and 37°C, washed thrice to remove unbound virus using PBA preheated to 23 or 37°C, then fixed in 10% Formalin (Sigma) for 20 min at room temperature, and then washed twice more with 23 or 37°C PBA prior to incubation with antibodies. Cells incubated at 23 or 37°C for longer than 20 min showed appreciable cell death (as great as 20%).

**MATERIALS AND METHODS**

**Cell lines.** Susceptible mouse NIH 3T3 cells and nonsusceptible human embryonic kidney 293 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) and 8% donor calf serum. The 293 cell line that stably expresses WT or mutant mATRC-1 tagged with HA1 epitope was maintained in DMEM with 8% donor calf serum and 250 µg of G418 (Sigma)/ml. The virus producer cell line Hi-BAG (293 cells stably expressing the recombinant MoMLV-derived BAG genome encoding β-galactosidase) was cultured in DMEM with 8% fetal bovine serum and 250 µg of G418/ml.

**Plasmid constructions.** Plasmid pDNA-MoMLV carrying gag, pol, and ectropic env genes has been described elsewhere (24) and was used as a backbone for construction and expression of the mutant Env. Specific substitutions on the env gene were generated by oligonucleotide-directed mutagenesis (QuickChange kit; Stratagene). After mutagenesis, the PmlI and BspEI fragment containing specific env mutations was subcloned into the PmlI and BspEI sites of pDNA-MoMLV, and the DNA sequence was confirmed. The construction of pcDNA mATRC1-HA encoding WT mouse Atrc-1 cDNA (accession number M26687) was described elsewhere (12). Briefly, the sequences encoding three copies of the HA1 epitope tag from influenza virus hemagglutinin protein (HA) were fused to the carboxyl-terminal residue of the receptor. For construction of the receptor mutants, substitutions were obtained by oligonucleotide-directed mutagenesis (QuikChange kit; Stratagene), the Hpal-Agel fragment containing each mutation was inserted into the Hpal-Agel site of pcDNA mATRC1-HA, and the sequence was confirmed.

**Virus production, titration, and Western blot analysis.** Hi-BAG cells were seeded at approximately 2 x 10⁶ cells per 100-mm dish and grown for 2 days until cells were about 60 to 70% confluent. Then, the cells were transfected using the CaPO₄ coprecipitation method (17) with 40 µg of pcDNA-MoMLV or its derivative plasmids containing mutant Env. After overnight incubation, the precipitates were replaced with 10 ml of fresh medium containing 8% fetal bovine serum. The virus supernatant was harvested 24 and 48 h later, and each harvest was filtered through a 0.45-µm-pore-size filter. An aliquot of 3 ml was used to quantitatively infect 1.2 x 10⁶ cells by end point dilution titration. Target cells (about 5 x 10⁴ per well) were seeded in 24-well plates and then exposed to a serial 10-fold dilution of virus in the presence of 20 µg of Polybrene/ml. After overnight incubation with virus, the medium was removed and replaced with fresh medium. The next day cells were fixed and assayed for β-galactosidase activity using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining. Viral titer was calculated as the 50% tissue culture infective dose (TCID₅₀) on October 30, 2017 by guest http://jvi.asm.org/ Downloaded from
234-to-alanine (rK234A) change should solve the problem by eliminating the side chain or its positive charge to give D84K and D84R infection. We previously established 293-derived cell lines that stably express rK234 mutant receptors at or slightly above the level of control 293/mATRC1 (16). These included rK234A, a mutant receptor with the small nonpolar side chain of an alanine replacing the positively charged lysine. This mutant mediates WT virus entry as efficiently as mATRC1 (Fig. 2) (16). D84K and D84R virus showed over 4,300- and 75-fold increases in infection via rK234A, consistent with the binding site becoming much more accessible to the mutant virus when the lysine is replaced by alanine.

Replacement of rK234 with aspartic acid complements the D84 down mutations in SU. These results raised the question: does D84 interact with rK234? To address this question, we quantified infection of D84K and D84R viruses via receptor mutant rK234D, effectively “swapping” charges on SU and receptor. We reasoned that if the swapped charges recapitulated an interaction similar to one occurring in binding, then infection would be increased. A decrease or lack of increase would argue against a natural interaction between the two residues.

As previously reported, WT virus infected 293 cells expressing a rK234D mutant receptor as efficiently as 293/mATRC1 (Fig. 3A) (16). The mean titers of D84K and D84R viruses on rK234D receptor-positive cells were $(5.3 \pm 3.3) \times 10^5$ (mean ± standard error) and $(3.3 \pm 0.9) \times 10^5$ infectious units (IFU)/ml, respectively, about sixfold lower than WT virus on mATRC1-positive cells (2 × 10⁶ IFU/ml) (Fig. 3A). Importantly, the level of complementation was substantially greater than complementation seen on rK234A-expressing cells, where infection was 500- and 80-fold lower than WT virus for D84K and D84R virus, respectively. We also quantified infection using a receptor mutant with another negative-for-positive-charge swap (glutamic acid; rK234E). Infection of the D84 down mutations via rK234E was only slightly improved compared to infection and D84R virus showed over 4,300- and 75-fold increases in infection via rK234A, consistent with the binding site becoming much more accessible to the mutant virus when the lysine is replaced by alanine.

FIG. 1. Substitution of D84 in SU with arginine but not with alanine results in marked reduction of infection. (A) Infection of NIH 3T3 cells. Serial 10-fold dilutions of viruses pseudotyped with Env carrying the indicated substitutions were incubated with quadruplicate wells of NIH 3T3 cells. Virus infection was calculated from the end point dilution ($n = 4$) in infectious units per milliliter, defined as the lacZ-transducing infectious units per milliliter. Mean results of three independent experiments are shown ± standard errors. No error bar is indicated for infection of D84K virus because a zero value was observed in all three independent experiments. (B) Infection of 293/mATRC1 cells, a 293-derived cell line stably expressing mATRC1 at twice the level of NIH 3T3 cells (4). Each value is the average of results from three independent experiments ± the standard error. No error bar is indicated for infection of D84K virus because a zero value was observed in all three independent experiments. (C) Western blot analysis of virions. Virus pellets were separated on an SDS–8% polyacrylamide gel and immunoblotted to anti-SU antiserum (top) and anti-CA antiserum (bottom).

FIG. 2. Replacing the side chain of rK234 in the receptor with an alanine gives infection by D84K or D84R virus. White bar, infection of 293/mATRC1; black bar, infection of 293 cells expressing rK234A mutant receptor. Values shown are the mean ± standard error of infection calculated from the end point dilution from three independent experiments. No error bar is indicated for infection of WT virus on mATRC1-positive cells because values shown for three independent experiments were normalized to the value of WT virus on these cells. In addition, the error bar for D84K infection of these cells reflects a value of zero in two of the three experiments and a value of 2 in the third experiment.
via mATRC1 (Fig. 3B). Here, infection was less than with aliquots of the same virus stocks on rK234A-expressing cells. Notably, infection when the side chain charges were swapped, i.e., D84K via the rK234D receptor ([5.3 ± 3.3] × 10^5 IFU/ml), was comparable to infection in the absence of interactive side chains at either position, i.e., D84A virus that via rK234A receptor ([7.0 ± 1.3] × 10^5 IFU/ml) (Fig. 3C). Both remained slightly below that of WT virus infection via these receptor mutants (2.0 × 10^6 IFU/ml).

Adding either rY235A or rE237A to a rK234D change in receptor abolished the complementation of D84K or D84R in SU. In our previous characterization of the virus binding site, we observed that combination of the rK234D and rY235A changes almost abolished virus infection, whereas combination of rK234D plus rE237A had little or no effect on infection (16). We asked if a similar difference would hold for the D84 substituted viruses. The results are shown in Fig. 4. In contrast to WT virus infection, D84R or D84K virus did not show substantial infection of cells expressing rK234D plus rE237A receptor. Interestingly, D84A virus showed a marked decrease via rK234D plus rE237A receptors, over 300-fold less than their infection via mATRC1.

**Virus binding studies.** Virus supernatant was concentrated under conditions that retain virion-associated SU while eluting shedded SU through a Centricon filter (16, 23, 24) and then used in a flow cytometry-based equilibrium binding assay (10). The restored infection seen in the virus titrations did not correlate with binding of the D84A, D84K, and D84R virus at 4°C using this assay (Table 1). As previously reported, WT virus infection of rK234D- and rK234E-expressing cells also did not correlate with binding under these conditions (16).

The lack of correlation between virus binding and infection could be the result of the temperature difference between the assays (4°C versus 37°C). It has been shown that temperature affects the association and dissociation rate of binding (22) and that WT virus binding to NIH 3T3 cells is increased at 37°C. Consequently, virus binding assays were performed again at two higher temperatures: 23 and 37°C.

The results are shown in Fig. 5. No significant difference was
observed in WT virus binding to 293/mATRC1 cells at 23 versus 37°C, and these levels were not greater than that seen at 4°C, the increase in mean fluorescence intensity (MFI) being 40-fold in each case. Notably, D84A virus showed a 20-fold increase in MFI at both 23 and 37°C on 293/mATRC1 cells, a level approximately 50% of WT virus binding. However, no

D84K virus binding to rK234D cells was evident at the higher temperatures.

DISCUSSION

The finding that the lysine 234-to-alanine change in the rK234A receptor restored infection of D84K and D84R viruses is strong evidence that the primary cause of the loss of infection is steric hindrance and charge-charge repulsion between these two residues. This suggests that D84 and rK234 are in close physical proximity in the SU-receptor complex. Complementation of D84K and D84R viruses by rK234D mutant receptor also strongly supports close proximity of the two residues. In addition, the higher infection of these two mutant viruses on rK234D than rK234A is consistent with a potential interaction between K84 or R84 and rD234.

Swapping charges between the Env and receptor residues is complementary, presumably because it maintains a relationship of size and charge similar to that normally present in the binary complex. The data for rK234E versus rK234A support the idea that charge and size influence complementation. We interpret higher complementation using rK234A as indicating that size has a greater influence than charge, that is, it is better to have no charge and small size, as in alanine, than to have charge and a less-than-optimal size, as in glutamic acid. Thus, the greatest level of complementation (infection) came from an exact swap between Env and the receptor, e.g., D84K Env and the rK234D receptor, which evidently have the most parsimonious size and charge.

Size may also explain why replacing D84 with lysine is more deleterious than replacing rK234 with aspartic acid. For example, the length of the side chains in residues 84 of Env and 234 of the receptor may influence the closest distance the two molecules can approach. Lysine side chains can extend almost twice the distance as aspartic acid side chains. The side chain of the critical aspartate extends 3.23 Å from its alpha-carbon in the Fr57 RBD structure (6), while Lys side chains extend about 5.17 to 6.22 Å for K106, -113, -131, -182, and -196 in the Fr57 RBD structure (6). Thus, the closest approach may be optimal for D84 with rK234, shorter for D84 with rK234D, and longer than optimal for D84K with rK234. If so, then a rK234D substitution would be expected to be less deleterious than a D84K change, since the strength of binding should be proportional to the distance between molecules. We favor this explanation, although others exist.

To date, no single residue of SU or of the receptor has been shown to be essential for virus infection. These results are consistent with the idea that SU-receptor binding involves multiple interactions. We favor that aspartic acid 84 contributes directly to infection and the Env binding interaction but is not essential to them, based on three observations. First, D84A virus was over 300-fold less infectious than WT virus on the rK234D plus E237A receptor (Fig. 4). Second, at 37°C, D84A virus binding to mATRC1 was only 50% of WT virus binding. If residue 84 made no direct contribution, then D84A infection and binding should have been comparable to that of WT virus on these receptors. Lastly, we interpret the lack of a substantial reduction in D84A virus infection via the rK234A receptor to be another indication that the contribution of residue 84 is not essential, that is, when its interactions are removed the remain-
ing interactions between SU and the receptor still allow for infection. By similar reasoning, the contribution of rK234 also appears to contribute directly to infection but is not essential to it. The loss of infection via double receptor mutants (Fig. 4) provides further support for this concept.

The phenotype of D84A MoMLV reported here is similar in infection of NIH 3T3 cells to that reported for D86A FrMLV (5) but differs slightly in receptor binding. D86A FrMLV was almost as infectious as WT virus (20-fold less infection [5]), similar to D84A, observed here to be 3- to 8-fold less (Fig. 1A). However, D86A SU showed loss of receptor-specific binding (5), while D84A MoMLV binding was detectable at 23 and 37°C, albeit at half the level of WT virus (Fig. 5). One possibility for this difference is that in FrMLV the two additional residues present just upstream of D86 might somehow result in a greater dependence on the critical aspartic acid residue.

Infection did not correlate with virus binding for most of the combinations of Env and receptor mutants. Yet infection provided de facto evidence that virus binding occurred. A similar lack of correlation has been reported for other mutant Env (5, 13) and for WT MoMLV infection via other receptor variants (1). One explanation may be the relative sensitivity of the two assays. For instance, virus infection assays can potentially detect a single virus infection of a single cell, whereas the binding assay relies on detection of multiple viruses attached to each cell (10). Alternatively, the mutations may increase the dissociation rate so that few viruses are attached to the host cells at any one moment. An increase in dissociation rate might have a less profound effect on infection. In human immunodeficiency virus infection, for example, Env-mediated fusion is a slow process, taking about 15 to 20 min for prebound Env to begin fusion (7, 14, 15, 20). In contrast, virus binding is a much more rapid event; half-maximal binding is often achieved within 2 min, and equilibrium can occur within 10 min (10).

Based on the location of the critical aspartic acid in the crystal structure of the FrMLV RBD (6), aspartic acid 86 and tryptophan 102 (homologous to aspartic acid 84 and tryptophan 100 in MoMLV) have been proposed as the binding partners of rY235 and rE237 in the receptor (5). Given the observations reported here, we propose the following refinements to this concept: rK234 positions directly adjacent to and interacts with D84. This location would place rY235 further from the aspartic acid residue than previously proposed. Figure 6 shows the configuration that we visualize for an rK234 interaction with D84. Dashed lines depict potential hydrogen bonds predicted by SwissModel between the lysine side chain and D84 and S82 in SU.

**FIG. 6.** Model for the close proximity of D84 and rK234 in the SU-receptor complex. The amino acid sequence of the MoMLV RBD (residues 1 to 234) was aligned with that of FrMLV (residues 1 to 236), and the alignment was used to model the structure of MoMLV sequences using SwissModel (8, 9). (A) Aspartic acid 84 (D84), serine 82 (S82), and tryptophan 100 (W100) are shown as stick diagrams, other residues in variable regions A and B are shown as space filled, and the remaining residues are depicted as ribbon diagrams rendered using RasMol (18). A stick diagram of a lysine residue representing rK234 in the virus receptor is shown to depict how we visualize its side chain closely juxtaposed to that of D84 on the RBD. Opaque space-filled versions are superimposed on stick diagrams. (B) A close-up of the region around D84 in the model, showing one possible configuration for the rK234 interaction with D84. Dashed lines depict potential hydrogen bonds predicted by SwissModel between the lysine side chain and D84 and S82 in SU.
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