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Variable Regions A and B in the Envelope Glycoproteins of Feline Leukemia Virus Subgroup B and Amphotropic Murine Leukemia Virus Interact with Discrete Receptor Domains

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The surface (SU) envelope glycoproteins of feline leukemia virus subgroup B (FeLV-B) and amphotropic murine leukemia virus (A-MLV) are highly related, even in the variable regions VRA and VRB that have been shown to be required for receptor recognition. However, FeLV-B and A-MLV use different sodium-dependent phosphate symporters, Pit1 and Pit2, respectively, as receptors for infection. Pit1 and Pit2 are predicted to have 10 membrane-spanning domains and five extracellular loops. The close relationship of the retroviral envelopes enabled us to generate pseudotype virions carrying chimeric FeLV-B/A-MLV envelope glycoproteins. We found that some of the pseudotype viruses could not use Pit1 or Pit2 proteins but could efficiently utilize specific chimeric Pit1/Pit2 proteins as receptors. By studying *Mus dunni* tail fibroblasts expressing chimeric Pit1/Pit2 proteins and pseudotype virions carrying chimeric FeLV-B/A-MLV envelopes, we show that FeLV-B and A-MLV VRA and VRB interact in a modular manner with specific receptor domains. Our results suggest that FeLV-B VRA interacts with Pit1 extracellular loops 4 and 5 and that residues Phe-60 and Pro-61 of FeLV-B VRA are essential for receptor choice. However, this interaction is insufficient for infection, and an additional interaction between FeLV-B VRB and Pit1 loop 2 is essential. Similarly, A-MLV infection requires interaction of A-MLV VRA with Pit2 loops 4 and 5 and VRB with Pit2 loop 2, with residues Tyr-60 and Val-61 of A-MLV VRA being critical for receptor recognition. Together, our results suggest that FeLV-B and A-MLV infections require two major discrete interactions between the viral SU envelope glycoproteins and their respective receptors. We propose a common two-step mechanism for interaction between retroviral envelope glycoproteins and cell surface receptors.

The murine leukemia viruses (MLVs), feline leukemia viruses (FeLVs), and gibbon ape leukemia viruses (GALVs) belong to the group of mammalian type C retroviruses (9). MLVs and FeLVs are further classified into subgroups based on their abilities to use different cell surface receptors for infection (17, 33, 36). Thus, MLVs are subgrouped into ecotropic (E-MLV), amphotropic (A-MLV), xenotropic (X-MLV), polytropic (MCF-MLV), and 10A1, while FeLVs are subgrouped into types A, B, and C. The entry of retroviruses into target cells is governed by the interaction of the retroviral surface (SU) envelope glycoprotein with specific cell surface receptors (42). Studies of naturally occurring and recombinant MLVs and FeLVs have identified the amino-terminal domain of the SU glycoprotein as responsible for receptor recognition and binding (5, 6, 24, 30). Within the amino-terminal domain, stretches of conserved residues are disrupted by three highly variable regions termed VRA, VRB, and a proline-rich region (PRR) located downstream of VRA and VRB. These variable regions are responsible for receptor recognition by MLV SUs (5, 6). Substitutions of the variable regions between MLV SUs have indicated that VRA and VRB of E-MLV and A-MLV contain all determinants necessary for receptor binding whereas MCF- and X-MLVs require additional sequences within PRR (5). The VRA and VRB of MLVs contain several cysteine residues that are conserved in all mammalian type C viral envelope glycoproteins, including FeLV and GALV envelopes. These conserved cysteines have been suggested to form disulfide bridges creating loop structures within the variable regions (21). Thus, VRA can form two disulfide-linked loops whereas VRB can form one loop. The first potential disulfide-linked loop within VRA is highly variable among mammalian type C envelopes. E-MLV envelope residues critical for receptor recognition have been localized to the first loop of VRA (4, 24). Critical residues responsible for receptor choice in FeLV-C SU have been mapped to a region called vr1 which corresponds to the VRA loop 1 of MLVs (7, 34). These findings suggest that residues critical for receptor choice in mammalian type C envelope glycoproteins may be located in similar loop structures. Other residues outside the predicted VRA loop 1 have also been implicated in receptor binding. PVC-211 MLV, a variant of Friend E-MLV, efficiently infects Chinese hamster ovary (CHO) cells, which are normally resistant to other E-MLVs (23). Two amino acids adjacent to the predicted first VRA loop of PVC-211 MLV SU have been implicated in its broadened receptor recognition. The 10A1 MLV SU glycoprotein, which differs from A-MLV SU by only six amino acids (30), has an extended ability to utilize Pit1 protein as a receptor (26, 43). All six amino acids lie outside the VRA loop 1.

The receptor proteins for E-MLV, GALV, FeLV-B, and A-MLV have been cloned and characterized (2, 25, 27, 39, 46). GALV and FeLV-B utilize the human protein Pit1 but not mouse Pit1 (38) as a receptor for infection, whereas the related protein Pit2 acts as the receptor for A-MLV (25, 46). Pit1 and Pit2 show 62% identity and are structurally and functionally related. The proteins are predicted to have 10 membrane-spanning domains with five extracellular loops, and they func-

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tion normally as sodium-dependent phosphate symporters (19, 28). Studies of chimeric Pit1 proteins made between human, mouse, and rat Pit1 have identified specific residues in Pit1 extracellular loop 4 that are critical for GALV and FeLV-B infections (18, 38). Recent studies have suggested that additional sequences within Pit1 loop 5 are required for FeLV-B infection but are not critical for GALV infection (31). Specific Pit2 residues critical for A-MLV infection are unknown; however, several regions of the receptor have been implicated (28, 34).

In this study, we show that VRA and VRB of FeLV-B and A-MLV interact with specific Pit1 and Pit2 extracellular loops and that both interactions are required for viral infections. We have identified FeLV-B and A-MLV envelope residues 60 and 61 located within the first disulfide loop of VRA as responsible for receptor specificity. Our results suggest that FeLV-B and A-MLV infections require a two-step interaction of the envelope glycoproteins with cell surface receptors, and we propose a common two-step interaction mechanism for retrovirus penetration into cells.

MATERIALS AND METHODS

Cell lines. *Mus dunni* tail fibroblast (MDTF) cells and CEAR13 cells (CHO cells expressing rat Pit2) were used as target cells for infection studies. The TELCeB6 cell line (kindly provided by Y. Takeuchi and F. L. Cosset) (10) contains a retroviral expression plasmid expressing Moloney MLV Gag and Pol proteins. This packaging cell line produces noninfectious viral particles carrying the nlsLacZ retroviral vector. CEAR13 cells were maintained in Dulbecco's modified alpha medium with 10% fetal bovine serum. All other cell lines were maintained in Dulbecco's modified Eagle medium with 10% fetal bovine serum. MDTF cells expressing chimeric Pit1/Pit2 proteins were generated by transfecting the cDNA expression vectors pLGRGSN, pLRGGSN, pLRRGSN, pLG GRSN, pLGRRSN, and pLGGrGSN (kindly provided by A. Dusty Miller) (26). Transfected cells were selected in G418 (1.5 mg/ml), and G418-resistant clones were analyzed for phosphate uptake. Clones showing the highest level of phosphate uptake were selected for infection assays. The clones were named after the chimeric Pit1/Pit2 cDNA transfected. MDTF cells expressing Pit1 were generated by transfecting Pit1 expression vector, pCDNA3/Pit1. pCDNA3/Pit1 was generated by isolating a *Hin*dIII-*Xho*I Pit1 fragment from pOJ9 (18) and cloning it into a *Hin*dIII-*Xho*I-digested pCDNA3 expression vector.

Construction of chimeric FeLV-B/A-MLV envelopes. The A-MLV envelope expression vector was provided by Y. Takeuchi and has been described before (10). The FeLV-B envelope expression vector was generated by using the envelope gene isolated from plasmid pFGB, which contains the infectious molecular clone of FeLV-B/Gardner-Arnstein (kindly provided by J. Neil) (15). A *Pst*I-*Hin*cII FeLV-B envelope fragment from pFGB was isolated and subsequently cloned into the *Pst*I-*Eco*RV-digested KS(+) Bluescript plasmid (Stratagene). The subsequent plasmid was digested with *Bam*HI-*Cla*I, and the FeLV-B envelope fragment was cloned into the *Bam*HI-*Cla*I site of the retroviral expression vector FBSALF (10). Chimeric envelopes between FeLV-B and A-MLV were generated by using the unique *Afl*II and *Eco*RI sites present in A-MLV envelope gene. These sites are absent in FeLV-B envelope gene and were therefore introduced by site-directed mutagenesis (Amersham). One mutation, made at bp 466 to 471 of FeLV-B, changed the sequence GTAAAA to CTTAAG, creating an *Afl*II site. A second mutation, at bp 598 to 603, changed the sequence CAA TTT to GAATTC to create an *Eco*RI site. These changes caused the substitution of FeLV-B residues valine 123 and glutamine 168 to leucine and glutamate, respectively (Fig. 1b). The leucine and glutamate residues are present in the corresponding positions of A-MLV SU glycoprotein. In addition, an *Afl*III site was introduced by PCR mutagenesis in the FeLV-B *env* cDNA at bp 338 to 343, changing the sequence from ATGTCT to ACGTGT. This mutation did not alter the amino acid sequence of FeLV-B envelope. The *Afl*III site is also present in the corresponding position of the A-MLV SU gene. Chimeric envelope cDNAs were then generated by using the unique *Afl*III, *Afl*II, and *Eco*RI sites to exchange the VRA and VRB between the FeLV-B and A-MLV envelopes and to make chimeric VRAs. All chimeric envelopes cDNAs were cloned between the *Bam*HI and *Cla*I sites of the retroviral expression vector FBSALF (10).

Mutagenesis of FeLV-B VRA amino acids. Specific FeLV-B VRA residues were mutated to A-MLV VRA residues by PCR mutagenesis. A *Bam*HI-*Afl*II PCR cDNA fragment encoding the N-terminal 123 amino acids which includes VRA mutations was ligated with an *Afl*II-*Cla*I FeLV-B envelope cDNA fragment (Fig. 1; see also Fig. 6) into *Bam*HI-*Cla*I-digested retroviral expression vector FBSALF. The N-terminal domains of the mutant envelope genes which encode residues for VRA, VRB, and PRR were sequenced to confirm the mutations.

Viruses and infection. The envelope gene expression vectors were transfected into TELCeB6 cells by calcium phosphate coprecipitation (Stratagene). Transfectants were selected with phleomycin (50 μg/ml), and resistant colonies were pooled 2 weeks after selection. Viral supernatants were harvested, and infection was carried out as previously described (38). Briefly, target cells were seeded in 24-well plates (3×10^4 cells/well) and incubated overnight at 37°C. The cells were then incubated with 1 ml of serially diluted viral supernatant for 4 h in the presence of Polybrene (8 μg/ml). The viral supernatant was then replaced with fresh medium, and the cells were incubated further for 2 days before X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) staining. LacZ pseudotype titers were determined by counting the number of LacZ-positive colonies, and titers were expressed as the number of CFU obtained per milliliter of viral supernatant.

Neutralization assays were carried out by treating the viral supernatant with 5% antiserum raised against FeLV-B gp70 for 1 h at 37°C prior to incubation with the target cells. Titers of infection were determined as described above.

Immunoblots. Approximately 10^7 virus producer cells were lysed in 200 μl of IPB lysis buffer (20 mM Tris-HCl [pH 7.5], 1% Triton X-100, 0.05% sodium dodecyl sulfate [SDS], 5 mg of sodium deoxycholate per ml, 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride). Cell lysates were incubated for 10 min at 4°C and were then centrifuged at $10,000 \times g$ for 10 min. Supernatants were frozen at −70°C. Viral pellets were obtained by ultracentrifugation of 8 ml of viral supernatant at 50,000 rpm for 2 h at 4°C with a 25% sucrose cushion. The viral pellets were suspended in 100 μl of phosphate-buffered saline and frozen at −70°C. Cell lysates (15 μl) and purified viral samples (15 μl) were mixed 1:1 (vol/vol) with 2× sample buffer (125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS, 0.2% bromophenol blue, 100 mM dithiothreitol), boiled for 5 min, and then run on a 10% polyacrylamide (SDS) gel. The proteins were then transferred to a nitrocellulose filter and immunostained with 1:500-diluted goat anti-gp70 (FeLV-B) serum (Quality Biotech Inc., Camden, N.J.) and then with 1:2,000-diluted rabbit anti-goat antibody conjugated to horseradish peroxidase (Organon Teknika Corp., West Chester, Pa.). The blots were developed by using an enhanced chemiluminescence kit (Dupont NEN).

RESULTS

Properties of native and chimeric FeLV-B and A-MLV envelope glycoproteins. Despite the fact that FeLV-B and GALV use Pit1 whereas A-MLV uses Pit2, sequence comparison of their SU envelope glycoproteins revealed a much closer homology between FeLV-B and A-MLV SUs than between FeLV-B and GALV SUs. We compared the N-terminal amino acids, including the VRA and VRB sequences, of FeLV-B, A-MLV, and GALV SU by using a dot matrix plot analysis (Fig. 1a). FeLV-B SU showed a high sequence identity to A-MLV SU (59% homology), even within VRA and VRB, with few breaks or shifts in the line of homology. Breaks or shifts in dot matrix plots are indicative of nonhomologous regions, insertions, or deletions. In contrast, there was considerable sequence divergence between FeLV-B and GALV SU (35% homology), with large gaps in VRA and VRB. The sequence alignment of the N-terminal 212 amino acids of FeLV-B and A-MLV is shown in Fig. 1b. This alignment shows that FeLV-B VRA and VRB are highly homologous in length and sequence to A-MLV VRA and VRB. The alignment also shows the conserved cysteine residues which have been suggested to form disulfide links (21).

Based on the close homology of the FeLV-B and A-MLV SU proteins, we generated chimeric FeLV-B/A-MLV envelopes by using the unique *Afl*II and *Eco*RI sites present in the A-MLV SU envelope gene (Fig. 1b and c). These sites are absent in the FeLV-B SU gene and were therefore introduced by site-directed mutagenesis. Introduction of these sites caused substitutions of FeLV-B valine 123 to leucine and glutamine 167 to glutamate (Fig. 1b), residues which are present in the corresponding positions of A-MLV SU. These substitutions did not alter the host range of FeLV-B, and antiserum raised against FeLV-B gp70 effectively neutralized the virus (data not shown). This antiserum also detected the A-MLV envelope glycoprotein in protein immunoblots (Fig. 2) and weakly neutralized A-MLV but did not affect the titers of GALV or RD114 (data not shown). This result shows that FeLV-B and

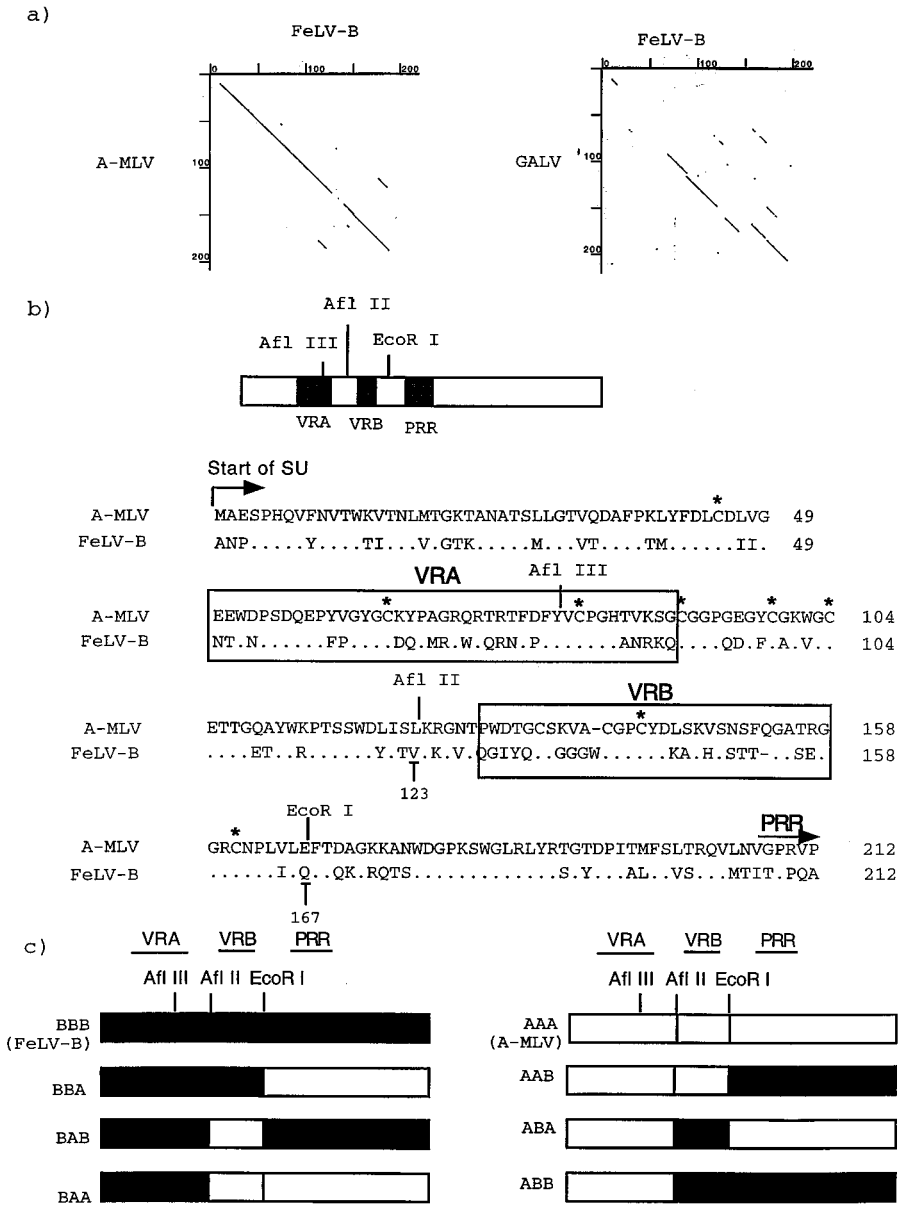

FIG. 1. Comparison of the N-terminal amino acids of SU envelope glycoproteins encoded by FeLV-B, A-MLV, and GALV. (a) Protein dot matrix plot comparing SU envelope amino acids between FeLV-B and A-MLV and between FeLV-B and GALV. (b) Alignment of FeLV-B and A-MLV amino acids showing VRA and VRB (boxed), the conserved cysteine residues (*), and the FeLV-B amino acids that were mutated to the corresponding A-MLV residues (underlined). Dots represents homologous amino acids. (c) Structures of the wild-type and chimeric FeLV-B and A-MLV envelope cDNAs. The variable regions and the restriction sites used to generate the chimeric envelope cDNAs are indicated.

A-MLV envelopes are not only sequence related but also antigenically related.

As diagrammed in Fig. 1c, six hybrid envelope cDNAs were generated by using the unique *Afl*II and *Eco*RI sites. The wild-type and hybrid envelope expression constructs were transfected into TELCeB6 retroviral packaging cells to produce pseudotype virions that encoded LacZ (see Materials and Methods). Pseudotype viruses produced from the transfected cells were named after the chimeric envelopes. Of the six pseudotype viruses with hybrid envelopes, only BBA and AAB were able to efficiently use the native Pit1 and Pit2 receptors, respectively. Specifically, AAA and AAB viruses could both infect MDTF and CHO cells expressing rat Pit2, whereas the BBB and BBA viruses could infect only MDTF cells expressing human Pit1. This result implies that the PRRs of these SU glycoproteins do not have a major influence on receptor specificity. Since the host ranges of BBA and AAB were identical to those of the wild-type BBB (FeLV-B) and AAA (A-MLV) viruses, respectively, these viruses were not used in further assays. The remaining four viruses (BAB, BAA, ABA, and ABB [Fig. 1c]) were negative or only weakly infectious for normal MDTF cells or for MDTF cells that expressed Pit1 (see below).

One possible explanation for the inability of the chimeric viruses to infect cells with the wild-type Pit1 or Pit2 receptor would be a lack of envelope glycoprotein synthesis or incorpo-

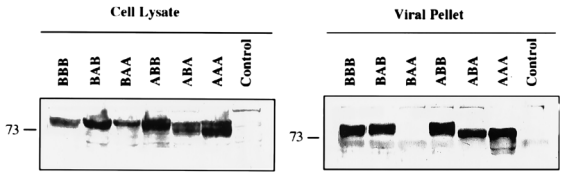

FIG. 2. Detection of wild-type and chimeric FeLV-B and A-MLV envelope glycoproteins. Protein immunoblots of cell lysates transfected with envelope cDNA expression vectors and of viral pellets are shown. The blots were stained with antiserum raised against FeLV-B gp70. Control lanes are cell lysate and supernatant from untransfected TELCeB6 packaging cells. 73, band at 73 kDa.

ration into virions. Consequently, we analyzed the expression of the hybrid envelope glycoproteins and their incorporation into viral particles by protein immunoblot assay (Fig. 2). In comparison with the untransfected control cells, we found that all of the envelope glycoproteins were efficiently expressed and incorporated into virions except for the BAA envelope glycoprotein. This hybrid envelope was expressed in transfected cells but was not incorporated into viral particles (Fig. 2). We repeated the transfection several times but could not generate virions that carried the BAA envelope. In agreement with this result, the BAA viral preparations were not infectious (data not shown).

Characteristics of the native and chimeric Pit1 and Pit2 receptors. Because the envelope glycoproteins of BAB, ABB, and ABA viruses were efficiently expressed and incorporated into virion particles but were unable to recognize native Pit1 or Pit2 receptors, we decided to determine whether they could infect MDTF cells expressing chimeric Pit1/Pit2 receptors. Figure 3a shows a topological model of Pit1 and Pit2 that includes the *Acc*I and *Pst*I sites that were used previously to construct chimeric Pit1/Pit2 cDNAs (26). These splice sites separate the receptor into three regions: an amino-terminal region containing extracellular loops 1 and 2, a mid-region containing extracellular loop 3, and a carboxyl-terminal region containing extracellular loops 4 and 5. The nomenclature of the chimeric receptors correspond to their structure, with GGG indicating human Pit1 and RRR indicating rat Pit2 (Fig. 3b). The GGrG receptor contains the nine-amino-acid sequence from Pit2 extracellular loop 4 substituted for the corresponding nine-amino-acid region A of Pit1 that has been shown to be critical for receptor function (19, 41).

VRA interacts with extracellular loops 4 and 5. The infectivities of the BBB, BAB, ABA, and ABB viruses for MDTF cells that stably express different receptors are shown in Fig. 4. The role of FeLV-B and A-MLV VRAs was first analyzed by substituting FeLV-B VRA with A-MLV VRA (Fig. 1c, ABB virus). The ABB virus could not infect normal MDTF cells or MDTF cells expressing Pit1 (GGG) (Fig. 4, ABB infection of MDTF and GGG) suggesting that the virus could not use Pit1 or Pit2. However, the virus did infect cells expressing the GGR receptor, which contains Pit2 extracellular loops 4 and 5. These cells were resistant to BBB virus (FeLV-B). These results suggest that A-MLV VRA interacts specifically with Pit2 extracellular loops 4 and 5 whereas FeLV-B VRA interacts with Pit1 extracellular loops 4 and 5. We analyzed the infectability of BBB and ABB viruses on cells expressing other chimeric Pit1/Pit2 proteins. The BBB virus could use only chimeric receptors which contained Pit1 loops 4 and 5 (Fig. 4, GRG and RRG), with the exception of the RGG receptor (see below). The BBB virus could not use receptors that contained Pit2 loops 4 and 5 (GGR and GRR) or the chimeric receptor in which the critical nine-amino-acid sequence (region A) of Pit1 loop 4 was replaced with Pit2 sequences (GGrG). These results confirm previous data showing that Pit1 loops 4 and 5 are critical for FeLV-B infection (34, 40) and provides further evidence that FeLV-B VRA interacts with these Pit1 loops. Conversely, the ABB virus could use only receptors that contained Pit2 loops 4 and 5 (GRR and GGR) and could not use the GGG, RGG, RRG, GRG, and GGrG receptors. The inability of this virus to use GGrG receptor suggests that both Pit2 loops 4 and 5 are essential for interaction with A-MLV VRA.

VRB interacts with extracellular loop 2. Although the BBB virus efficiently infected cells expressing the GGG receptor, it only weakly infected cells with the RGG receptor. However, substitution of FeLV-B VRB with A-MLV VRB (BAB virus) enhanced the titer of infection on cells expressing the RGG receptor more than 1,000-fold (Fig. 4). The RGG receptor differs from the GGG receptor by having Pit2 extracellular loops 1 and 2 (Fig. 3). Comparison of the amino acid sequences of Pit1 and Pit2 loops 1 and 2 shows that loops 1 are identical whereas loops 2 are highly divergent. This suggests that A-MLV VRB most likely interacts with Pit2 loop 2 (Fig. 4; compare BBB and BAB infections of RGG). To determine whether FeLV-B VRB interacts with Pit1 loop 2, the VRB sequence of A-MLV (AAA virus) was substituted for FeLV-B VRB sequence to produce the ABA virus. The ABA virus weakly infected normal MDTF cells (Fig. 4) and CHO/RRR cells (CHO cells expressing rat Pit2) (Fig. 5), suggesting that the ABA virus could weakly utilize Pit2. However, the virus could efficiently infect cells with receptors containing Pit1 loops 1 and 2 (GRR and GGR), suggesting that FeLV-B VRB likely interacts with Pit1 loop 2. In addition, AAB virus could efficiently use RRR (Pit2) (data not shown), whereas ABB could use the GRR receptor but not the RRR receptor present in normal MDTF cells. This result further suggests that FeLV-B VRB specifically interacts with Pit1 loop 2.

The PRR of the A-MLV envelope glycoprotein may enhance virion stability or efficiency. As mentioned above, the ABB virus was able to infect only cells expressing receptors with Pit2 loops 4 and 5 (Fig. 4), implying that A-MLV VRA interacts with Pit2 loops 4 and 5. A further substitution of PRR in ABB

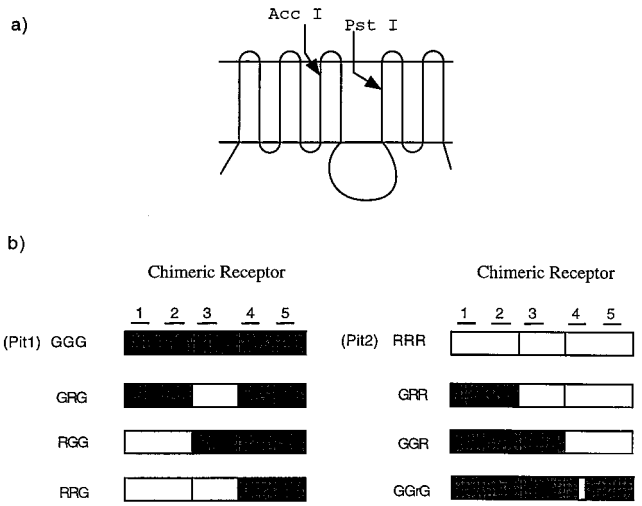

FIG. 3. Structures of the chimeric Pit1 and Pit2 receptors. (a) Topological model of the sodium-dependent phosphate symporters Pit1 and Pit2. The unique *Acc*I and *Pst*I sites, used to generate chimeric Pit1/Pit2 receptors, are indicated by arrows. (b) Diagrams of the wild-type and chimeric Pit1 and Pit2 receptor cDNAs.

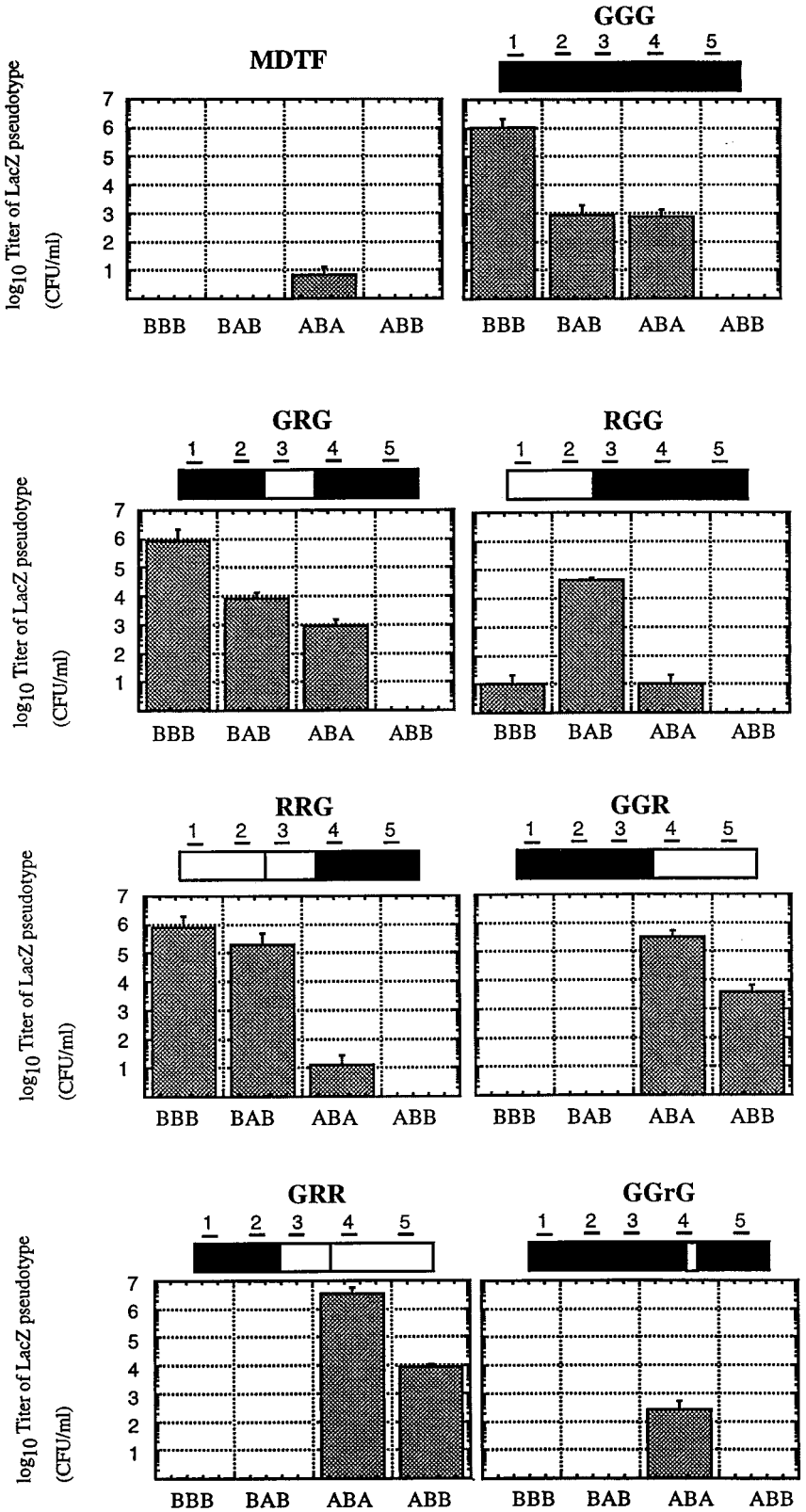

FIG. 4. Transduction of LacZ pseudotype viruses carrying chimeric FeLV-B/A-MLV envelopes on MDTF and MDTF cells expressing chimeric Pit1/Pit2 receptors. The chimeric receptors expressed by MDTF cells are illustrated above the histograms.

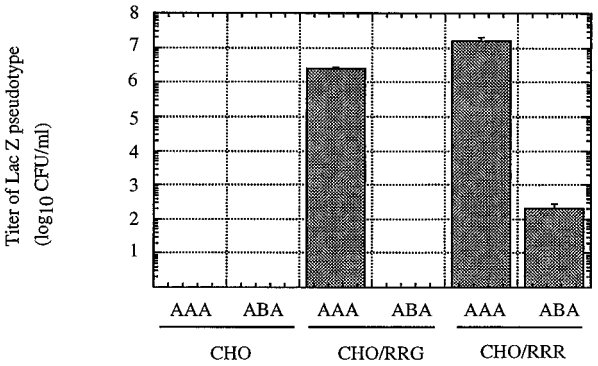

FIG. 5. Transduction of LacZ AAA and ABA pseudotype viruses on CHO cells expressing the RRR (rat Pit2) or RRG receptor.

to give ABA virus not only enhanced the titers of infection on GGR and GRR cells but also extended the ability of the pseudotype virus to weakly use Pit2 (Fig. 4, ABA infection of MDTF; Fig. 5, ABA infection of CHO/RRR) and Pit 1 chimeras which contained Pit1 loops 1 and 2 (ABA infection of GGG, GRG, and GGrG). This would be consistent with the possibility that the PRR of A-MLV interacts weakly with both Pit1 and Pit2. However, the enhancing influence of the A-MLV PRR on viral infection appears to be common to all receptors rather than specific to any of the chimeras. Consequently, it is possible that the A-MLV PRR merely increases stability of the envelopes or facilitates a structural change that enables the envelopes to function more efficiently. Previous evidence suggested that the A-MLV PRR was required only for envelope stability (5, 6).

FeLV-B may interact with extracellular loop 3 of Pit2. Surprisingly, the BBB virus efficiently infected cells with the RRG receptor (Fig. 4), yet this hybrid receptor lacks the Pit1 loop 2 sequence. The BBB virus, however, only weakly infected cells expressing the RGG receptor. The RRG receptor differs from RGG by having Pit2 extracellular loop 3 and the large cytoplasmic loop (Fig. 3), consistent with the possibility that Pit2 extracellular loop 3 provides a site of interaction for FeLV-B. However, a role of the large cytoplasmic loop of the receptors cannot be excluded; it is conceivable that this loop influences overall receptor folding.

FeLV-B residues Phe-60 and Pro-61 and A-MLV Tyr-60 and Val-61 are critical for receptor recognition. To define specific residues within VRA critical for receptor recognition, regions of FeLV-B VRA were substituted with A-MLV VRA sequences. Our aim was to change the host range of the BBB virus to that of the ABB virus and to thereby identify specific residues critical for receptor choice. The mutated viruses were tested on cells expressing either the GGG receptor or the GGR receptor. These two receptors were indicative of BBB and ABB host ranges. FeLV-B and A-MLV VRA contain several cysteine residues that are not only conserved between the two VRAs but also conserved in other VRAs of MLVs, GALV, and FeLV (6). These cysteine residues have been suggested to form disulfide links creating loop structures (21). FeLV-B and A-MLV VRA can form two such loops. In this report, these disulfide loops are referred as VRA1 and VRA2.

To determine which of these loops was responsible for receptor choice, we generated a chimeric envelope in which FeLV-B VRA2 (*Afl*III-*Afl*II fragment) was replaced with A-MLV VRA2 (Fig. 6a, baBB virus). The baBB virus-infected cells expressing GGG but not GGR (Fig. 6b), thus showing the same host range as the BBB virus. This result suggested that sequences upstream of VRA2 were responsible for receptor specificity. Further substitutions were made within BBB VRA1 as well as sequences between VRA1 and VRA2 (residues 66 to 78). Substitution of FeLV-B residues NTWN (50 to 53) or FeLV-B residues 66 to 70 to corresponding A-MLV residues (Fig. 6a, baBB1 and baBB2 viruses, respectively) produced virions with a host range similar to that of BBB (Fig. 6b), although the titer of the baBB2 virus was 100-fold lower than that of the BBB virus. Thus, by a process of elimination, these results indicated that FeLV-B residues Phe-60 and Pro-61 and A-MLV Tyr-60 and Val-61 may be the most critical for receptor choice. To prove that these residues were responsible for receptor specificity, FeLV-B Phe-60 and Pro-61 were simultaneously mutated to tyrosine and valine (baBB3 virus). The resulting pseudotype virus was unable to infect cells with the GGG receptor but was able to infect cells with the GGR receptor with titers similar to those of the ABB virus. To further determine the role of the individual residues in receptor recognition, single amino acids were mutated (Fig. 6). Mutation of Phe-60 to Tyr (baBB4 virus) or Pro-61 to Val (baBB5 virus) caused a considerable reduction in viral titers with no significant change in the host range of the viruses. Together these results suggest that FeLV-B Phe-60 and Pro-61 are critical for Pit1 recognition whereas A-MLV Tyr-60 and Val-61 are critical for Pit2 recognition.

DISCUSSION

Because the envelope glycoproteins of retroviruses consist of related SU and transmembrane subunits, it is likely that they fold similarly and employ common mechanisms for receptor recognition and membrane fusion. For example, the SU envelope glycoproteins of mammalian type C retroviruses contain eight conserved cysteine residues and stretches of homologous amino acids interrupted by three variable regions, VRA, VRB, and PRR. VRA and VRB are believed to contain disulfide-bonded loops that are highly variable in length and sequence, and they have been implicated in receptor recognition and binding. Previous studies of chimeric MLV SU made by using A-, X-, and MCF-MLVs showed that VRA and VRB were critical for receptor recognition and that some chimeric MLVs were unable to infect cells that express natural receptors for A-, X-, and MCF-MLVs (6). However, it was not possible to further study these chimeric MLV envelopes because the X- and MCF-MLV receptors are unknown and therefore chimeric receptors were unavailable. Similarly, chimeric cell surface receptors have been analyzed. Generally, these receptor chimeras were made between the homologous proteins of species that are susceptible and resistant to infection in order to identify receptor sites that control infection, and the conclusions were then tested by site-directed mutagenesis (1, 18, 38, 45). In addition, chimeras have been made between Pit1 and Pit2 proteins in order to identify sites in these receptors that are necessary for infections by A-MLV, GALV, and FeLV-B (26, 31). These investigations have indicated that a stretch of nine amino acids in extracellular loop 4 of Pit1 is essential for infections by GALV and FeLV-B and that additional amino acids within loop 5 contribute to infections by FeLV-B. In contrast, results with A-MLV have been relatively ambiguous and have suggested that multiple regions of Pit2 may be important (26, 31).

By using chimeras between the closely related FeLV-B and A-MLV envelope glycoproteins (Fig. 1) and between their even more closely related Pit1 and Pit2 cell surface receptors, we have been able to simultaneously investigate the critical sites for interaction in both the SU glycoproteins and their

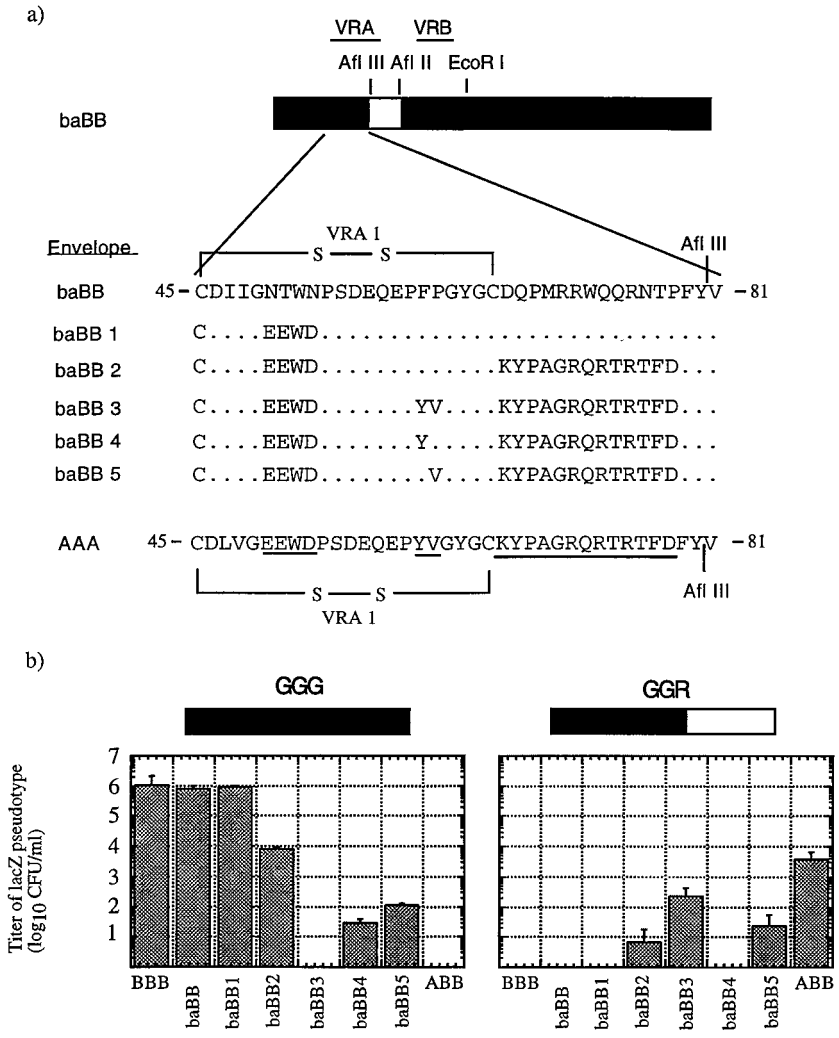

FIG. 6. Mutagenesis of FeLV-B VRA1. (a) The chimeric baBB envelope cDNA was generated by using the unique *Afl*III and *Afl*II restriction sites. This envelope contains FeLV-B sequence (black shading) with A-MLV VRA2 sequence (white box). The predicted disulfide-bridged VRA1 is shown. Mutant baBB envelope cDNAs were generated by PCR mutagenesis. Unchanged amino acids are represented by dots. (b) Infection of MDTF cells expressing the GGG (Pit1) or GGR receptor with LacZ pseudotype viruses carrying mutant baBB envelope glycoproteins.

corresponding receptors. Interestingly, several viruses pseudotyped with FeLV-B/A-MLV chimeric SU envelope glycoproteins were unable to infect MDTF cells (these contain the A-MLV receptor Pit2) or MDTF/GGG cells (which express the FeLV-B receptor human Pit1) but were able to efficiently infect MDTF cells that express specific Pit1/Pit2 receptor chimeras. By studying the utilization of these receptors by chimeric pseudotype viruses, several clear patterns were observed that had not been revealed in previous investigations. An important result was the striking similarity in the interactions of FeLV-B and A-MLV envelope glycoproteins with their respective receptors. For both viruses, our results suggest that the VRA and VRB of their SU glycoproteins function as discrete modules that can be spliced together in novel combinations and that these two modules recognize discrete sites in the corresponding receptors. Specifically, the VRA modules recognize sequences within extracellular loops 4 and 5 of the receptors. For FeLV-B SU, Phe-60 and Pro-61 in the first disulfide-bonded loop of VRA are critical for this interaction, whereas for A-MLV, the corresponding Tyr-60 and Val-61 residues are essential (Fig. 6). Similarly, VRB modules specifically recognize sequences in extracellular loop 2 of the receptors, although we cannot exclude the possibility that extracellular loop 1 also interacts with VRB. Our interpretation of these results is compatible with evidence obtained by Pedersen et al. (31) and with mutations of Pit2 loop 2 residues that render the receptor nonfunctional for A-MLV (20a). For both viruses, both VRA and VRB interactions appeared to be necessary for infections.

Our observation that residues F60 and P61 in FeLV-B and Y60 and V61 in A-MLV SU are critical for receptor recognition suggests that the first disulfide-bonded loop of VRA may be the most important for receptor binding specificity. In agreement with this conclusion are recent reports that have localized residues critical for receptor recognition by E-MLVs to the first disulfide-bonded loop within VRA (4, 22). It is not clear from our results which Pit1 or Pit2 amino acids in loops 4 and 5 interact with the critical envelope residues at positions 60 and 61. Sequences of naturally occurring Pit1 and mutational studies have suggested that residues 550 and 552 of Pit1 extracellular loop 4 are critical for receptor function (38). Basic amino acids at these positions render Pit1 inactive for FeLV-B,

whereas neutral or acidic amino acids are favorable. These results raise the possibility that positively charged amino acids in the FeLV-B SU glycoprotein such as the basic amino acids at positions 70 to 75 may interact with the Pit1 receptor site. We emphasize that our results identifying SU amino acids 60 and 61 as critical for receptor recognition do not exclude the possibility that additional amino acids also contribute to receptor binding.

Although our results strongly suggest that the natural infections by FeLV-B and A-MLV require both loop 2 and loop 4–5 interactions with the envelope glycoproteins, we cannot exclude the possibility that other receptor regions provide nonspecific sites for envelope interaction. Two observations would be compatible with this interpretation. First, our results show that the presence of A-MLV PRR causes an enhancement of viral infectivity that is independent of the receptor being analyzed (Fig. 4). For example, the ABA virus appears to be more infectious than the ABB virus on all cells. Although this could imply that A-MLV PRR interacts with a receptor site that is common to both Pit1 and Pit2, it is also possible that the A-MLV PRR increases envelope stability or efficiency of infection. Because such proline-rich sequences would not be expected to adopt stable secondary structures, it seems likely that they would act as flexible hinges rather than as sites for receptor recognition. However, in some MLV strains, the proline-rich sequences may contribute to receptor recognition (5). Second, we were surprised to find that the BBB virus (i.e., FeLV-B) could efficiently infect MDTF cells that express the RRG receptor, which lacks Pit1 loop 2 sequence. However, this virus weakly infected cells expressing the RGG receptor, which is consistent with other evidence that Pit1 loop 2 is important for FeLV-B infection. Similarly, it has been reported that both A-MLV and GALV efficiently infect cells expressing the RRG receptor but weakly infect cells expressing the RGG receptor (26, 31). This promiscuous activity of the RRG protein in the infections of FeLV-B, A-MLV, and GALV suggests that the viruses may be able to weakly interact with the Pit2 loop 3 region in certain chimeric receptors in a manner that can enhance infection (see Results). These issues require additional investigation.

Based on the evidence described in this report, we propose that VRA and VRB of the SU envelope glycoproteins of mammalian type C retroviruses function as separate modules that bind to their corresponding target sites on cell surface receptors by a two-step process. Although our data do not establish whether VRA and VRB interactions occur simultaneously or sequentially, these interactions are discrete, and we have shown that they can be analyzed in FeLV-B/A-MLV recombinants. Based on physical chemical principles, it is unlikely that discrete interactions by separable modules could occur absolutely simultaneously. Moreover, a two-step recognition and docking mechanism is reasonable because it would allow an initial reversible binding to be followed by a proofreading or commitment step that would increase the affinity and reduce dissociation. According to our hypothesis, the initial interaction may induce a conformational change in the SU glycoprotein that facilitates the second interaction and leads to virus penetration of the cell. Our studies of receptor recognition are consistent with the possibility that the initial interaction occurs between VRA and extracellular loops 4 and 5 and that this is followed by a second interaction between VRB and extracellular loop 2. This possibility is consistent with recent studies which have shown that sequences downstream of VRA are involved in a postprimary stage of binding (10a). It is intriguing that this two-step mechanism for receptor recognition and infection by FeLV-B and A-MLV is clearly analogous to the two-step mechanism for cell surface attachment by the human immunodeficiency virus (HIV). HIV initially binds to its primary receptor CD4 by its SU envelope glycoprotein gp120 (11, 20), but this is insufficient for infection. Recent reports have established that the binary HIV gp120-CD4 complexes associate with a coreceptor that normally functions as a G-protein-coupled receptor for proinflammatory chemokines (3, 8, 12–14, 16). The C3 and C4 regions of gp120 have been implicated in binding to CD4 (29), whereas the gp120 V3 loop, which is exposed only after CD4 binding, has been implicated in interactions with coreceptors (32, 40, 44). Our results support the hypothesis that all retroviruses may use a similar two-step process for cell surface attachment and membrane fusion.

Several groups have previously attempted to target retroviral vectors to specific cells by incorporating ligands or antibodies into the SU envelope glycoproteins; however the resulting viral vectors have had low infectivities, despite showing high levels of binding onto the target receptors (35, 37, 41). Our results provide a possible explanation for this problem and imply that infections by type C retroviruses may require interactions of discrete modular envelope regions with discrete regions of the receptor. Similarly, our results may help to explain why retroviruses have tended to coevolve with their receptors and why jumps to novel receptors or species appear to have been rare.

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