Equine infectious anemia virus (EIAV), a member of the lentivirus subfamily, establishes a persistent infection in horses and causes a uniquely dynamic lentiviral disease characterized by recurrent waves of viremia and disease cycles with clinical signs that include fever, diarrhea, edema, lethargy, anemia, thrombocytopenia, and occasional encephalitis or ataxia (24). EIAV disease in horses is apparently related to an exclusive infection of monocytes and macrophages, making EIA a relevant model for studying lentiviral pathogenicity from macrophage infections without the complications of lymphocyte infections associated with the immunodeficiency lentiviruses. Toward defining EIAV-macrophage interactions, we recently identified a tumor necrosis factor receptor (TNFR) family protein, designated equine lentivirus receptor 1 (ELR1), as a functional cellular receptor for both primary and cell-adapted strains of EIAV (35). This finding of a single functional receptor protein for EIAV is in distinct contrast to the common use of dual coreceptors reported for immunodeficiency lentiviruses (human, simian, and feline immunodeficiency viruses) (3, 12, 13, 30, 33) but similar to the single receptor usage reported for simple retroviruses, such as murine or avian leukemia viruses (1). Of particular interest is the fact that feline immunodeficiency virus (FIV) also uses a TNFR protein, CD134, as a coreceptor with CD4 for infection of target cells (12, 30). The single TNFR protein receptor target for EIAV combined with various chimeric receptor proteins derived from exchanges between the functional ELR1 and the non-binding homolog, mouse herpesvirus entry mediator (murine HveA). Complementary exchanges of the respective cysteine-rich domains (CRD) between the ELR1 and murine HveA proteins revealed CRD1 as the predominant determinant of functional gp90 binding to ELR1 and also to a chimeric murine HveA protein expressed on the surface of transfected Cf2Th cells. Mutations of individual amino acids in the CRD1 segment of ELR1 and murine HveA indicated the Leu70 in CRD1 as essential for functional binding of EIAV gp90 and for virus infection of transduced Cf2Th cells. The specificity of the EIAV SU binding domain identified for the ELR1 receptor is fundamentally identical to that reported previously for functional binding of feline immunodeficiency virus SU to its coreceptor CD134, another TNFR protein. These results indicate unexpected common features of the specific mechanisms by which diverse lentiviruses can employ TNFR proteins as functional receptors.

The equine lentivirus receptor 1 (ELR1), a member of the tumor necrosis factor receptor (TNFR) protein family, has been identified as a functional receptor for equine infectious anemia virus (EIAV). Toward defining the functional interactions between the EIAV SU protein (gp90) and its ELR1 receptor, we mapped the gp90 binding domain of ELR1 by a combination of binding and functional assays using the EIAV SU gp90 protein and various chimeric receptor proteins derived from exchanges between the functional ELR1 and the non-binding homolog, mouse herpesvirus entry mediator (murine HveA). Complementary exchanges of the respective cysteine-rich domains (CRD) between the ELR1 and murine HveA proteins revealed CRD1 as the predominant determinant of functional gp90 binding to ELR1 and also to a chimeric murine HveA protein expressed on the surface of transfected Cf2Th cells. Mutations of individual amino acids in the CRD1 segment of ELR1 and murine HveA indicated the Leu70 in CRD1 as essential for functional binding of EIAV gp90 and for virus infection of transduced Cf2Th cells. The specificity of the EIAV SU binding domain identified for the ELR1 receptor is fundamentally identical to that reported previously for functional binding of feline immunodeficiency virus SU to its coreceptor CD134, another TNFR protein. These results indicate unexpected common features of the specific mechanisms by which diverse lentiviruses can employ TNFR proteins as functional receptors.

The goal of the current study was to identify amino acid residues in ELR1 that are critical for EIAV gp90 binding, as a part of ongoing studies that utilize EIAV as a model to define the mechanisms of retrovirus entry into target cells. Following the exemplary mapping studies described by Elder and colleagues, we employed domain exchanges and single amino acid substitutions between ELR1 and murine HveA to identify the specific cysteine-rich domain(s) critical for functional interactions with EIAV gp90. Chimeric receptor proteins were evaluated both for their ability to bind EIAV gp90 in a cell-cell binding assay and their ability to support virus infection in transduced target cells. The results of these studies for the first
time reveal the binding sites on ELR1 and highlight a remarkable conservation of structure and function among TNFR proteins that serve as receptors for different viruses.

**MATERIALS AND METHODS**

**ELR1 mutagenesis.** Murine HveA was cloned by reverse transcriptase PCR (RT-PCR) from mouse thymus cells using primers based on the published murine HveA sequence (27, 32). ELR1 and murine HveA domain exchange mutants or point mutations were generated using an overlapping PCR strategy (19). Briefly, 5’ and 3’ halves of the appropriate receptor genes were amplified in separate PCRs. The internal primers were designed to overlap the position selected for mutation. The agarose gel-purified PCR products were then used to amplify the full-length receptor gene using two outer primers. The full-length PCR products were then digested using EcoRI and BamHI and inserted into a pFB-Neo-LacZ vector (Stratagene, La Jolla, CA) with the same digestion sites. As outlined below in Fig. 2, all chimeric constructs contained the ELR1 leader sequence, transmembrane domain, and cytoplasmic domain with a hemagglutinin (HA) tag. For the point mutation constructs based on murine HveA, all receptor gene sequences were from wild-type murine HveA.

**Construction of expression vectors for GFP-labeled EIAV gp90.** We constructed cell-adapted EIAV, an molecular clone EIAVUK described in detail by Cook et al. seeded in T25 flasks 1 day before retrovirus infection. Cells were infected with pseudotyped retroviruses expressing the receptor and its mutant derivatives were made as described previously (35). EIAVUK nonpermissive Cf2Th cells were harvested from flasks using 10 mM EDTA–PBS and then fixed in 1% paraformaldehyde for 1 h at 4°C. The cells were then washed three times with wash buffer (PBS containing 5% heat-inactivated fetal bovine serum) and then incubated with 2 μl rabbit anti-ELR1 polyclonal antibody or 1 μl anti-gp90 monoclonal antibody in 100 μl wash buffer for 1 h at 4°C. The cells were then washed three times with wash buffer and incubated with 1 μl secondary conjugate in 100 μl wash buffer for 1 h at 4°C. Following three washes with wash buffer, antibody labeling of ELR1 and gp90 labeling were analyzed by flow cytometry using a BD FACSCalibur (BD Biosciences, San Jose, CA). HA tag staining was performed following the protocol described previously (35).

**Cell-cell binding assay.** The cell-cell binding protocols used to assay the level of binding between various ELR1 constructs and EIAV gp90, individually expressed on surfaces of different cells, are based on procedures previously described for the study of binding between the S protein of severe acute respiratory coronavirus and its cellular receptor (8). For the cell-cell binding assay, Cf2Th cells transduced with ELR1 or its mutant derivatives were used as the target monolayer, and CHO cells transduced with the EIAV gp90-GFP served as the ligand cells. Approximately 2 × 10⁵ receptor-transduced Cf2Th cells expressing individual ELR1 protein constructs were seeded in each well of six-well plates (Becton Dickinson Labware, Bedford, MA). Cells were cultured at 37°C for 2 days and then transferred to 4°C for 30 min. Immediately prior to use, the transduced gp90-GFP CHO or GFP CHO cell monolayers were detached by adding 10 mM EDTA–PBS and washed three times with ice-cold minimal essential medium plus 10% fetal bovine serum. About 3 × 10⁵ transduced CHO cells (ligand) were then added to each well of receptor-transduced Cf2Th cells (target) at 4°C and incubated for 1.5 h. Unbound CHO cells were then removed by washing three times with PBS and one time with PBS containing 0.5 M NaCl. Plates were then moved to room temperature, and the attached cells were removed by pipetting using wide-bore tips with 0.5 ml PBS in each well. Resuspended cells were subjected to HA tag staining and flow cytometry analysis to measure bound Cf2Th cells.

**Virus replication and RT assay.** The extracellular RT activity in cell-free culture medium was measured to determine the levels of virus production, as described previously (18). Reported RT levels represent the averages of duplicate samples.

**Modeling of ELR1.** Homology modeling was conducted using MODELLER 8.0 (www.saliab.org/modeler/modeller.html) on a Macintosh platform. Sequence alignments for input into MODELLER (28) were generated by CLUSTALW (www.ebi.ac.uk/clustalw). The refined structure of HveA at 2.65 Å resolution (Protein Data Bank ID 1IMA) was used as a template. The amino acid sequence of murine HveA and ELR1 (NCBI PIDS AAAY21177 and AA04055, respectively) were obtained from the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/entrez). Only CRD1, a modular folding unit within the full-length receptor, was used in modeling studies. Single point mutations to murine HveA and ELR1 corresponding to position 70 in ELR1 were also modeled. In all cases, the homology domain with the lowest MODELLER objective function score was selected and visualized using VMD 1.86 (14).

**RESULTS**

**Validation of cell-cell binding assay.** The data in Fig. 1 demonstrate the specificity of the cell-cell binding assay for measuring ELR1 binding with EIAV gp90. In the cell-cell binding assay, only the CHO cells expressing gp90GFP bound to the target Cf2Th/ELR1 cells in flow cytometry (Fig. 1D). In
contrast, there was no significant binding of the CHO gp90-GFP cells to Cf2Th cells expressing the nonfunctional mouse herpes simplex virus receptor mHveA (Fig. 1C), and CHO cells expressing only GFP also failed to significantly bind the target Cf2Th/ELR1 cells (Fig. 1B). The specificity of the binding observed between the ligand CHO gp90-GFP cells and the target Cf2Th/ELR1 cells was further tested by determining the ability of a reference polyclonal anti-ELR1 antibody to block the cell-cell binding under the standard assay conditions. As summarized in Fig. 1E, the reference polyclonal immune serum to ELR1 inhibited the cell-cell binding, and the observed inhibition was directly related to the dilution of the serum added to the binding assay. In distinct contrast, normal rabbit serum failed to reduce the level of cell-cell binding. Thus, these data indicate that the conditions employed for the cell-cell binding assays specifically measure interactions mediated by EIAV gp90 and ELR1 expressed on the cell surfaces.

**EIAV gp90 binds to the C terminus of CRD1 of ELR1.** Based on a comparison of TNFR family protein sequences using CLUSTALW, ELR1 is most closely related to the herpes simplex virus entry mediator HveA and it mouse version, murine HveA (data not shown). A closer comparison revealed that ELR1 and murine HveA contain predicted ectodomains and analogous CRD segments, as described by Naismith and Sprang (26). In preliminary studies, murine HveA expressed on the surface of transduced Cf2Th cells did not bind EIAV gp90 or support infection (data not shown). The lack of EIAV-specific receptor function is in contrast to the gp90 binding and productive virus infection mediated by ELR1 expressed on the surface of transduced Cf2Th cells. Thus, we pursued an initial mapping of the functional binding domains of ELR1 by two complementary domain exchange strategies. In the first strategy (loss of function), we substituted selected CRD segments from murine HveA into ELR1 and measured the chimeric receptor proteins for gp90 binding and for their ability to mediate infection of Cf2Th cells transduced with the respective chimeric receptor. In the second strategy (gain of function), we substituted selected CRD segments from ELR1 into the murine HveA backbone and assayed the product chimeric receptors for their ability to bind gp90 and to mediate infection of transduced Cf2Th cells. The panel of chimeric receptor constructs is summarized in Fig. 2.

Prior to assaying the functional properties of the various chimeric receptors, the total expression of each receptor construct in transduced Cf2Th cells was evaluated by flow cytometry using the HA tag contained in each receptor protein. These assays indicated that 85 to 95% of the transduced cells for each receptor construct were positive for HA, indicating similar levels of expression for each receptor protein (data not shown). Surface expression of the chimeric receptors was confirmed by flow cytometry of the various transduced cell lines labeled with the rabbit immune serum to ELR1 (data not shown). While qualitatively confirming the surface expression of each receptor construct, these assays could not provide a quantitative comparison of surface expression levels, as the amount of potential antibody-reactive ELR1 sequences varied markedly among the constructs. However, for those point mutation constructions based on ELR1, we observed similar levels of surface expression compared to wild-type ELR1.

We next assayed each cell surface-expressed receptor construct in the cell-cell binding assay for its capacity to bind EIAV gp90 expressed on the surface of transduced CHO cells (Fig. 2). Figure 2B summarizes the loss-of-function experiments in which selected CRD segments of murine HveA were substituted into the ELR1 protein backbone. Cell-cell binding assays indicate about 57% binding for the parental ELR1 and less than 1% binding for the murine HveA, demonstrating the specificity of the assay procedures. In our analysis, wild-type ELR1 binding capacity was set as 100%, and the binding capacities of the various mutant receptor constructs were calculated as a proportion of ELR1 binding. Substitution of the
CRD1 from murine HveA into ELR1 (M1E2E3E4) reduced the binding capacity by about 50% compared to the binding level observed with parental ELR1. Individual substitutions of the murine HveA CRD2, CRD3, or CRD4 into ELR1 resulted in 15%, 19%, and 5% loss of binding capacity, respectively, compared to parental ELR1.

The results of the cell-cell binding assays for the murine HveA receptor proteins containing individual CRD segments from ELR1 are also summarized in Fig. 2B. These data demonstrate that the chimeric receptor containing the CRD1 from ELR1 (E1M2E3M4) gained 85% of the binding capacity compared to ELR1. In contrast, the chimeric receptors with substitutions of CRD2, CRD3, or CRD4 from ELR1 into the murine HveA backbone recovered only 14%, 3%, and 2% of ELR1 binding capacity, respectively. Taken together, the loss of binding function and gain of binding function assays both indicate a predominant role for CRD1 in gp90 binding, with only a relatively minor influence of the other three CRD segments.

Having established the gp90 binding properties of the chimeric receptor constructs, we next assayed the functional sequences by measuring the ability of each chimeric construct to support EIAV infection and replication in transduced Cf2Th cells. Figure 2C summarizes the replication properties of EIAV in the panel of Cf2Th cells stably transduced with the various ELR1 constructs containing a CRD substitution from murine HveA. Figure 2D summarizes the replication data for the complementary set of substitution mutations into the murine HveA protein backbone. In the assays of the murine CRD substitutions into ELR1 that measure loss of function, virus replication levels in the cells transduced with the M1E2E3E4 chimeric receptor were reduced by about 60% compared to virus replication in ELR1-transduced cells. Virus replication levels produced by the remaining chimeric receptor proteins...
with substitutions in CRD2, CRD3, or CRD4 were reduced to various intermediate levels between those observed with M1E2E3E4 and ELR1 (Fig. 2C). In the assays of the equine CRD substitutions into murine HveA that measured gain of function, virus replication in the E1M2M3M4-transduced cells was equal to that observed in the ELR1-transduced cells; no other substitution in this panel of receptor constructs was able to support EIAV replication (Fig. 2D). Therefore, these data clearly demonstrate that domain exchanges between ELR1 and mHveA did not alter the overall structure of the receptors as binding ligands. Importantly, the binding chimeric receptors also support virus entry and subsequently virus replication in transduced cells, at various levels between ELR1 and mHveA.

The virus replication assays further highlight the role of the CRD1 domain in receptor function, with the remarkable observation that substitution of this domain into the murine HveA protein produced a fully functional receptor for EIAV.

To further define the critical sequences in the ELR1 CRD1 that mediate receptor function, we next employed the same domain exchange strategy, but with smaller segments of the equine CRD1 substituted into the murine HveA protein backbone (Fig. 3A). The constructs were then assayed for gp90 binding (Fig. 3B) and virus replication (Fig. 3C) in transduced Cf2Th cells. As summarized in Fig. 3B, only receptor constructs containing the C-terminal sequences of the ELR1 CRD1 segment gained gp90 binding and EIAV infection functions; the chimeric receptor (D1A) containing the N-terminal sequences of the ELR1 CRD1 failed to bind gp90 or to support virus replication. Specifically, the D1B and D1.5B mutants both displayed gp90 binding levels equal to ELR1 (Fig. 3B). The D1B and D1.5B receptors also supported EIAV replication as effectively as ELR1, while the D1.5A chimeric receptor failed to support virus replication. Taken together, these data indicated that the gp90 binding and EIAV infection functions were associated with the C-terminal half of the CRD1 of ELR1.

Mapping of CRD1 residues critical for receptor-envelope interactions. Even though ELR1 and murine HveA differ markedly in their EIAV gp90 binding capacity, the critical sequences identified in the D1B subdomain of the CRD1 of these respective receptor proteins differ by only a few amino acids (Fig. 4A). To further define the specific residues in CRD1 that mediate gp90 binding, a panel of receptor mutations containing single amino acid substitutions were constructed, each converting the equine amino acid to its corresponding murine amino acid (Fig. 4A). To eliminate the influence of other CRD sequences in ELR1 on functional properties, we introduced these point mutations into the backbone of the fully functional E1M2M3M4 chimeric receptor that contained only the CRD1 sequences from ELR1 substituted into the murine HveA protein.

Following the same procedure as used for the CRD exchange mutants, the various point mutations in the context of stably transduced Cf2Th cells were compared for their abilities to bind EIAV gp90 and to support virus replication. As shown in Fig. 4B, CRD1 mutations of K56M caused a 20% decrease in gp90 binding activity compared to ELR1, while the S58N, R62H, A66V, and G68S mutations displayed gp90 binding levels similar to ELR1. In contrast, the mutant L70H displayed an 80% reduction in gp90 binding capacity. Interestingly, all of the CRD1 point mutants were able to support EIAV replication in transduced Cf2Th cells (Fig. 4C), although the observed levels of virus replication differed among the individual mutants. Interestingly, the virus replication levels produced with
each mutant were not tightly correlated to the gp90 binding levels observed with the various point mutations. For example, the R62H, A66V, and G68S mutants that displayed wild-type gp90 levels supported virus replication as well as the ELR1 protein. Similarly, the L70H mutant, which displayed a greatly reduced gp90 binding capacity, mediated virus replication levels that were about 10-fold less than the replication levels observed with ELR1. However, the S58N mutant displayed a wild-type gp90 binding capacity but produced virus replication levels that were also about 10-fold lower than ELR1. While the mechanistic explanations for the lack of correlation between gp90 binding and virus replication capacities remain to be defined, the current results do emphasize the importance of the ELR1 L70 as a determinant for gp90 binding and suggest that S58 may play an important role in virus replication following the initial binding events.

Conversion of murine HveA to a functional EIAV receptor by a single point mutation. In order to further explore the roles of the individual variant amino acids of CRD1 of ELR1 and murine HveA, a series of point mutations were made in the murine HveA backbone to convert the residue to its corresponding amino acid in ELR1 (Fig. 5A). The receptor mutants were tested for their gp90 binding capacities and abilities to support virus replication. As shown in Fig. 5B, mutations in the murine HveA protein at position 56, 58, 62, 66, or 68 failed to bind gp90. In contrast, mutant H70L receptor protein displayed gp90 binding levels that were equivalent to the ELR1 protein. Consistent with the observed gp90 binding capacity of the murine HveA point mutations, only the H70L receptor protein was able to support virus replication in transduced Cf2Th cells; the remaining point mutants were defective for virus replication (Fig. 5C). This ability of a single amino acid mutation (mH70L) to transform a nonfunctional receptor into a functional receptor further confirmed the important role of Leu70 in the receptor binding site for EIAV gp90.

Requirement of a small hydrophobic amino acid at ELR1 residue 70 for functional binding. The previous experiments indicated Leu70 as critical for functional binding to EIAV gp90. To explore the nature of the side chain at this position that is necessary for receptor function, we mutated H70 in murine HveA to a series of selected amino acids with different chemical properties and then assayed the gp90 binding and virus replication properties of the mutated receptors. As summarized in Fig. 6, substitution of the murine HveA H70 with relatively small hydrophobic amino acids, valine or isoleucine, produced receptor proteins that displayed gp90 binding levels that were about 50% of the activity observed for ELR1 (Fig. 6B). However, the receptors with these two point mutations were both functional for supporting EIAV replication in the transduced Cf2Th cells (Fig. 6C). In contrast to this gain of function with the valine and isoleucine substitutions, the H70F and the H70E receptors both failed to bind gp90 (Fig. 6B) or to support EIAV replication (Fig. 6C).

Modeling of ELR1. Given the critical role of L70 of ELR1 for gp90 binding and the support of viral replication, the CRD1 domain of the receptor protein was examined by homology modeling. For comparative evaluation, the same domain of murine HveA was also modeled using the crystal structure of the corresponding domain of HveA as a template. The highly conserved sequences of ELR1 and murine HveA show 89% and 64% identity in CRD1, respectively, to HveA. Since mutations at position 70 of ELR1 are critical for activity, the inactivating mutant L70H ELR1 was also modeled, as was the analogous mutation in murine HveA (H70L), which fully
supported receptor binding. Given the high sequence conservation, it is unsurprising that this domain in all models retained nearly identical overall structures to that of the CRD1 of HveA. All of the modeled structures retained similar backbone coordinates, with root mean square deviations differing less than 0.41 Å from the template, suggesting that the differing
activities of the various constructs are not due to any change in the global fold of the domain. Closer examination of the β-turn containing L70 of ELR1 (Fig. 7A) shows that the surface-accessible volume of this loop is primarily defined by the acidic Glu at position 69 and the hydrophobic Leu at position 70. In all four models (Fig. 7A to D), the Glu is conserved. The most striking differences between the four model structures are the alterations in polarity and surface profile attributable to the Leu/His at position 70.

**DISCUSSION**

In the current study we have used domain exchange and point mutation strategies between the ELR1 protein with a high binding capacity for EIAV gp90 and the murine HveA protein, which lacks substantial gp90 binding capacity, despite a high degree of homology between the two TNFR family proteins. The results of these studies, employing both loss-of-function and gain-of-function assays, clearly identify the C-terminal half of the CRD1 segment of ELR1 as the critical domain mediating gp90 binding and Leu70 as the critical residue within the CRD1 necessary for functional binding of ELR1 to gp90. In addition, the data presented here demonstrate that the CRD2, CRD3, and CRD4 sequences in ELR1, while not essential for gp90 binding, can influence the level of gp90 binding, presumably through allosteric effects on overall receptor protein conformation and interactions with the gp90 protein.

The current observations suggest that the relatively small number of variant amino acid residues between the functional ELR1 and the nonfunctional murine HveA proteins, including L70, may actually represent contact points with the EIAV gp90, rather than producing marked differences in the conformation of the respective receptor proteins. In this regard, the homology modeling of CRD1 (Fig. 7) indicates remarkably similar conformations for the respective CRD1 segments of the ELR1 and murine HveA receptor proteins. The observation that no single point mutation in ELR1 completely abrogated gp90 binding suggests further that receptor binding to the viral envelope protein likely involves multiple contact points with residues within and outside of the CRD1 sequences. Thus, these data are consistent with other reports that multiple amino acid point mutations are required to completely eliminate receptor activity for FIV (11), avian sarcoma-leukosis virus (16, 17), human immunodeficiency virus (23), and murine leukemia virus (21).

A remarkable observation in these studies was that the sin-
molecular modeling studies of the CRD1 domain indicate that binding and functioned as a receptor for EIAV infection. The chains failed to produce gp90 binding, while substitutions with properties revealed a strong influence of amino acid structure substitutions by various amino acids with different chemical notype to the murine HveA protein. Our evaluation of His70 for this critical residue to provide a functional receptor phe-


tioned interaction between herpes simplex virus (HSV) gD protein and its HveA receptor (7) have demonstrated a critical role for the C-terminal segment of CRD1 of the HveA protein for binding to the viral gD envelope protein (9, 34). These similarities between EIAV, FIV, and HSV envelope-receptor interactions appear to reveal common themes in the use of TNFR family proteins as virus receptors and also suggest common structural motifs in the envelope proteins of these diverse viruses that are utilized for receptor binding. In the cases of FIV and HSV, the natural ligand binding sites of the receptor proteins are distinct from their respective viral binding sites (11, 29, 34). The ligand specificity of ELR1 and its relationship to the EIAV gp90 binding site remain to be determined.

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