Efficient Subgroup C Avian Sarcoma and Leukosis Virus Receptor Activity Requires the IgV Domain of the Tvc Receptor and Proper Display on the Cell Membrane

Audelia Munguia and Mark J. Federspiel*

Department of Molecular Medicine, Mayo Clinic, Rochester, Minnesota 55905

Received 7 July 2008/Accepted 28 August 2008

We recently identified and cloned the receptor for subgroup C avian sarcoma and leukemia viruses [ASLV(C)], i.e., Tvc, a protein most closely related to mammalian butyrophilins, which are members of the immunoglobulin protein family. The extracellular domain of Tvc contains two immunoglobulin-like domains, IgV and IgC, which presumably each contain a disulfide bond important for native function of the protein. In this study, we have begun to identify the functional determinants of Tvc responsible for ASLV(C) receptor activity. We found that the IgV domain of the Tvc receptor is responsible for interacting with the glycoprotein of ASLV(C). Additional experiments demonstrated that a domain was necessary as a spacer between the IgV domain and the membrane-spanning domain for efficient Tvc receptor activity, most likely to orient the IgV domain a proper distance from the cell membrane. The effects on ASLV(C) glycoprotein binding and infection efficiency were also studied by site-directed mutagenesis of the cysteine residues of Tvc as well as conserved amino acid residues of the IgV Tvc domain compared to other IgV domains. In this initial analysis of Tvc determinants important for interacting with ASLV(C) glycoproteins, at least two aromatic amino acid residues in the IgV domain of Tvc, Trp-48 and Tyr-105, were identified as critical for efficient ASLV(C) infection. Interestingly, one or more aromatic amino acid residues have been identified as critical determinants in the other ASLV(A-E) receptors for a proper interaction with ASLV glycoproteins. This suggests that the ASLV glycoproteins may share a common mechanism of receptor interaction with an aromatic residue(s) on the receptor critical for triggering conformational changes in SU that initiate the fusion process required for efficient virus infection.

The envelope glycoproteins of retroviruses are comprised of the surface (SU) glycoprotein, which is responsible for interacting with the host cell receptor, and the transmembrane glycoprotein (TM), which anchors SU to the viral membrane, contains the fusion peptide, and is responsible for the fusion of the viral and cellular membranes (19). The first step in retrovirus infection is the binding of the SU glycoprotein to a cell-specific protein that serves as a receptor. A proper interaction between SU and the receptor triggers a conformational change in the SU glycoprotein, exposing the fusion peptide and initiating the multistep fusion process between the viral and cellular membranes resulting in entry of a subviral complex into the cytoplasm. For most retroviruses, this occurs at the cell surface at neutral pH. The avian sarcoma and leukemia virus (ASLV) group of retroviruses accomplishes entry in at least two distinct steps (4). As with other retroviruses, ASLV requires the proper interaction between SU and a specific receptor, triggering a conformational change in the Env trimers and initiating the fusion process. However, ASLV also requires a low-pH environment to complete the fusion process and release of the viral genetic material into the host cell. For all retroviruses, the interaction of the SU glycoprotein with the host cell receptor is an extremely complex process involving many determinants in both proteins that affect receptor specificity and binding affinity. Regardless of this complexity, families of retroviruses have evolved by altering their glycoproteins to utilize different host cellular proteins as receptors and still retain efficient entry.

The subgroup A to E ASLVs [ASLV(A-E)] are a homologous group of retroviruses that provide a powerful model system for examining how retroviral glycoproteins may have evolved to utilize different cellular proteins as receptors to initiate infection (4, 36). The ASLV subgroups are divided based on host range, interference patterns, and cross-reactivity with neutralizing antibodies. The envelope glycoproteins of ASLV(A-E) are highly conserved except for the five variable regions (vr1, vr2, hr1, hr2, and vr3) in the SU glycoprotein. The hr1 and hr2 domains contain determinants important for receptor interaction, while the vr3 domain is involved in determining receptor specificity. These differences in the SU glycoproteins of ASLV(A-E) allow the subgroups to utilize different proteins as receptors.

Three very different families of cell surface proteins that act as receptors for ASLV(A-E) have been identified and cloned (4). The receptor for ASLV(A) is Tva, a protein most related to the family of low-density lipoprotein receptors (LDLR) (5, 37). The functional ASLV(A) receptor interaction domain of Tva is a 40-amino-acid cysteine-rich domain in the extracellular region known as the LDL-A module, which is related to similar cysteine-rich domains in other LDLR (6, 31, 38). At least two regions of LDL-A are important for mediating efficient ASLV(A) entry. Amino acid residue Trp-48 in the C terminus of the LDL-A domain appears to be a primary interaction determinant for high-affinity binding to the SU(A) glycoprotein and for inducing the fusion process (33, 39). In addition, several studies have shown an additional residue...
in the middle of the domain, residue 31, which is glutamine in quail Tva and leucine in chicken Tva, to also be important for binding and entry of ASLV(A) (28, 32).

The Tvb receptors confer susceptibility to ASLV(B,D,E) and are related to members of the tumor necrosis factor receptor (TNFR) family, which contain three cysteine-rich domains (CRD1 to -3) (1, 3, 7). The chicken TvbS1 receptor confers susceptibility to ASLV(B,D,E), while the chicken TvbS3 receptor is a receptor for ASLV(B,D). The turkey TvbT protein is a receptor for ASLV(E) only (2). The difference between TvbS1 and TvbS3 is a cysteine at amino acid residue 62 between TvbS1 and TvbS3 is a cysteine at amino acid residue 62, possibly altering the receptor conformation, which appears to be necessary for ASLV(E) infection. Studies have shown that amino acid residues 32 to 46 in CRD1 of TvbS1 are sufficient to act as an ASLV(B,D) receptor: specifically, Leu-36, Gln-37, and Tyr-42 appear to be essential amino acids (1, 21, 22). Furthermore, amino acid residues Tyr-67, Asn-72, and Asp-73 in CRD2 of TvbS1 appear to be necessary for efficient ASLV(E) binding and receptor function. We recently published a study showing a possible role of CRD3 of Tvb in efficient binding and entry of ASLV(B,D,E) (29). An inbred line of chickens contained a mutant tvb allele, tvb2, which encodes a TvbS1 receptor with a substitution of C125S in the extracellular domain of Tvc. This fragment was subcloned into the EcoRI and BamHI sites of the pBluescript II KS (+) vector to create pBS-Tvc. Next, the IgV domain was removed from CLA12NCO (described below) as a PstI-BamHI fragment and was subcloned into the PstI site of pBS-Tvc to create pBS-IgV. Finally, pBS-IgV was digested with EcoRI and BamHI, and this fragment was cloned into the EcoRI and

**MATERIALS AND METHODS**

**Vector construction.** The chicken Tvc expression plasmid (pTvc) was described previously (12). Briefly, pTvc is a pSG5 expression vector that encodes a Tvc receptor truncated at amino acid residue 335 in the cytoplasmic domain. The pSG5 expression plasmid has been described previously (14). In this study, a hemagglutinin (HA) epitope tag (YPYDVPDYA) followed by 10 histidine residues was fused in frame to the BglII site in the vector to create pTvc-HA. His. pTvc-HA/His was used as a template to create receptor proteins that contained mutations encoded in the tvc gene. pTvc-HA/His was digested with EcoRI and BamHI to create a 740-bp fragment encoding the extracellular domain of Tvc. This fragment was subcloned into the EcoRI and BamHI sites of the pBluescript II KS (+) vector to create a plasmid with a potential N-linked glycosylation site. Structural studies on proteins with IgV and IgC domains in the immunoglobulin family showed that these cysteine residues form intradomain disulfide bonds to stabilize the structure of each domain of the protein. Similar to the butyrophilins, the cytoplasmic tail domain of the Tvc receptor contains a B30.2 domain, a domain not required for ASLV(C) infection.

The functional determinants of Tvc responsible for ASLV(C) receptor activity have not been identified. In this study, we determined that the IgV domain of the Tvc receptor is responsible for interacting with the glycoprotein of ASLV(C). Although we could find no evidence that the IgC domain of the Tvc receptor interacted with the ASLV(C) glycoproteins, tvc-negative cells expressing the wild-type Tvc protein were more efficiently infected by ASLV(C) than were those expressing a mutant Tvc receptor with the IgC domain deleted. After conducting further experiments, we concluded that a domain appears to be necessary as a spacer between the IgV domain and the membrane-spanning domain for efficient Tvc receptor activity, most likely to orient the IgV domain a proper distance from the cell membrane. The effects on ASLV(C) glycoprotein binding and infection efficiency were also studied by site-directed mutagenesis of the cysteine residues of Tvc as well as conserved amino acid residues of the IgV Tvc domain compared to other IgV domains.
BamHI sites of pTvc-HA/His to create plgV-HA/His. To express only the IgC domain, pTvc-HA/His was digested with convenient PsI sites within the rve gene to remove the IgV domain to generate plgC-HA/His. The Tve-HA/His, IgV-HA/His, and IgC-HA/His coding regions were PCR amplified and then subcloned into the BamHI and BamHI sites of the CLA12NCO adaptor plasmid. The tvc-ht hvis, igv-ht hvis, and igc-ht hvis genes were removed from the unique Clal site of the RCASBP(B) retroviral vector. The adaptor plasmid and RCASBP(B) retroviral vector were described previously (13, 18).

To create the ig/va gene, a QuiChang site-directed mutagenesis kit (Stratagene) was used following the manufacturer’s protocol to introduce Nhel and EcoRI sites into plgV-HA/His, at nucleotides 153 and 234, to produce plgV-HA/His. The chicken Tva expression plasmid (pTvaV) was used as a template to amplify the coding sequence for the Tva extracellular domain (amino acid residues 1 to 88) (10). An Nhel site was introduced into pTvaVs at nucleotide 234, and the extracellular domain of chicken Tva was removed as an Nhel-EcoRI fragment and cloned into the Nhel and EcoRI sites of plgV-HA/His to create plgV-Tva-HA/His. To construct the ig/va-ht hvis gene, an Nhel site was introduced into plgC-HA/His, using a QuiChang site-directed mutagenesis kit, to produce plgC-HA/His. To engineer the ntg-ht hvis gene, plgV-Tva-HA/His was digested with PsI to remove the IgV domain, creating pTva-HA/His. Using plgV-HA/His, an Igal site was inserted at nucleotide 140, just prior to the start of the IgV domain. The IgV domain was removed as an EagI-BamHI fragment and inserted into the EagI and BamHI sites of pTva-HA/His to produce pTvaVigHa/His. The chimera Tvc/ve genes were subcloned into the CLA12NCO adaptor plasmid, and the genes were subcloned as Clal fragments into the Clal site of the RCASBP(B) vector. Amino acid mutations were introduced into the ve genes in the CLA12NCO adaptor plasmid by use of a QuiChang site-directed mutagenesis kit (Stratagene) following the manufacturer’s protocol. The tvc-ht hvis genes cassette containing the point mutations were isolated as Clal fragments and cloned into the Clal site of RCASBP(B).

The construction of RCASBP(A)AP and RCASBP(C)AP retroviral vectors, comprising the avian leukosis virus (ALV) replication-competent vector containing the subgroup A or C env gene and the human heat-stable alkaline phosphatase (AP) gene, has been described previously (13). The suicide vector encoding the SU glycoprotein of RCASBP(C) linked to the constant region of rabbit immunoglobulin G (rIgG), cloned into the CLA12NCO adaptor plasmid, has been described previously (12).

Chicken lines. The inbred White Leghorn chicken line L15 was originally developed at the Northern Poultry Breeding Station (Reaseheath, Cheshire, United Kingdom) and imported to Prague, Czech Republic, in 1977 (27). Line L15 is resistant to ASLV(A) infection but susceptible to ASLV(B) infection. Line Rh-C was bred at the Avian Disease and Oncology Laboratory (East Lansing, United States) and imported to Prague, Czech Republic, in 1977 (27). Line L15 has been described previously (12).

Cell culture and virus propagation. Chicken embryo fibroblasts (CEFs) were prepared from 10-day-old embryos of chicken lines L15 and Rh-C (13). CEFs and DF-1 cells, a continuous fibroblastic cell line derived from line 0 CEFs (15, 34), were maintained in Dulbecco’s modified Eagle’s medium (Cellgro) supplemented with 10% fetal bovine serum (Gibco BRL), 100 U/ml of penicillin, and 100 µg/ml of streptomycin (Quality Biologicals, Inc., Gaithersburg, MD) at 39°C and 5% CO2. Hamster NIL-2 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% CO2. CEFs prepared from 10-day-old embryos of chicken lines L15 and Rh-C (13). CEFs (10, 12).

RESULTS

The IgV domain of Tve confers susceptibility to ASLV(C) infection. To determine if the IgV domain, IgC domain, or both extracellular domains in the Tve receptor contain determinants important for ASLV(C) receptor function, expression plasmids encoding only the IgV domain (plgV-HA/His) or the IgC domain (pIgC-HA/His) were constructed by using previously described pTve expression plasmid (12). All of the membrane proteins constructed in these studies contained a deletion of most of the intracellular B30.2 domain plus the addition
of an HA epitope and 10-histidine tag to aid in the detection of protein expression in cells (Fig. 1). The expression plasmids encoding Tvc, IgV, or IgC receptor constructs or an empty vector (M) were transiently transfected into mammalian NIL-2 cells. The transfection mixture contained 8.0 μg of expression plasmid DNA and 0.8 μg of an EGFP expression plasmid. (A) Western immunoblot analysis of the levels of Tvc, IgV, and IgC proteins expressed in transfected NIL-2 cells. Equal amounts of total protein (10 μg) isolated from cell lysates were separated by SDS-10% PAGE and transferred to a nitrocellulose membrane. The membrane was probed with an anti-HA-tag monoclonal antibody (12CA5), followed by anti-mouse IgG-horseradish peroxidase conjugate, and the bound protein complexes were visualized by chemiluminescence. (B) NIL-2 cells transiently expressing the Tvc, IgV, or IgC receptor protein were challenged with serial 10-fold diluted RCASBP(A)AP or RCASBP(C)AP. Bar M, cells that were transfected with an empty expression vector. As controls, clonal NIL-2 cell lines which stably express Tva (NIL-Tva) or Tvc (NIL-Tvc), as well as chicken DF-1 cells, were challenged with RCASBP(A)AP and RCASBP(C)AP. Asterisks indicate that the virus titer was below the limit of detection (<10 infectious units/ml). (C and D) The Tvc, IgV, or IgC expression gene or no gene (M) was delivered and expressed, using the RCASBP(B) vector, in line L15 CEFs (see panel B for details). Line L15 CEFs expressing the Tvc, IgV, or IgC receptor protein or no receptor (M) were challenged with 10-fold serial dilutions of RCASBP(A)AP or RCASBP(C)AP. Virus titers were determined by AP assay. Molecular sizes in kilodaltons are shown on the left in panels A and C. The virus titer results in panels B and D are the averages and standard deviations for three separate experiments. ifu, infectious units.
observed for expression of any of the ASLV Tva, Tvb, and Tvc receptors in mammalian cells, the highest possible titer achieved in the mammalian cells was always 10- to 1,000-fold lower than the titer obtained in chicken cells. This difference was also seen with clonal cell lines stably expressing the ASLV receptor. In this experiment, we used NIL-2 cell lines stably expressing either Tva or Tvc as controls to evaluate whether the low transfection efficiency affected the experiment. We did observe a major difference in the transient delivery of Tvc versus stable expression: ASLV(C) infection was 10-fold less efficient in NIL-2 cells transiently expressing the Tvc or IgV protein than in the NIL-Tvc cell line.

Since we were concerned about the effect that the low transfection efficiency may have on these experiments, we tested an alternative method to efficiently express the Tvc, IgV, and IgC membrane proteins in line L15 CEFs. Line L15 CEFs are resistant to ASLV(C) infection due to a mutation in the tvc gene, which encodes a Tvc protein with a premature stop codon. We have shown that the replication-competent ASLV-based retroviral vector with the subgroup B env gene [RCASBP(B)] is very efficient at delivering a gene of interest and expressing high levels of an experimental protein in infected cells. Therefore, we cloned the tvc, igv, and igc genes into the RCASBP(B) retroviral vector. To initiate virus propagation, we monitored in infected cell culture supernatants by using ELISA to detect ASLV CA protein; the production of this protein peaked at ~8 days posttransfection (data not shown). The expression levels of the Tvc and IgC proteins appeared to be similar but were higher than the expression level of the IgV membrane protein, which could be detected by Western analysis in these experiments (Fig. 2C). At 8 days posttransfection, the infected line L15 CEF cultures were challenged with 10-fold serial dilutions of RCASBP(C)AP and RCASBP(A)AP virus stocks. The price for the improved delivery of genes encoding Tvc proteins with RCASBP(B) was that background infection of line L15 CEFs averaged ~8 x 10^4 infectious units/ml in uninfected (data not shown) and RCASBP(B) vector-infected L15 CEFs. Expression of the wild-type Tvc receptor protein in L15 CEFs by RCASBP(B) gene delivery restored ASLV(C) infection efficiency to a level similar to the titer in DF-1 cells (Fig. 2D). As observed using NIL-2 cells, the expression of the IgV receptor protein in line L15 cells did confer susceptibility to ASLV(C) infection: virus titers were ~50-fold higher than those in mock-infected cells. However, the level of ASLV(C) infection using the IgV receptor was ~30-fold less efficient than that in cells expressing the wild-type Tvc receptor. Similar to NIL-2 cells, line L15 cells which expressed the IgC protein were not efficiently infected with ASLV(C) (Fig. 2D).

The IgV domain of Tvc binds to ASLV(C) glycoprotein with low-nanomolar affinity. The results from the studies above show that the expression of the IgV domain of Tvc, but not the IgC domain, confers susceptibility to ASLV(C) infection. Therefore, we hypothesized that the IgV domain would contain the interaction determinants necessary for a high binding affinity for the ASLV(C) SU glycoprotein. To test this hypothesis, we estimated the binding affinities of the Tvc, IgV, and IgC receptors by using a soluble form of the ASLV(C) SU glycoprotein, SU(C)-rIgG. The production and integrity of soluble SU(C)-rIgG were described previously (12). To assay binding affinities, DF-1 cells naturally expressing the wild-type Tvc receptor and line L15 CEFs alone or expressing the Tvc, IgV, or IgC protein were incubated with different amounts of SU(C)-rIgG, and binding levels were measured by FACS. The wild-type Tvc receptor expressed at natural levels on DF-1 cells bound to SU(C)-rIgG with an estimated affinity of 1.3 ± 0.26 nM (mean ± standard deviation for three experiments). The estimated binding affinity for SU(C)-rIgG of Tvc expressed on line L15 CEFs was 0.08 ± 0.03 nM, ~15-fold higher than that of Tvc on DF-1 cells. More than likely, the L15 CEFs express much higher levels of the Tvc receptor after delivery with RCASBP(B) than the natural level on DF-1, and this difference may account for the increase in the estimated binding affinity in L15 CEFs expressing Tvc due to the increase in avidity of the viral glycoproteins interacting with multiple receptors. As hypothesized, the IgV receptor protein expressed on L15 CEFs bound to the ASLV(C) SU glycoprotein at a low-nanomolar affinity, ~0.86 ± 0.22 nM, ~10-fold lower than that of wild-type Tvc (0.08 nM). As predicted by our data, we could not detect binding above background of SU(C)-rIgG to the IgC receptor protein expressed on L15 CEFs with the SU(C)-rIgG concentrations used in the analysis. Like the case for infection, the IgV domain of Tvc contained the determinants critical for high-affinity Tvc binding to the ASLV(C) SU glycoprotein. Also, as observed for infection, the IgV domain expressed in the truncated Tvc receptor does not appear to bind to SU(C)-rIgG as well as wild-type Tvc does.

Mutational analysis of conserved cysteine residues in the IgV and IgC domains of Tvc. Similar to other proteins in the immunoglobulin superfamily, the extracellular domain of the Tvc protein contains four conserved cysteine residues, two in the IgV domain (Cys-33 and Cys-107) and two in the IgC domain (Cys-147 and Cys-201). To determine if the cysteine residues are necessary for Tvc to function as an ASLV(C) receptor, each cysteine residue was mutated to alanine singly and in combination. The Tvc receptors containing the cysteine mutations were delivered by RCASBP(B) vectors to line 15 CEFs, resulting in easily detectable levels of receptor expression (data not shown). The mutant Tvc receptors with single cysteine mutations in the IgV domain (C33A or C107A) were approximately as efficient at mediating ASLV(C) infection as the wild-type Tvc receptor (Table 1). Surprisingly, the Tvc receptor containing double cysteine mutations in the IgV domain (C33A/C107A) also mediated efficient ASLV(C) infection at levels similar to those for wild-type Tvc. However, all of the other mutant Tvc receptors, both those that contained the single mutation C147A or C201A, located in the IgC domain (10- to 50-fold decrease), and all other combinations of cysteine mutations (100- to 500-fold decrease), were significantly impaired in mediating ASLV(C) infection compared to wild-type Tvc (Table 1). The cysteine residues in the IgV domain in the full-length Tvc receptor do not appear to be necessary for efficient ASLV(C) receptor function, but the C33A, C107A, and C33A/C107A mutations constructed in the truncated IgV-only membrane-bound receptor did eliminate the rescued ASLV(C) infectivity (Table 1). The cysteine mutations in the IgC domain or between the IgV and IgC domains significantly
decreased the efficiency of the receptor in mediating ASLV(C) infection. These results suggest that the IgC domain may play a role in efficient Tvc receptor function.

**Increasing the IgV domain distance from the cell membrane affects the efficiency of ASLV(C) infection.** One possible function of the IgC domain in Tvc could be as a type of spacer between the IgV domain and the membrane surface that provides the proper orientation and distance for ASLV(C) to use Tvc as an efficient receptor. This function would fit all of the data from the above experiments: the IgC domain would have no identifiable determinants for ASLV(C) glycoprotein binding or virus infection, but mutations in the IgC domain would still alter receptor function. To test this hypothesis, we constructed chimeric Tvc receptors, replacing either the IgC domain or the IgV domain with the extracellular domain (88 residues) of the chicken Tva receptor (Fig. 3A). The chimeric Tvc/Tva receptors were delivered to line L15 CEFs by use of RCASBP(B) vectors and generated cells with similar levels of chimeric receptor protein expression (Fig. 3B). The expression of the IgV/Tva chimeric receptor enabled efficient ASLV(C) infection, at levels comparable to those with wild-type Tvc and 10-fold higher than those with the receptor with only the IgV domain (Table 2). Interestingly, if the extracellular domains were reversed (Tva/IgV), the chimeric receptor did not function as an ASLV(C) receptor. The IgC/Tva chimeric protein also did not function as an ASLV(C) receptor, as shown above for the IgC domain alone. As expected, expressing the Tva domain alone enabled efficient ASLV(A) infection and did not mediate ASLV(C) infection above background levels. Only the wild-type Tvc receptor, the IgV domain alone, and the IgV/Tva chimeric receptor protein bound SU(C)-rIgG at detectable levels; these were the same receptor proteins that conferred susceptibility to ASLV(C) infection at levels above background (Table 2).

**To determine if the chimeric membrane proteins could also function as ASLV(A) receptors, the proteins were expressed in Rh-C CEFs. Line Rh-C CEFs are resistant to ASLV(A) infection due to a Cys40Trp mutation in the Tva receptor (10).**

**FIG. 3.** Tvc and Tvc/Tva chimeric receptor protein expression in line L15 CEFs. (A) Schematic representation of the Tvc, IgV/Tva, IgC/Tva, Tva, and Tva/IgV receptors expressed on the cell surface and used to determine receptor function. (B) Western immunoblot analysis of the expression levels of the chimeric proteins delivered and expressed by RCASBP(B) vector infection of line L15 CEFs (as described in the legend to Fig. 2). M, CEFs infected with an empty RCASBP(B) vector.

**TABLE 1. Effects of cysteine mutations in the Tvc receptor, delivered and expressed by RCASBP(B) vectors in line L15 CEFs, on infection with RCASBP(C)AP**

| Tvc receptor construct | IgV domain at position | IgC domain at position | Virus titer (avg ± SD)* |
|------------------------|------------------------|------------------------|------------------------|
|                        | 33 107                 | 147 201                |                        |
| Wild type              | C C                    | C C                    | (3.4 ± 2.3) × 10^5     |
| C33A                   | A C                    | C C                    | (1.7 ± 0.3) × 10^4     |
| C107A                  | C A                    | C C                    | (9.1 ± 3.8) × 10^4     |
| C147A                  | C A                    | A C                    | (6.9 ± 1.9) × 10^3     |
| C201A                  | C C                    | C A                    | (3.3 ± 3.0) × 10^4     |
| C33A/C107A             | A A                    | C C                    | (1.2 ± 0.4) × 10^5     |
| C33A/C147A             | A C                    | A C                    | (2.2 ± 1.6) × 10^3     |
| C33A/C201A             | A A                    | C A                    | (2.7 ± 0.8) × 10^3     |
| C107A/C147A            | C A                    | A A                    | (3.7 ± 2.1) × 10^2     |
| C107A/C201A            | C A                    | C A                    | (1.6 ± 1.7) × 10^3     |
| C147A/C201A            | C C                    | A A                    | (5.7 ± 3.2) × 10^2     |
| C33A/C107A/C147A/C201A | A A                    | A A                    | (2.3 ± 1.7) × 10^3     |
| Mockb                  | (4.5 ± 1.8) × 10^2     |
| WT                     | C C                    | Deleted                | (2.9 ± 1.2) × 10^5     |
| IgV                    | C C                    | Deleted                | (2.3 ± 1.1) × 10^4     |
| IgV/C33A              | A C                    | Deleted                | (2.6 ± 2.2) × 10^3     |
| IgV/C107A             | C A                    | Deleted                | (2.5 ± 1.7) × 10^3     |
| IgV/C33A/C107A        | A A                    | Deleted                | (1.2 ± 0.7) × 10^3     |
| Mockb                  | (1.8 ± 1.4) × 10^3     |

*Virus titers were determined by AP assay and are infectious units/ml. The averages and standard deviations are for three separate experiments.

b Line L15 CEFs infected with an empty RCASBP(B) vector.
Similar expression levels of the chimeric receptor proteins to those in line L15 CEFs were detected in Rh-C CEFs (data not shown). There was little or no detectable ASLV(A) infection in line Rh-C CEFs alone or in Rh-C CEFs which expressed only the IgV domain, the IgC domain, or the wild-type Tvc receptor protein. All of the Tvc/Tva chimeric receptor proteins expressed in Rh-C CEFs conferred a significant increase in ASLV(A) infection efficiency compared to that for Rh-C CEFs expressing the wild-type or mutant Tvc receptors. The IgC/Tva and Tva/IgV chimeric receptor proteins appeared to function as well as wild-type Tva as an ASLV receptor. The IgV/Tva chimeric receptor protein expressed on the cell surface than the normal endogenous levels of receptor. Since changes in binding affinity of ASLV glycoprotein-receptor interaction may not be apparent due to unnatural high-avidity virion-receptor interactions in the experiment, the SU(C)-rIgG immunoadhesin glycoprotein was used to quantitate binding efficiencies for wild-type or mutant Tvc receptors expressed on L15 CEFs, and the bound SU(C) was quantitated by FACS. Unexpectedly, detectable SU(C)-rIgG binding over background was measured only on CEFs expressing wild-type Tvc or the Tvc-D83A mutant receptor; both receptors had similar estimated binding affinities to SU(C)-rIgG at detectable levels; both proteins were estimated to bind SU(C)-rIgG at low-nanomolar affinities (0.67 nM).

Identification of amino acid residues in the IgV domain of Tvc important for interacting with the ASLV(C) glycoprotein and/or ASLV(C) infection. The amino acid sequence of the IgV domain of Tvc was compared to those of other IgV domains of butyrophilins (Fig. 4A) and CD80/CD86 proteins (Fig. 4B), the closest related members of the Ig superfamily (23, 26). In addition to the two cysteines at positions 33 and 107 in Tvc, nine other amino acid residues conserved in the IgV domains were chosen for mutagenesis to alanine (L-31, W-48, Y-74, R-77, D-83, L-92, D-101, G-103, and Y-105) to define other residues in the IgV domain responsible for interacting with the ASLV(C) glycoprotein. The Tyr residue in CD80 (Tyr-74 in Tvc) has been identified as an essential residue for CD80 to efficiently interact with CTLA4 and CD28 molecules. Wild-type Tvc and Tvc receptors containing mutations (L31A, W48A, Y74A, R77A, D83A, L92A, D101A, G103A, and Y105A) were constructed and expressed in line L15 CEFs at detectable levels, as analyzed by Western immunoblotting (Fig. 5A), although the wild-type Tvc and Tvc-D83A receptor proteins were expressed at the highest levels. An ASLV(C) virus stock, RCASBP(C)AP, was titrated on L15 CEFs expressing the wild-type or mutant Tvc receptors to evaluate the mutations’ effects on the efficiency of virus infection (Fig. 5B). Two mutations of aromatic residues, the Tvc-W48A and Tvc-Y105A mutations, reduced the ASLV(C) infection efficiency >100-fold, to near background infection levels. Three mutations, the Tvc-Y74A, Tvc-D83A, and Tvc-D101A mutations, did not alter ASLV(C) infection efficiency compared to that with wild-type Tvc. The other four mutant Tvc receptors conferred susceptibility to ASLV(C) infection, but at reduced levels (two- to eightfold) compared to that with wild-type Tvc.

The ASLV receptors are normally expressed at low levels in avian cells. Experimentally, the efficient delivery and expression of ASLV receptors, using either a transient transfection system or the RCASBP vector system, result in higher levels of receptor protein expressed on the cell surface than the normal endogenous levels of receptor. Since changes in binding affinity of ASLV glycoprotein-receptor interaction may not be apparent due to unnatural high-avidity virion-receptor interactions in the experiment, the SU(C)-rIgG immunoadhesin glycoprotein was used to quantitate binding efficiencies for wild-type or mutant Tvc receptors expressed on L15 CEFs, and the bound SU(C) was quantitated by FACS. Unexpectedly, detectable SU(C)-rIgG binding over background was measured only on CEFs expressing wild-type Tvc or the Tvc-D83A mutant receptor; both receptors had similar estimated binding affinities to SU(C)-rIgG (data not shown). Since we could detect the mutant Tvc proteins by Western immunoblotting (Fig. 5A), we also performed a pull-down assay and immunoblot analysis to determine if the mutant Tvc receptors could bind and pull down the SU(C)-rIgG protein (Fig. 5C). In this assay, wild-type Tvc and the three Tvc mutants that conferred wild-type ASLV(C) infectivity to L15 CEFs, Tvc-Y74A, Tvc-D83A, and Tvc-D101A, were efficiently bound and pulled down by SU(C)-rIgG, i.e., the Tvc proteins were detected at approximately the same levels as those before the pull-down assay (Fig. 5A). However, SU(C)-rIgG could not pull down detectable levels of the other Tvc mutant proteins. We concluded from these experiments that Tvc-D83A and possibly Tvc-Y74A and Tvc-Y105A mutations reduce the ASLV(C) infection efficiency compared to that with wild-type Tvc.

---

**Table 2. ASLV infection susceptibilities and estimated binding affinities of chimeric Tvc and Tva receptor proteins**

| Protein | ASLV titer (avg ± SD)a,b | Apparent KD (nM)c,d |
|---------|--------------------------|---------------------|
| Tvc     | (3.5 ± 2.3) × 10^1       | 0.08 ± 0.03         |
| IgV     | (2.8 ± 1.1) × 10^4       | —                   |
| IgC     | (1.2 ± 0.3) × 10^3       | —                   |
| IgV/Tva | (4.1 ± 0.7) × 10^5       | —                   |
| IgC/Tva | (1.3 ± 0.7) × 10^3       | —                   |
| Tva/IgV | (9.5 ± 6.7) × 10^1       | —                   |
| Tva(Tvc) | (9.0 ± 2.6) × 10^1     | 0.67 ± 0.8          |
| Chicken Tva | —        | —                   |
| Mock    | (1.0 ± 0.1) × 10^3       | —                   |

---

a Virus titers were determined by AP assay and are infectious units/ml. The averages and standard deviations are for three separate experiments.
b Each result is the average and standard deviation for three experiments.
c Virus titers were determined in line L15 CEFs.
d Virus titers were determined in line Rh-C CEFs.

---

*Vol. 82, 2008 Tvc FUNCTIONAL DETERMINANTS 11425*
effect on the level of ASLV(C) infectivity conferred (Fig. 5B), at least at the levels that the receptor proteins were expressed in these experiments. We hypothesize that the Tvc-W48A and Tvc-Y105A receptors have the highest SU(C)-rIgG binding defect since these receptors are also significantly reduced in conferring ASLV(C) infectivity.

**DISCUSSION**

We have shown here that the major determinants in the Tvc receptor for binding of the ASLV(C) SU glycoprotein and for ASLV(C) infection are located in the IgV domain. We could not detect any significant direct interaction between the IgC domain and ASLV(C). However, expression of the IgC-deleted Tvc receptor reduced both the ASLV(C) binding affinity and infection efficiency to \( \frac{1}{100} \) of those of wild-type Tvc. In addition, the IgC domain could be replaced with the extracellular domain of the ASLV(A) receptor Tva in the Tvc receptor, and this IgV/Tva chimeric receptor conferred maximum ASLV(C) infection efficiency and recovered the majority of the SU(C) binding affinity despite no homology between Tva and the IgC domain. ASLV evolved to use different cell surface proteins as receptors, and logically, the wild-type receptor protein should be most efficient at conferring virus entry.

The distance of the virus binding determinants of the receptor from the cell surface, the availability/accessibility of these binding surfaces to virus glycoprotein binding, and combinations of these and other possible scenarios could radically affect the efficiency of virus glycoprotein-receptor interactions that lead to virus entry (Fig. 6).

The fact that only the IgV/Tva combination of Tvc IgV domain and Tva chimeric receptors could function efficiently as both an ASLV(C) and ASLV(A) receptor highlights the importance of how the functional receptor domains are presented on the cell surface (Fig. 6). While the Tva/IgV receptor did confer susceptibility to ASLV(A) infection (\( \frac{1}{100} \) fold above background), the ASLV(A) infection efficiency was \( \frac{1}{100} \) fold lower than those of wild-type Tva and IgV/Tva and did not support ASLV(C) infection. Displaying the IgC domain on the N terminus of Tva resulted in a further reduction in ASLV(A) infection efficiency, possibly due to the IgC domain physically blocking access of virions to the Tva domain. It was reported previously that varying the length and presumably the distance from the cell surface of CD46, the receptor for measles virus, altered the efficiency of measles virus binding, fusion, and entry into the cells (8). It is difficult to predict the effect that mutations may have on the overall structure of the receptor proteins without direct crystal structures. In addition, there is very little information on how and with which other proteins, lipoproteins, and/or lipid domains the receptor proteins are normally displayed on the cell surface.

Structural studies of proteins belonging to the immunoglobulin superfamily have shown that the conserved cysteine resi-
The efficiency of the mutant Tvc receptor cysteine mutations in the IgC domain affected the infection bond for recognition. However, all single and combination ing the ASLV(C) SU glycoprotein do not require the cysteine residues form intradomain disulfide bonds to stabilize the structure of the protein. For ASLV(C) receptor function, the cysteine residues of the IgV domain do not appear to be required, at least in the complete Tvc extracellular domain, implying that the residues/regions of IgV responsible for binding the ASLV(C) SU glycoprotein do not require the cysteine bond for recognition. However, all single and combination cysteine mutations in the IgC domain affected the infection efficiency of the mutant Tvc receptor >10-fold compared to the wild-type Tvc receptor. These data, in combination with the domain deletion and substitution data, again highlight the importance of the proper display of the IgV domain: a cysteine mutation(s) in the IgC domain likely alters the IgC structure, thereby changing the distance of IgV from the cell surface and/or displaying the IgV domain in a way that blocks ASLV(C) interaction.

The normal functions of the Tva, Tvb, and Tvc proteins remain unknown. In studies of naturally occurring mutations in the ASLV receptor alleles that confer resistance to ASLV infection, besides mutations that result in the absence or truncation of the receptor protein, several mutations code for a substitution of a single cysteine residue in the receptor, resulting in drastically lower binding affinities for the ASLV envelope glycoprotein: these include Cys40Trp in the LDLR-related region of Tva (10), Cys125Ser in CRD3 of Tvb (29), and the Cys62Ser substitution previously identified as the Tvb (3) receptor (1). Presumably, these Cys substitutions alter the structure of the receptor protein to either lower the binding affinity for the viral glycoprotein, reduce the accessibility of the binding region(s) on the receptor to interaction with the viral glycoproteins, and/or result in a nonoptimal distance of the receptor interaction domain(s) from the membrane (Fig. 6). The phenotype of the Cys125Ser mutation in the Tvb receptor is very similar to the phenotype of the Tvc receptor with cysteine mutations in the IgV domain (29). Previous analysis of the Tvb receptor showed that the cysteine residues in CRD1 and CRD2 did not appear to be important for efficient ASLV(B) infection. The Tvb receptor CRD3 was thought to be dispensable for ASLV(B) receptor function since the deletion of CRD3 did not alter receptor function (1, 21, 22). However, the Tvb receptor with the Cys125Ser mutation has a significantly lower binding affinity for the ASLV(B) glycoproteins, reducing virus infection efficiency. This may be another example of a mutation changing the structure of the receptor by altering the distance of the binding region(s) from the cell surface and/or altering the accessibility of the binding region(s) to interaction with ASLV virion glycoproteins.

The ASLV receptors Tva, Tvb, and Tvc belong to three very different families of proteins. Therefore, it is not obvious that these receptors would share a homologous region for interacting with the ASLV(A-E) glycoproteins. In this initial analysis of Tvc determinants important for interacting with ASLV(C) glycoproteins, at least two aromatic amino acid residues in the IgV domain of Tvc, Trp-48 and Tyr-105, were identified as critical for efficient ASLV(C) infection. Interestingly, one or more aromatic amino acid residues have been identified as critical determinants in the other ASLV(A-E) receptors for proper interaction with ASLV glycoproteins: in Tva, residue Trp-48 is critical for ASLV(A) receptor function, and in Tvb, residues Tyr-42 and Tyr-67 are critical for ASLV(B,D,E) in-
fection. Aromatic residues have also been shown to be important determinants of other retroviral glycoprotein-receptor interactions (24). This suggests that the ASLV glycoproteins may share a common mechanism of receptor interaction with an aromatic residue(s) on the receptor critical for triggering conformational changes in SU that initiate the fusion process required for efficient virus infection. Since there is no requirement for the ASLV receptor protein to retain its normal cellular function, the native structures of the Tva, Tvb, and Tvc proteins may be fairly pliable in relation to their use as ASLV receptors. It may also be an advantage for ASLV to evolve to bind a region(s) of cellular proteins that is not critical for their normal cellular function, as binding of the natural ligands to the native receptors would not compete with ASLV binding.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grant AI48682 and by the Mayo Foundation (to M.J.F.). We thank Jan Svoboda, Jiri Hejnar, Jiri Plachy, and Josef Geryk (Academy of Sciences of the Czech Republic) for discussions and for critical readings of the manuscript.

REFERENCES

1. Adkins, H. B., S. C. Blacklow, and J. A. T. Young. 2001. Two functionally distinct forms of a retroviral receptor explain the nonreciprocal receptor interference among subgroups B, D, and E avian leukosis viruses. J. Virol. 75:3520–3526.
2. Adkins, H. B., J. Brojtatsch, J. Naughton, M. M. Rolls, J. M. Pesola, and J. A. T. Young. 1997. Identification of a cellular receptor for subgroup E avian leukosis virus. Proc. Natl. Acad. Sci. USA 94:11617–11622.
3. Adkins, H. B., J. Brojtatsch, and J. A. T. Young. 2000. Identification and characterization of a shared TNFR-related receptor for subgroup B, D, and E avian leukosis viruses reveal cysteine residues required specifically for subgroup E viral entry. J. Virol. 74:3572–3578.
4. Barnard, R. J., D. Elleder, and J. A. T. Young. 2006. Avian sarcoma and leukaemia virus-receptor interactions: from classical genetics to novel insights into virus-cell membrane fusion. Virology 344:25–29.
5. Bates, P., J. A. T. Young, and H. E. Varmus. 1993. A receptor for subgroup A Rous sarcoma virus is related to the low density lipoprotein receptor. Cell 74:1043–1051.
6. Belanger, C., K. Zingler, and J. A. Young. 1995. Importance of cysteines in the LDLR-related domain of the subgroup A avian leukosis and sarcoma virus receptor for viral entry. J. Virol. 69:1019–1024.
7. Brojtatsch, J., J. Naughton, M. M. Rolls, K. Zingler, and J. A. T. Young. 1996. CAR1, a TNFR-related protein, is a cellular receptor for cytopathic avian leukosis-sarcoma viruses and mediates apoptosis. Cell 87:845–855.
8. Buchholz, C. J., U. Schneider, P. Devaux, D. Gerlier, and R. Cattaneo. 1993. Isolation of a chicken gene encoding an envelope protein required for the regulated secretion of milk-lipid droplets. Proc. Natl. Acad. Sci. USA 90:8084–8088.
9. Butyrophilin (Btn1a1) in lactating mammary gland is essential for the regulated secretion of milk-lipid droplets. Proc. Natl. Acad. Sci. USA 95:8467–8472.
10. Butyrophilin (Btn1a1) in lactating mammary gland is essential for the regulated secretion of milk-lipid droplets. Proc. Natl. Acad. Sci. USA 95:8467–8472.
11. Adkins, H. B., S. C. Blacklow, and J. A. T. Young. 2001. Two functionally distinct forms of a retroviral receptor explain the nonreciprocal receptor interference among subgroups B, D, and E avian leukosis viruses. J. Virol. 75:3520–3526.
12. Adkins, H. B., J. Brojtatsch, J. Naughton, M. M. Rolls, J. M. Pesola, and J. A. T. Young. 1997. Identification of a cellular receptor for subgroup E avian leukosis virus. Proc. Natl. Acad. Sci. USA 94:11617–11622.
13. Adkins, H. B., J. Brojtatsch, and J. A. T. Young. 2000. Identification and characterization of a shared TNFR-related receptor for subgroup B, D, and E avian leukosis viruses reveal cysteine residues required specifically for subgroup E viral entry. J. Virol. 74:3572–3578.
14. Barnard, R. J., D. Elleder, and J. A. T. Young. 2006. Avian sarcoma and leukaemia virus-receptor interactions: from classical genetics to novel insights into virus-cell membrane fusion. Virology 344:25–29.
15. Bates, P., J. A. T. Young, and H. E. Varmus. 1993. A receptor for subgroup A Rous sarcoma virus is related to the low density lipoprotein receptor. Cell 74:1043–1051.
16. Belanger, C., K. Zingler, and J. A. Young. 1995. Importance of cysteines in the LDLR-related domain of the subgroup A avian leukosis and sarcoma virus receptor for viral entry. J. Virol. 69:1019–1024.
17. Brojtatsch, J., J. Naughton, M. M. Rolls, K. Zingler, and J. A. T. Young. 1996. CAR1, a TNFR-related protein, is a cellular receptor for cytopathic avian leukosis-sarcoma viruses and mediates apoptosis. Cell 87:845–855.
18. Buchholz, C. J., U. Schneider, P. Devaux, D. Gerlier, and R. Cattaneo. 1993. Isolation of a chicken gene encoding an envelope protein required for the regulated secretion of milk-lipid droplets. Proc. Natl. Acad. Sci. USA 90:8084–8088.
19. Butyrophilin (Btn1a1) in lactating mammary gland is essential for the regulated secretion of milk-lipid droplets. Proc. Natl. Acad. Sci. USA 95:8467–8472.
20. Butyrophilin (Btn1a1) in lactating mammary gland is essential for the regulated secretion of milk-lipid droplets. Proc. Natl. Acad. Sci. USA 95:8467–8472.
21. Adkins, H. B., S. C. Blacklow, and J. A. T. Young. 2001. Two functionally distinct forms of a retroviral receptor explain the nonreciprocal receptor interference among subgroups B, D, and E avian leukosis viruses. J. Virol. 75:3520–3526.
22. Adkins, H. B., J. Brojtatsch, J. Naughton, M. M. Rolls, J. M. Pesola, and J. A. T. Young. 1997. Identification of a cellular receptor for subgroup E avian leukosis virus. Proc. Natl. Acad. Sci. USA 94:11617–11622.
23. Adkins, H. B., J. Brojtatsch, and J. A. T. Young. 2000. Identification and characterization of a shared TNFR-related receptor for subgroup B, D, and E avian leukosis viruses reveal cysteine residues required specifically for subgroup E viral entry. J. Virol. 74:3572–3578.
24. Barnard, R. J., D. Elleder, and J. A. T. Young. 2006. Avian sarcoma and leukaemia virus-receptor interactions: from classical genetics to novel insights into virus-cell membrane fusion. Virology 344:25–29.
25. Bates, P., J. A. T. Young, and H. E. Varmus. 1993. A receptor for subgroup A Rous sarcoma virus is related to the low density lipoprotein receptor. Cell 74:1043–1051.
26. Belanger, C., K. Zingler, and J. A. Young. 1995. Importance of cysteines in the LDLR-related domain of the subgroup A avian leukosis and sarcoma virus receptor for viral entry. J. Virol. 69:1019–1024.
27. Brojtatsch, J., J. Naughton, M. M. Rolls, K. Zingler, and J. A. T. Young. 1996. CAR1, a TNFR-related protein, is a cellular receptor for cytopathic avian leukosis-sarcoma viruses and mediates apoptosis. Cell 87:845–855.
28. Buchholz, C. J., U. Schneider, P. Devaux, D. Gerlier, and R. Cattaneo. 1993. Isolation of a chicken gene encoding an envelope protein required for the regulated secretion of milk-lipid droplets. Proc. Natl. Acad. Sci. USA 90:8084–8088.
29. Butyrophilin (Btn1a1) in lactating mammary gland is essential for the regulated secretion of milk-lipid droplets. Proc. Natl. Acad. Sci. USA 95:8467–8472.