Model of the TVA Receptor Determinants Required for Efficient Infection by Subgroup A Avian Sarcoma and Leukosis Viruses

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ABSTRACT

The study of the interactions of subgroup A avian sarcoma and leucosis viruses [ASLV(A)] with the TVA receptor required to infect cells offers a powerful experimental model of retroviral entry. Several regions and specific residues in the TVA receptor have previously been identified to be critical determinants of the binding affinity with ASLV(A) envelope glycoproteins and to mediate efficient infection. Two homologs of the TVA receptor have been cloned: the original quail TVA receptor, which has been the basis for most of the initial characterization of the ASLV(A) TVA, and the chicken TVA receptor, which is 65% identical to the quail receptor overall but identical in the region thought to be critical for infection. Our previous work characterized three mutant ASLV(A) isolates that could efficiently bind and infect cells using the chicken TVA receptor homolog but not using the quail TVA receptor homolog, with the infectivity of one mutant virus being >500-fold less with the quail TVA receptor. The mutant viruses contained mutations in the hr1 region of the surface glycoprotein. Using chimeras of the quail and chicken TVA receptors, we have identified new residues of TVA critical for the binding affinity and entry of ASLV(A) using the mutant glycoproteins and viruses to probe the function of those residues. The quail TVA receptor required changes at residues 10, 14, and 31 of the corresponding chicken TVA residues to bind wild-type and mutant ASLV(A) glycoproteins with a high affinity and recover the ability to mediate efficient infection of cells. A model of the TVA determinants critical for interacting with ASLV(A) glycoproteins is proposed.

IMPORTANCE

A detailed understanding of how retroviruses enter cells, evolve to use new receptors, and maintain efficient entry is crucial for identifying new targets for combating retrovirus infection and pathogenesis, as well as for developing new approaches for targeted gene delivery. Since all retroviruses share an envelope glycoprotein organization, they likely share a mechanism of receptor triggering to begin the entry process. Multiple, noncontiguous interaction determinants located in the receptor and the surface (SU) glycoprotein hypervariable domains are required for binding affinity and to restrict or broaden receptor usage. In this study, further mechanistic details of the entry process were elucidated by characterizing the ASLV(A) glycoprotein interactions with the TVA receptor required for entry. The ASLV(A) envelope glycoproteins are organized into functional domains that allow changes in receptor choice to occur by mutation and/or recombination while maintaining a critical level of receptor binding affinity and an ability to trigger glycoprotein conformational changes.

For enveloped viruses to infect cells, they must fuse their viral membrane with a cellular membrane. For retroviruses, the interaction of the viral envelope glycoproteins with a cellular surface protein receptor initiates the entry and fusion process (1, 2). Retroviral envelope glycoproteins are trimers of surface (SU) glycoprotein and transmembrane (TM) glycoprotein heterodimers, with the SU glycoprotein containing the domains important for interaction with the receptor and the TM glycoprotein containing the domains responsible for the fusion process and tethering the glycoprotein to the viral surface (3, 4). The initial interaction of the retroviral glycoprotein with a specific cell surface receptor results in a conformational change in the trimeric glycoprotein structure exposing the TM glycoprotein domains. For most retroviruses, this initial viral glycoprotein receptor interaction triggering structural rearrangements and subsequent steps of viral and cellular membrane fusion occur at the cell surface in a neutral pH environment. In a variation of this mechanism, the envelope glycoproteins of HIV require two receptor interactions to initiate the fusion process: an initial binding with CD4 that triggers a structural change in the glycoproteins that then allows the interaction with a second receptor, CCR5 or CXCR4. Other enveloped viruses that employ class I fusion proteins use the viral glycoprotein re-

ceptor interaction to traffic the viral particle to an endocytic compartment, where low pH is required to trigger conformational changes in the viral glycoproteins initiating the fusion process. The avian sarcoma and leukosis virus (ASLV) family of retroviruses uses a third mechanism of entry: the initial interaction of the ASLV glycoproteins with their receptor triggers an initial conformational change at the cell surface but then requires exposure to a

Received 13 August 2014 Accepted 25 November 2014

Accepted manuscript posted online 3 December 2014

Citation Melder DC, Pike GM, VanBrocklin MW, Federspiel MJ. 2015. Model of the TVA receptor determinants required for efficient infection by subgroup A avian sarcoma and leukosis viruses. *J Virol* 89:2136–2148. doi:10.1128/JVI.02339-14.

Editor: K. L. Beemon

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low-pH environment to complete the conformational changes that can enable the completion of the fusion process (5, 6).

ASLVs have been divided into 10 envelope glycoprotein subgroups, A through J, on the basis of interference patterns, host range, and cross-reactivity to neutralizing antibodies (3, 4). Five highly related ASLV envelope subgroups that infect chickens, ASLV subgroup A [ASLV(A)] through ALSV(E), likely evolved from a common ancestor: their envelope glycoproteins are highly conserved, except for five variable domains in SU glycoproteins (vr1, vr2, hr1, hr2, and vr3) (5–13). A variety of studies have identified hr1 and hr2 to be the principal binding domains between the viral glycoprotein and receptor, with vr3 contributing to the specificity of the receptor interaction for initiating a productive infection. Three very different families of proteins have been identified to be receptors for these five ASLV subgroups. TVA proteins, which are proteins related to the low-density lipoprotein (LDL) receptors (LDLRs), are receptors for ASLV(A) (14, 15). TVB proteins, which are proteins related to the tumor necrosis factor receptors, are receptors for ASLV(B), ASLV(D), and ASLV(E) (16–19). TVC proteins, which are proteins related to mammalian butyrophilins, members of the immunoglobulin protein family, are receptors for ASLV(C) (20). These five ASLV subgroups are examples of the ability of retroviruses to evolve their highly conserved envelope glycoproteins to efficiently use different cell surface proteins as functional receptors for infection (5, 6, 9).

In experiments designed to identify important functional determinants for a productive interaction between ASLV(A) envelope glycoproteins and their receptor, TVA, a genetic selection system was used to select mutant ASLV(A) that could evade the antiviral effect of a secreted form of the TVA receptor, sTVA-mIgG, the extracellular domain of the quail TVA receptor fused to a mouse IgG (mIgG) domain, in chicken DF-1 cells (21). As hypothesized, several ASLV(A) mutants that had mutations in an envelope glycoprotein variable domain that significantly lowered the binding affinity between the mutant glycoprotein and the quail sTVA-mIgG competitive inhibitor and that escaped receptor interference were selected (22). The viruses contained the E149K, Y142N, or Y142N/E149K mutation in the hr1 variable region in the SU(A) glycoprotein subunit (Fig. 1A). The binding affinity of the mutants for quail sTVA-mIgG was directly related to their infection efficiency using the quail TVA receptor. However, these ASLV(A) mutants retained the ability to bind with a high affinity and efficiently use the chicken TVA homolog for entry at nearly wild-type levels. This was unexpected, since earlier studies had shown that the major interaction region between ASLV(A) Env and TVA was in a region identical in both the quail and chicken TVA homologs (23–27). Therefore, these mutant glycoproteins must differentially bind to TVA in the nonconserved regions of the N-terminal half of the 40-amino-acid cysteine-rich domain (the LDL-A domain) of the TVA receptor.

The chicken and quail TVA homologs are ~65% identical, with most of the 11 amino acid differences being clustered at the LDL-A domain (Fig. 1B) (28). Data previously presented by others determined that only the LDL-A region of TVA is necessary and sufficient to confer susceptibility to infection by ASLV(A) (23, 26). Two regions in the identical C-terminal halves of the TVA LDL-A domain contain the major functional determinants required for infection by ASLV(A) centered around a tryptophan residue, W48, in the mature quail TVA and the 6 residues required for the coordination of calcium, the acidic side chains of D36,

D40, D46, and E47 and the backbone carbonyl oxygen atoms of L34 and H38 (23–27). Two solution structures of the quail TVA LDL-A domain that may be used to model possible alterations in structure by different mutations have been published (29, 30). Unfortunately, the two structures differ substantially in the N terminus.

In this study, we identified several residues in the amino terminus of the TVA LDL-A domain that are critical for high-affinity binding with ASLV(A) glycoproteins and the infectivity of the viruses. We used the ability of the E149K, Y142N, or Y142N/E149K mutant ASLV(A) glycoproteins to preferentially bind the chicken TVA receptor homolog rather than the quail TVA homolog to identify other receptor regions important for high-affinity binding. The binding affinities of chicken and quail sTVA-mIgG receptor chimeras for wild-type and mutant ASLV(A) glycoproteins expressed on the surface of DF-1 cells were quantitated using a fluorescence-activated cell sorting (FACS) binding assay. The ability of membrane-bound forms of the chimeric TVA receptors to mediate efficient virus infection of chicken cells was also quantitated. This analysis identified the amino acid at position 31 of the quail TVA LDL-A to be a major determinant conferring the preferential utilization of one TVA receptor over the other by mutant virus; changes at position 31 did not alter binding with wild-type ASLV(A). TVA LDL-A residue 31 is in a region that is structurally very different between the two TVA solution structures: residue 31 is completely exposed to solvent in the structure determined by the Agard group (the Agard structure) (29), while this residue is mostly buried in the structure determined by the Rong group (the Rong structure) (30). Additional experiments identified residues neighboring position 31 in the TVA structure that further define this critical determinant of high-affinity binding between TVA and ASLV(A) glycoproteins and efficient viral infection.

MATERIALS AND METHODS

sTVA-mIgG constructions. Genes encoding soluble forms of quail sTVA-mIgG (21) and chicken sTVA-mIgG (cksTVA-mIgG) (22) were described previously. The sTVA-mIgG genes encode the extracellular domain of the particular TVA receptor fused to the constant region of a mouse IgG heavy chain and are in the CLA12NCO adaptor plasmid (31, 32). Specific amino acid mutations in TVA were constructed by using a QuikChange site-directed mutagenesis kit (Stratagene). The nucleotide sequence of the entire gene region was then verified by sequencing. The sTVA-mIgG gene cassettes were then isolated as ClaI fragments and subcloned into the ClaI site of the RCASBP retroviral vector with the subgroup C envelope glycoprotein [RCASBP(C)]. The replication-competent RCAS vector system has been reviewed previously (31, 32).

Membrane-bound TVA constructs. The construction of the chicken TVA expression plasmid pTvaS was described previously (28). In short, the sequence encoding the complete chicken TVA receptor was truncated in the intracellular tail, and a hemagglutinin tag and His₆ tag were added by PCR. The TvaS gene was subcloned into the NcoI to EcoRI sites of the CLA12NCO RCAS adaptor plasmid, using an NcoI to EcoRI fragment, to create CLA12NCO-TvaS. The extracellular region of TVA flanked by the NcoI and EagI sites from any sTVA-mIgG construct could then be subcloned into the NcoI and EagI sites of CLA12NCO-TvaS to create a membrane-bound form. The membrane-bound TVA gene cassettes were then isolated as ClaI fragments and subcloned into the ClaI site of the RCASBP(B) retroviral vector.

Cell culture and virus propagation. Cells of the DF-1 cell line, a continuous fibroblastic cell line derived from line O chicken embryo fibroblasts (CEFs) (33, 34), and CEFs from line Rh-C embryos obtained from

FIG 1 (A) Schematic representation of the ASLV EnvA glycoprotein. The variable regions of the subgroup A to E SU glycoproteins (vr1, vr2, hr1, hr2, and vr3), the internal fusion peptide, and the membrane-spanning domain of the TM glycoproteins are indicated. The sequences of the hr1 regions of the wild-type EnvA glycoprotein (WTA) and mutant EnvA glycoproteins are shown, and examples of their corresponding affinities of binding to chicken and quail TVA immunoadhesins (sTVA-mIgG) from this study are provided. (B) Extracellular domains of the chicken and quail TVA receptors. The different N termini (position +1) of the mature chicken TVA 88-amino-acid extracellular domain and the mature quail TVA 83-amino-acid extracellular domain are indicated. The mature quail TVA extracellular region is numbered (+1 to 83), while the LDL-A module is highlighted (gray box) and the three disulfide bonds are indicated by brackets above the TVA sequences. Identical residues are denoted by dots; gaps in the alignment are denoted by hyphens. Schematic representations of the quail and chicken TVA immunoadhesin chimeras initially tested are shown below the TVA sequences, with the dark gray boxes representing wild-type chicken (wtCK) TVA sequences and the light gray boxes representing wild-type quail (wtQ) TVA sequences. (C) Analysis of the ability of ASLV(A) to use the chicken, quail, and chicken-quail chimeric TVA receptors to efficiently infect avian cells. A subset of membrane-bound forms of chicken-quail TVA receptor chimeras and the wild-type chicken and wild-type quail TVA receptors were delivered by RCASBP(C) vectors into cultures of line Rh-C CEFs, which normally lack a functional TVA receptor. Controls included line Rh-C cells infected with the RCASBP(B) vector alone [(B) RHC cells] and DF-1 cells to accurately quantitate the virus titers on normal chicken cells with the wild-type TVA receptor (DF-1 cells). The cell cultures were infected with 10-fold serial dilutions of the ASLV(A) stocks with wild-type [(A)AP] or mutant [EK(A)AP, YN(A)AP, YNEK(A)AP] envelope glycoproteins, and the titer was determined by AP assay. Each of the results is the average of three different experiments. Error bars show standard deviations. ifu, inclusion-forming units.

the USDA Avian Disease and Oncology Laboratory were grown in Dulbecco's modified Eagle's medium (Gibco/BRL) supplemented with 10% fetal bovine serum (Gibco/BRL), 100 units of penicillin per ml, and 100 μ g of streptomycin per ml (Quality Biological, Inc., Gaithersburg, MD) at 39°C in 5% CO₂. HEK293 cells, HEK293T cells, Vero cells, D17 cells, CHO cells, and NIH 3T3 cells were grown as described above, except that they were grown at 37°C.

In standard transfections, 10 μ g of purified plasmid DNA containing the replication-competent RCAS vector in proviral form was introduced into DF-1 cells by the calcium phosphate precipitation method. Viral spread was monitored by assaying culture supernatants for the ASLV capsid (CA) protein by enzyme-linked immunosorbent assay (ELISA) (35). Virus and sTVA-mIgG stocks were generated from cell supernatants that had been cleared of cellular debris by centrifugation at 2,000 $\times$ g for 10 min at 4°C and stored in aliquots at -80°C. RCASBP(A)AP [(A)AP], the RCASBP retroviral vector with the wild-type subgroup A envelope glycoprotein and alkaline phosphatase transgene (AP), the RCASBP(A)AP E149K(A)AP [EK(A)AP], Y142N(A)AP [YN(A)AP], and Y142N/E149K(A)AP [YNEK(A)AP] mutants, and the assay for detecting alkaline phosphatase (AP) activity in infected DF-1 cells were described previously (21). RCASBP(C) vectors were used to deliver the wild-type and mutant sTVA-mIgG genes in DF-1 cell cultures to produce the sTVA-mIgG proteins used in the determination of the affinity of TVA binding to Env proteins of ASLV(A) (EnvA proteins) (see below). RCASBP(B) vectors were used to deliver and express the wild-type and mutant membrane-bound TVA receptors in line Rh-C CEFs that lacked a functional TVA receptor, to determine the efficiency of infection by wild-type RCASBP(A)AP and mutant E149K(A)AP, Y142N(A)AP, and Y142N/E149K(A)AP viruses.

ELISA. The ASLV CA p27 protein was detected in culture supernatants by an ELISA based on an assay described previously (35). Currently, we use rabbit anti-p27 antibodies conjugated with horseradish peroxidase (HRP) (anti-p27-HRP) or not conjugated with HRP (anti-p27); the antibodies were obtained from Charles River Laboratories (Morrisville, NC). Ninety-six-well plates were coated with anti-p27 antibodies in 50 mM sodium carbonate-sodium bicarbonate buffer (pH 9.5) overnight at 4°C. The wells were washed with phosphate-buffered saline (PBS) containing 0.1% Tween 80 and were incubated in a solution of 5% nonfat dried milk in PBS at 37°C for 1 h to block nonspecific binding. Samples were prepared by adding Tween 80 to the cell culture supernatants (to a final concentration of 0.005%), followed by three cycles of freezing at -70°C and thawing at 37°C. The anti-p27-coated wells were incubated with the supernatants at 37°C for 1 h. The wells were then washed with PBS containing 0.1% Tween 80 and incubated with anti-p27-HRP in 5% milk-PBS. The antigen-antibody complexes were detected using the 1-step Turbo tetramethylbenzidine ELISA substrate from Pierce Biotechnology (Rockford, IL). The color reaction was stopped with 1 M H₂SO₄ after 20 min and read on a Tecan Genios plate reader with a 450-nm filter.

The levels of sTVA-mIgG proteins in culture supernatants were quantitated by ELISA for the mouse IgG tag, as previously described (21). The linear range for a standard assay was between 0.5 and 50 ng of ImmunoPure mouse IgG Fc fragment per ml.

FACS analysis. DF-1 cells infected with (A)AP, EK(A)AP, YN(A)AP, or YNEK(A)AP viruses were removed from the culture with trypsin de Larco (Quality Biological, Inc.) and washed with Dulbecco's PBS. The cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min and then washed with PBS. Approximately 1 $\times$ 10⁶ DF-1 cells in PBS supplemented with 1% calf serum (PBS-CS) were incubated with supernatant containing one of the sTVA-mIgG proteins on ice for 30 min. The cells were then washed with PBS-CS and incubated with 5 μ l of goat anti-mouse IgG (H+L) linked to phycoerythrin (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in PBS-CS (total volume, 1 ml) on ice for 30 min. The cell-sTVA-mIgG-Ig-phycoerythrin complexes were then washed with PBS-CS, resuspended in 0.5 ml PBS-CS, and analyzed with a

Becton, Dickinson FACSCalibur flow cytometer using CellQuest (version 3.1) software.

Apparent K_d calculations. The maximum possible fluorescence and the dissociation constant (K_d) value for each data set obtained from the FACS binding assays were estimated by fitting the data via nonlinear least squares to a log logistic growth curve function (f): $f(y) = M/[1 + e^{-r(\log x - \log K_d)}]$ where y is the mean fluorescence; M is the maximum fluorescence; r is the rate; x is the concentration of sTVA-mIgG; and K_d is the dissociation constant, defined as the concentration of sTVA-mIgG at half-maximal binding (22).

Protein modeling. The modeling of the published TVA nuclear magnetic resonance (NMR) structures and the generation of the structures of mutant TVA proteins were done using the Sybyl program (version 8.0), which was originally from Tripos but which is now Centara.

RESULTS AND DISCUSSION

Partial recovery of function with chimeras of the quail and chicken TVA receptor. To identify regions or residues of the quail TVA receptor responsible for the loss of binding affinity of the EK, YN, and YNEK envelope glycoproteins to the quail TVA receptor but not the chicken TVA receptor, chimeric sTVA-mIgG proteins in which either regions or specific residues of the quail receptor were replaced with the corresponding regions or residues from the chicken TVA receptor were produced (Fig. 1B). While previous studies have determined that only the 40-amino-acid LDL-A region of TVA is required to confer susceptibility to ASLV(A) infection, there may still be important determinants in other regions of the extracellular domain that affect binding and/or the efficiency of the entry process. The extracellular domains of the TVA receptors were divided into six regions using convenient restriction sites to generate nine quail-chicken sTVA-mIgG chimeras (Fig. 1B). Initial experiments determined that there was no difference in the binding of wild-type chicken sTVA-mIgG (wtCK) and the quail N-terminal chicken sTVA-mIgG chimera (Q-CK) to the wild-type and mutant EnvA glycoproteins (data not shown).

The binding affinities of the wild type and these initial chimeric sTVA-mIgGs for the YN and YNEK glycoproteins expressed on the surface of infected DF-1 cells were estimated using a FACS-based assay (Table 1). The chimeras with a significantly improved binding affinity for the mutant EnvA glycoproteins, Q-CK-3, -4, -7, and -8, all shared only region 4 of chicken TVA in common. The binding affinities of chimeras Q-CK-3, -4, and -8 were improved so that they were at or near the levels of binding of wtCK for the YN and YNEK glycoproteins, while the binding affinity of chimera Q-CK-7 for the YN and YNEK glycoproteins was significantly improved. The chimeras with region 4 from quail TVA, Q-CK-1, -2, -5, and -6, had lower binding affinities for the mutant envelope glycoproteins, similar to the findings for wild-type quail sTVA-mIgG. These chimeras also had binding affinities similar to the binding affinity for wild-type EnvA, except for chimera Q-CK-5, which had an ~5-fold-lower binding affinity for wild-type EnvA than wild-type quail sTVA-mIgG (wtQ). These results with Q-CK-7 and Q-CK-5 may indicate that TVA regions 2 and 3 function most efficiently if both regions are from the same receptor homolog.

The efficiencies with which the chimeric TVA receptors mediate infection of cells compared to those of wild-type chicken and quail TVA receptors were assayed by delivering and expressing membrane-bound forms of the TVA receptors in cultured line Rh-C CEFs using RCASBP vectors with the subgroup C envelope glycoprotein [RCASBP(C)]. Line Rh-C CEFs do not express func-

TABLE 1 Estimated binding affinities of quail and chicken TVA immunoadhesin chimeras for wild-type and mutant EnvA glycoproteins^c

sTVA-mIgG	DF-1 cells with WTA ^d		DF-1 cells with YN mutant glycoprotein		DF-1 cells with YNEK mutant glycoprotein	
	Apparent <i>K_d</i> (nM) ^a	% maximum binding ^b	Apparent <i>K_d</i> (nM)	% maximum binding	Apparent <i>K_d</i> (nM)	% maximum binding
wtQ	0.167 ± 0.112		>1,000	37.2 ± 4.8	>1,000	21.1 ± 1.0
Q-CK	0.083 ± 0.009		0.095 ± 0.034		0.146 ± 0.074	
Q-CK-1	0.029 ± 0.006		6.59 ± 4.84	47.1 ± 8.6	>1,000	17.4 ± 1.9
Q-CK-2	0.042 ± 0.012		43.7 ± 71.6	44.8 ± 8.5	>1,000	23.4 ± 7.6
Q-CK-3	0.105 ± 0.010		0.224 ± 0.113		0.644 ± 0.579	
Q-CK-4	0.068 ± 0.015		0.077 ± 0.017		0.181 ± 0.096	
Q-CK-5	0.849 ± 0.320		445 ± 793	37.1 ± 6.3	>1,000	31.0 ± 9.7
Q-CK-6	0.295 ± 0.154		39.6 ± 34.6	35.7 ± 6.8	>1,000	21.9 ± 1.6
Q-CK-7	0.075 ± 0.018		0.522 ± 0.232		9.29 ± 7.32	
Q-CK-8	0.110 ± 0.059		0.208 ± 0.046		0.798 ± 0.472	
wtCK	0.073 ± 0.043		0.074 ± 0.051		0.183 ± 0.197	

^a The apparent *K_d* values were estimated by fitting the data via the nonlinear least-squares method to a logistic growth curve function, as described in Materials and Methods. Each result is the average and standard deviation from 3 to 5 experiments.
^b If the apparent *K_d* values were estimated from binding curves that did not average at least 50% maximum total binding, the average percent maximum binding and standard deviation obtained are shown. The maximum concentration of sTVA-mIgG proteins was 7.8 nM.
^c See Fig. 2.
^d WTA, wild-type EnvA glycoprotein.

tional TVA receptors. The cell cultures expressing the TVA receptors were then challenged with 10-fold serial dilutions of wild-type RCASBP(A)AP [(A)AP] or each of the three mutant EnvA viruses, EK(A)AP, YN(A)AP, and YNEK(A)AP, and the AP-positive foci were quantitated (Fig. 1C). All four viruses had approximately the same titers from DF-1 cells with the normally expressed chicken TVA receptor and from line Rh-C CEFs expressing wtCK TVA, as expected. Also as expected, the titers of mutant YN(A)AP and YNEK(A)AP viruses from line Rh-C CEFs expressing wtQ TVA were 10- to 30-fold and 300- to 1,000-fold lower, respectively, than the viral titers obtained using line Rh-C CEFs expressing wtCK TVA. The ~8-fold lower binding affinity of the EK(A)AP mutant glycoprotein for wtQ TVA did not result in a detectable difference in the titer of the EK(A)AP virus on any of the TVA-expressing cells. The TVA receptor chimeras that had an improved binding affinity for the mutant glycoproteins Q-CK-3, -4, -7, and -8 also had an improved efficiency of infection of cells expressing the TVA chimera than those expressing wtQ TVA: an ~10- to 20-fold improvement for the YN(A)AP mutant and an ~100-fold improvement for the YNEK(A)AP mutant. While these TVA chimeras recovered nearly all of the wild-type binding affinities and infection efficiencies for the YN mutant, there were still 10- to 30-fold deficiencies in YNEK infections, despite the recovered binding affinities.

The residue at quail TVA LDL-A position 31 is the major determinant of the level of binding affinity for the mutant EnvA glycoproteins. From the initial results presented above, the two amino acid differences between the quail and chicken TVA homologs in region 4, residues 31 and 32 in the quail LDL-A domain, account for the majority of the differences in binding affinity for ASLV mutant EnvA glycoproteins. To test if one or both amino acids at positions 31 and 32 of the quail TVA LDL-A domain are required for the optimal affinity of TVA binding to the mutant EnvA glycoproteins, sTVA-mIgG immunoadhesins were constructed with the mutations Qu-Q31L (in which quail TVA LDL-A glutamine residue 31 was replaced with the leucine from chicken TVA), Qu-D32E, and Qu-Q31L-D32E in an otherwise

wild-type quail sTVA-mIgG background and the reciprocal mutations, CK-L31Q (in which chicken TVA leucine residue 31 was replaced with the glutamine from quail TVA), CK-E32D, and CK-L31Q-E32D, in the wild-type chicken sTVA-mIgG background (Fig. 2A). These sTVA-mIgG mutants could be used to test the hypothesis that this region is critical for overall ASLV EnvA binding to the TVA receptor. The reciprocal of the mutations that would rescue the binding affinity in the quail sTVA-mIgG for the mutant EnvA glycoproteins would be expected to reduce the binding affinity in the chicken sTVA-mIgG immunoadhesins. The overall result of this analysis was that the residue at position 31 in the quail TVA LDL-A domain is critical for wild-type levels of binding affinity for the mutant EK, YN, and YNEK glycoproteins (Table 2). Qu-Q31L improved the binding affinity of the Qu-Q31L sTVA-mIgG for the mutant envelope glycoproteins to wild-type levels. The reciprocal change, CK-L31Q, reduced the binding affinity of CK-L31Q sTVA-mIgG for the mutant envelope glycoproteins to quail TVA levels.

The efficiencies with which the membrane-bound forms of these mutant TVA receptors mediated infection of wild-type and mutant ASLV(A)s were assayed as described above. While the Qu-Q31L mutation in sTVA-mIgG recovered the binding affinity for all of the viruses to wild-type levels, cells expressing this TVA receptor (Qu-Q31L) clearly increased the titers of the mutant viruses 10- to 30-fold but did not recover all of the infectious titer for the YNEK(A)AP mutant; the Qu-Q31L mutant TVA receptor did enable nearly wild-type titers for the YN(A)AP mutant virus (Fig. 2C). Cells expressing the TVA receptor with mutations at both Qu-Q31L and Qu-D32E did not improve the infectivity. The reciprocal CK-L31Q mutation in the chicken TVA homolog sTVA-mIgG reduced the affinity of binding to the mutant envelope glycoproteins to the quail sTVA-mIgG level. However, the CK-L31Q TVA receptor mediated almost wild-type levels of YN(A)AP mutant infection and only a 10- to 20-fold lower infectious titer of the YNEK(A)AP mutant when a 500- to 1,000-fold lower titer was expected, as for wtQ TVA. Again, the CK-L31Q-E32D double mutation did not improve mutant virus infectivity.

FIG 2 Schematic representation of the LDL-A regions of the quail and chicken TVA immunoadhesin chimeras, testing the LDL-A residues at positions 31 and 32 (A) and testing the LDL-A residues at positions 12 and 31 (B). Dark gray boxes, wild-type chicken (wtCK) TVA sequence; light gray boxes, wild-type quail (wtQ) TVA sequence. The quail TVA LDL-A region is shown and numbered using the mature quail TVA extracellular domain numbering, and the three disulfide bonds are indicated by brackets above the TVA sequence. Identical residues are denoted by dots; gaps in the alignment are denoted by dashes. (C) Analysis of the ability of ASLV(A) to use the chicken, quail, and chicken-quail chimeric TVA receptors to efficiently infect avian cells. A subset of membrane-bound forms of these chicken-quail TVA receptor chimeras and the wild-type chicken and wild-type quail TVA receptors were delivered by RCASBP(C) vectors into line Rh-C CEF cultures, as described in the Fig. 1 legend. Each of the results is the average of three different experiments. Error bars show standard deviations.

The importance of the quail LDL-A residue 31 Q31L mutation for improving the infectivity of the YN and YNEK mutant ASLV(A)s was observed previously but was observed in combination with a change of the quail proline at residue 12 to the chicken TVA residue serine (P12S), Qu-P12S-Q31L (36). The previous investigators were not able to determine binding affinity differences in any of the chimeric quail-chicken TVA receptors that they constructed. Quail sTVA-mIgG immunoadhesins were constructed with the chicken residues at positions 12 and 31 (Qu-P12S and Qu-P12S-Q31L), and the chicken sTVA-mIgG immunoadhesin was constructed with the quail residues (CK-S12P and CK-S12P-L31Q) (Fig. 2B). The immunoadhesins with the single mutations at residue 12, Qu-P12S and CK-S12P, bound to wild-type and mutant EnvA glycoproteins at levels similar to those for wild-type quail and chicken immunoadhesins (Table 3). The P12S mutation in combination with Q31L, Qu-P12S-Q31L, did not alter the improvement in binding affinity for the mutant envelope

glycoproteins; however, the reciprocal mutations in the chicken TVA receptor, CK-S12P-L31Q, may reduce the binding affinity for only the EK mutant glycoproteins. Membrane-bound TVA receptors were constructed and expressed with the combination Qu-P12S-Q31L mutations and CK-S12P-L31Q mutations to determine if the combined mutations would significantly alter the infectivity of the mutant viruses compared to that of viruses with the single mutations at residue 31 alone. In contrast to the published report (36), the addition of the P12S mutation to the Q31L mutation did not improve the infectivity of the YNEK mutant (Fig. 2C). Therefore, the specific residue at quail TVA position 12 does not appear to be a major interaction determinant in mediating binding to ASLV(A) glycoproteins or infectivity.

There does appear to be a general correlation between the estimated binding affinities measured using the sTVA-mIgG FACS assay and the efficiency with which the same mutant TVA receptors as membrane-bound receptors mediate infection of the mu-

TABLE 2 Estimated binding affinities of quail and chicken TVA immunoadhesin chimeras for wild-type and mutant EnvA glycoproteins^c

sTVA-mIgG	DF-1 cells with WTA ^d		DF-1 cells with EK mutant glycoprotein		DF-1 cells with YN mutant glycoprotein		DF-1 cells with YNEK mutant glycoprotein	
	Apparent K_d (nM) ^a	% maximum binding ^b	Apparent K_d (nM)	% maximum binding	Apparent K_d (nM)	% maximum binding	Apparent K_d (nM)	% maximum binding
wtQ	0.180 ± 0.060		2.80 ± 0.29		76.8 ± 50.9	38.9 ± 4.9	>1,000	22.0 ± 5.2
Qu-Q31L	0.086 ± 0.030		0.058 ± 0.026		0.129 ± 0.093		0.232 ± 0.176	
Qu-D32E	0.180 ± 0.046		ND ^e		91.9 ± 69.0	37.1 ± 6.1	>1,000	20.0 ± 6.9
Qu-Q31L-D32E	0.119 ± 0.040		ND		0.361 ± 0.264		0.622 ± 0.433	
CK-L31Q-E32D	0.462 ± 0.138		ND		10.6 ± 7.23		9.55 ± 4.19	
CK-E32D	0.128 ± 0.029		ND		0.058 ± 0.010		0.068 ± 0.024	
CK-L31Q	0.284 ± 0.048		2.77 ± 1.34		42.5 ± 40.9	44.4 ± 10.8	29.5 ± 11.8	42.4 ± 5.6
wtCK	0.055 ± 0.015		0.155 ± 0.075		0.034 ± 0.014		0.037 ± 0.020	

^a The apparent K_d values were estimated by fitting the data via the nonlinear least-squares method to a logistic growth curve function, as described in Materials and Methods. Each result is the average and standard deviation from 4 experiments.

^b If the apparent K_d values were estimated from binding curves that did not average at least 50% maximum total binding, the average percent maximum binding and standard deviation obtained are shown. The maximum concentration of sTVA-mIgG proteins was 14.3 nM.

^c See Fig. 4.

^d WTA, wild-type EnvA glycoprotein.

^e ND, not done.

tant viruses. However, for the YNEK mutant, although the binding affinity improved significantly to nearly wild-type levels, the ability to mediate infection improved significantly but did not reach chicken TVA levels. It should be noted that the binding affinity assay uses the sTVA-mIgG proteins, which are likely dimers due to the mIgG domain and which may skew the affinity estimate toward a higher affinity because the two domains increases the avidity of the interaction with the EnvA glycoprotein trimer. One interpretation of the discrepancies is that the interactions of quail TVA receptors and the ASLV(A) envelope glycoproteins are not as robust as the interactions of chicken TVA receptors and the ASLV(A) envelope glycoproteins. The subgroup A ASLVs were originally isolated from chickens and most likely evolved from a primordial isolate to use different cell surface chicken proteins as receptors. Therefore, the interactions between the chicken TVA receptor and the ASLV(A) envelope glycoprotein were maximized, while they were not as efficient as those obtained when the quail TVA homolog was used.

Other TVA residues important for improving YNEK infectivity. While the mutant with the Q31L mutation in the quail TVA immunoadhesin recovered the wild-type binding affinity for all of

the mutant ASLV(A) glycoproteins, EK, YN, and YNEK (Table 2), the mutant with the Q31L mutation in a membrane-bound quail TVA did not recover all of the infectivity of the YNEK mutant (Fig. 2C, Qu-Q31L). However, the quail-chicken TVA chimeras Q-CK-3 (with chicken TVA regions 2, 3, and 4) and Q-CK-4 (with chicken TVA regions 2, 3, 4, and 5) recovered both the binding affinity and nearly wild-type infectivity for the mutant viruses (Table 1; Fig. 1C). To assess the importance of other differences between the quail and chicken TVA receptors in these regions on infectivity, membrane-bound TVA receptors were constructed and expressed with additional mutations in the Qu-Q31L TVA receptor. Five receptors, Qu-S9A-Q31L, Qu-R10Q-Q31L, Qu-G14E-Q31L, Qu-R17H-Q31L, and Qu-Q31L-ASGS (where ASGS represents chicken residues 52 to 55), were constructed and expressed in Rh-C lineage CEFs, and the CEFs were challenged with wild-type (A)AP and YNEK(A)AP mutant viruses. All of these receptors mediated efficient infection of wild-type (A)AP, as expected (Fig. 3). The YNEK(A)AP virus infected control DF-1 cells and CEFs expressing the wtCK TVA receptor efficiently, while the titers of virus infecting cells expressing the wtQ receptor were 1,000-fold lower than those of cells expressing the wtCK TVA

TABLE 3 Estimated binding affinities of quail and chicken TVA immunoadhesin chimeras for wild-type and mutant EnvA glycoproteins^c

sTVA-mIgG	DF-1 cells with WTA ^d		DF-1 cells with EK mutant glycoprotein		DF-1 cells with YN mutant glycoprotein		DF-1 cells with YNEK mutant glycoprotein	
	Apparent K_d (nM) ^a	% maximum binding ^b	Apparent K_d (nM)	% maximum binding	Apparent K_d (nM)	% maximum binding	Apparent K_d (nM)	% maximum binding
wtQ	0.051 ± 0.018		3.44 ± 0.76		8.95 ± 6.17		161.4 ± 96.0	27.9 ± 5.3
Qu-P12S	0.063 ± 0.033		3.08 ± 0.43		10.9 ± 9.3		193.7 ± 169.4	24.2 ± 3.3
Qu-P12S-Q31L	0.191 ± 0.004		0.367 ± 0.027		0.629 ± 0.332		2.58 ± 0.77	
CK-S12P-L31Q	0.087 ± 0.040		156.9 ± 60.5	28.4 ± 2.8	50.4 ± 42.2	42.1 ± 10.0	100.7 ± 41.3	31.1 ± 1.4
CK-S12P	0.081 ± 0.012		0.400 ± 0.213		0.166 ± 0.064		0.535 ± 0.174	
wtCK	0.074 ± 0.010		0.233 ± 0.047		0.121 ± 0.057		0.393 ± 0.145	

^a The apparent K_d values were estimated by fitting the data via the nonlinear least-squares method to a logistic growth curve function, as described in Materials and Methods. Each result is the average and standard deviation from 3 experiments.

^b If the apparent K_d values were estimated from binding curves that did not average at least 50% maximum total binding, the average percent maximum binding and standard deviation obtained are shown. The maximum concentration of sTVA-mIgG proteins was 16 nM.

^c See Fig. 4.

^d WTA, wild-type EnvA glycoprotein.

FIG 3 Analysis of the ability of ASLV(A) to use the chicken, quail, and chicken-quail chimeric TVA receptors to efficiently infect avian cells. Membrane-bound forms of the quail Q31L mutant TVA receptor alone or in combination with other quail-to-chicken TVA receptor mutations and the wild-type chicken and wild-type quail TVA receptors were delivered by RCASBP(C) vectors into line Rh-C CEF cultures, as described in the Fig. 1 legend. Each of the results is the average of three different experiments. Error bars show standard deviations.

receptor, the titers of virus infecting cells expressing Q 31L were improved ~50-fold, and the titers of virus infecting cells expressing the Q-CK-4 TVA receptor were only 5- to 10-fold lower, as expected. Both the TVA receptors with the R10Q and Q31L mutations and the TVA receptors with the G14E and Q31L mutations improved the infectivity of YNEK(A)AP virus for cells expressing the Q31L TVA receptor alone by ~10-fold, although it was still not improved to the level of that for cells expressing Q-CK-4 (Fig. 3). Therefore, in addition to the conserved tryptophan 48, this study has identified residues 10, 14, and 31 in the quail TVA receptor to be critical for efficient infection by ASLV(A).

Effect of quail TVA LDL-A residues 22 to 27 on interactions with EnvA glycoproteins. To further assess the possible effects of

differences in region 3 of the quail and chicken TVA receptors on binding affinity with EnvA glycoproteins, a quail sTVA-mIgG immunoadhesin was constructed with chicken TVA residues 22 to 27 in an otherwise quail TVA immunoadhesin (Q-CK-9) and quail TVA residues 22 to 27 in an otherwise chicken sTVA-mIgG (Q-CK-10) (Fig. 4A). Surprisingly, only replacement of chicken residues 22 to 27 in the quail sTVA-mIgG immunoadhesin had an effect on binding interactions with EnvA glycoproteins (Table 4). There was no effect on the ability of Q-CK-10 to bind wild-type or mutant EnvA glycoproteins. However, the Q-CK-9 chimera was significantly impaired in binding to even wild-type EnvA glycoproteins (~50-fold), and binding to each of the EK, YN, and YNEK mutant glycoproteins was almost knocked out entirely. However, when region 3 was combined with chicken region 2 of the LDL-A domain, as in the Q-CK-9 chimera, this sTVA-mIgG immunoadhesin (Q-CK-5) was only slightly impaired (2- to 3-fold) in binding to wild-type EnvA glycoproteins compared to the binding ability of wild-type quail immunoadhesin (Table 1). Unexpectedly, despite the significantly lower binding affinities of quail TVA with the exchange of these 6 residues, the exchange did not alter the ability of membrane-bound forms of the chimeric TVA receptors to mediate infection even with wild-type (A)AP or the reduced binding to the Q-CK-9 TVA receptor (Fig. 4B).

The Q31L mutation modeled on the quail TVA solution structures. The TVA receptors are related to LDLRs that contain at least one and usually multiple 40-amino-acid cysteine-rich ligand binding domains (LBDs). These conserved LBDs have 6 invariant cysteine residues in three disulfide bonds (which bind the 1st and 3rd, 2nd and 5th, and 4th and 6th cysteine residues), with these 6 residues including 4 highly conserved acidic residues in the C-terminal portion of the LBD critical for calcium coordination. Three-dimensional structures of multiple LDLR LBDs have been determined using X-ray crystallography or nuclear magnetic resonance (NMR) techniques. The extracellular domain of quail TVA (83 amino acids) contains one region related to LBD, LDL-A.

FIG 4 (A) Schematic representation of the quail and chicken LDL-A regions of the TVA immunoadhesin chimeras, testing the LDL-A residues at positions 22 to 27. Dark gray boxes, wild-type chicken TVA sequence (wtCK); light gray boxes, wild-type quail TVA sequence (wtQ). The quail TVA LDL-A region is shown and numbered using the mature quail TVA extracellular domain numbering, and the three disulfide bonds are indicated by brackets above the TVA sequence. Identical residues are denoted by dots; gaps in the alignment are denoted by dashes. (B) Analysis of the ability of ASLV(A)s to use the chicken, quail, and chicken-quail chimeric TVA receptors to efficiently infect avian cells. Membrane-bound forms of these chicken-quail TVA receptor chimeras and the wild-type chicken and wild-type quail TVA receptors were delivered by RCASBP(C) vectors into line Rh-C CEF cultures, as described in the Fig. 1 legend. Each of the results is the average of three different experiments. Error bars show standard deviations.

TABLE 4 Estimated binding affinities of quail and chicken TVA immunoadhesin chimeras for wild-type and mutant EnvA glycoproteins

	DF-1 cells with WTA ^c		DF-1 cells with EK mutant glycoprotein		DF-1 cells with YN mutant glycoprotein		DF-1 cells with YNEK mutant glycoprotein	
	Apparent <i>K_d</i> (nM) ^a	% maximum binding ^b	Apparent <i>K_d</i> (nM)	% maximum binding	Apparent <i>K_d</i> (nM)	% maximum binding	Apparent <i>K_d</i> (nM)	% maximum binding
sTVA-mIgG								
wtQ	0.076 ± 0.023		2.80 ± 0.29		22.8 ± 12.6		>1,000	31.8 ± 2.8
Q-CK-9	4.60 ± 0.56		>1,000	22.5 ± 5.5	>1,000	17.4 ± 9.2	>1,000	20.6 ± 3.7
Q-CK-10	0.065 ± 0.031		0.180 ± 0.121		0.129 ± 0.068		0.244 ± 0.122	
wtCK	0.054 ± 0.020		0.155 ± 0.075		0.135 ± 0.045		0.215 ± 0.097	
wtQ	0.029 ± 0.006		2.60 ± 0.73		5.44 ± 2.08		>1,000	27.2 ± 0.28
Qu-P22R	0.027 ± 0.022		1.03 ± 0.88		0.219 ± 0.247		14.09 ± 12.55	
Q-CK-9	1.97 ± 0.07		>1,000	10.2 ± 1.0	>1,000	17.4 ± 4.9	>1,000	19.5 ± 1.1
Q-CK-9-R22P	0.018 ± 0.013		5.29 ± 0.11		19.35 ± 4.17		>1,000	26.1 ± 7.1
Q-CK-10-P22R	0.026 ± 0.024		0.136 ± 0.018		0.051 ± 0.010		0.123 ± 0.028	
Q-CK-10	0.021 ± 0.018		0.063 ± 0.028		0.028 ± 0.009		0.055 ± 0.008	
CK-R22P	0.043 ± 0.029		0.077 ± 0.001		0.129 ± 0.084		0.157 ± 0.088	
wtCK	0.015 ± 0.001		0.052 ± 0.009		0.017 ± 0.001		0.049 ± 0.019	

^a The apparent *K_d* values were estimated by fitting the data via the nonlinear least-squares method to a logistic growth curve function, as described in Materials and Methods. Each result is the average and standard deviation from 4 experiments.
^b If the apparent *K_d* values were estimated from binding curves that did not average at least 50% maximum total binding, the average percent maximum binding and standard deviation obtained are shown. The maximum concentration of sTVA-mIgG proteins was 33.9 nM.
^c WTA, wild-type EnvA glycoprotein.

Using nuclear magnetic resonance techniques, two different groups have published a three-dimensional structure of the major solution conformation of the quail TVA LDL-A region expressed as a secreted sTVA protein (Fig. 5A) (29, 30). Both structures have the expected disulfide bond pattern and orientation of the acidic residues involved in calcium coordination according to those in other LDLR LBD structures. However, there is a significant disparity in the resulting structures of the two groups: the quail TVA LDL-A structure of the Agard group (29) closely resembles the reported structures of other LDLR LBDs, while the quail TVA LDL-A structure of the Rong group (30) has a carboxyl terminus similar to that in other LDLR LBD structures but has an amino terminus that adopts a very different conformation (Fig. 5A). LDL-A residue Q31 is prominently accessible to a binding interaction in the Agard structure, while residue Q31 is almost completely buried in the Rong structure and not likely to be accessible to a strong binding interaction with a domain on the ASLV(A) envelope glycoprotein.

The 11 residues that were different between the quail and chicken TVA LDL-A region were modeled into both the Agard quail TVA structure and the Rong quail TVA structure using the protein modeling software Sybyl (version 8.0) (Fig. 5B). The chicken L31 residue is still prominently accessible to the ASLV(A) glycoprotein interaction in the Agard-modeled chicken TVA structure, while L31 is completely buried by the E14 residue in the Rong-modeled chicken TVA structure. These observations and the fact that the Agard sTVA LDL-A structure is similar to all of the other LDLR LBD structures lead to the conclusion that the Agard solution may more accurately reflect the structure of the quail TVA LDL-A domain.

A model of the structure of the Q-CK-9 LDL-A domain obtained using the Agard quail TVA LDL-A domain structure was generated (Fig. 5C, middle) and compared to the Agard-modeled chicken TVA structure (Fig. 5C, left). The 6 chicken TVA residues at positions 22 to 27 appear to extend toward regions in the receptor important for interaction with the EnvA glycoproteins, Q31 and W48, whereas the wild-type quail residues 22 to 27 extend

toward those regions only when just the 6 chicken residues are modeled on the Agard quail TVA structure and not when the entire chicken TVA LDL-A region is modeled. If this model is accurate, this extension may inhibit the interaction with the viral glycoproteins, but only for the Q-CK-9 chimeric sTVA-mIgG, explaining the observation that only this chimera alters the binding affinity. To test this hypothesis, four additional mutant immunoadhesins were constructed by exchanging the amino acid at position 22 to obtain quail sTVA-mIgG with the P22R mutation (Qu-P22R), Q-CK-9 with the R22P mutation (Q-CK-9-R22P), Q-CK-10 with the P22R mutation (Q-CK-10-P22R), and chicken sTVA-mIgG with the R22P mutation (CK-R22P), and their binding affinities for ASLV(A) were estimated. Changing R22 to P22 in Q-CK-9 would be predicted to remove any steric block created (Fig. 5C, right) and thereby recover the binding affinity to wild-type quail sTVA-mIgG levels, and this is what was observed (Table 4). Mutating P22R in the otherwise wild-type quail sTVA-mIgG also recovered some of the affinity of binding to YN and YNEK mutant glycoproteins, leading to a possible conclusion that residue 22 may also be important for efficient TVA ASLV(A) glycoprotein binding. However, no effects on binding affinity were observed with the reciprocal mutation in chicken sTVA-mIgG (CK-R22P).

The structurally adjacent LDL-A residues F16, L31, and L34 define a major determinant of the binding affinity between the chicken TVA receptor and the EnvA glycoproteins. To determine if LDL-A residues structurally near residue 31 contribute significantly to the affinity of TVA receptor binding to ASLV EnvA glycoproteins, the residues were replaced by alanine, a neutral, nonpolar amino acid, and the binding affinities were quantitated. In the quail TVA LDL-A NMR structures, 2 residues conserved between quail and chicken TVA homologs, phenylalanine at position 16 (F16) and leucine at position 34 (L34), are adjacent to the residue at position 31 (Fig. 5A and B). Single alanine substitution mutations, double alanine substitution mutations, and one triple alanine substitution mutation were constructed in the chicken sTVA-mIgG immunoadhesin at residues 16, 31, and 34 in the

FIG 5 Three-dimensional models of the published quail sTVA NMR structures. (Top) Sequence of the LDL-A region and flanking residues of the wild-type quail (wtQ) TVA receptor compared to the sequences of the 42-residue quail sTVA protein (TVA-42) synthesized by the Agard group (Agard Quail-42) (29) and the 47-residue quail sTVA protein (TVA-47) synthesized by the Rong group (Rong Quail-47) (30). (A) Three-dimensional models of each quail sTVA NMR structure are shown, with the positions of certain critical residues being highlighted. (B) Using the two quail TVA NMR structures, the same region of the chicken TVA receptor was modeled using Sybyl (version 8.0) software. The residues at the same positions in both the quail and the chicken three-dimensional models are highlighted. The L31 residue in the Rong model of the chicken TVA-47 structure is not exposed to solvent, being completely buried by the position of E14, so it is noted by (L31). (Middle) Schematic representation of the LDL-A region comparing the differences between the chicken and quail TVA regions. Identical residues are denoted by dots, gaps in the alignment are denoted by dashes, the conserved cysteine residues and disulfide bonds are highlighted in red, residues 22 to 27 are highlighted with red brackets, and the residues highlighted in the structures in panel C are highlighted by red arrows. (C) Models of the Agard TVA structures. (Left) The Agard modified chicken TVA-42 structure from panel B rotated 90°; (middle) the Agard quail TVA-42 structure with the chicken RDPQTD exchange; (right) the Agard quail TVA-42 structure with only the chicken DPQTD exchange.

FIG 6 Schematic representation of the chicken LDL-A regions of the TVA immunoadhesins with alanine substitutions at position 16, 31, and/or 34. The chicken and quail TVA LDL-A regions are shown and numbered using the mature quail TVA extracellular domain numbering, and the three disulfide bonds are indicated by brackets above the TVA sequence. Identical residues are denoted by dots; gaps in the alignment are denoted by dashes. The dark gray boxes below show the wild-type chicken (wtCK) TVA sequence and the sequences with alanine substitutions.

LDL-A domain (Fig. 6). The chicken immunoadhesin was used as the base sTVA-mIgG since it bound wild-type and EK, YN, and YNEK mutant glycoproteins with equal affinities. All three immunoadhesins with a single alanine substitution, CK-L31A, CK-L34A, and CK-F16A, bound wild-type EnvA glycoproteins at levels similar to those for wild-type chicken sTVA-mIgG (Table 5). However, the single alanine substitutions significantly reduced the binding affinity for the YN and YNEK EnvA mutant glycoproteins to CK-L21Q levels. This was a bit surprising, since both leucines, which are neutral nonpolar amino acids, were replaced with an-

other neutral, nonpolar residue. It would appear that the YN and YNEK mutations interact with the alkyl-group side chains with alanine substitutions. Any combination of these three alanine substitutions significantly reduced or eliminated detectable binding between the mutant immunoadhesins and wild-type and mutant EnvA glycoproteins (Table 5).

The Y142N mutation in ASLV(A) envelope glycoproteins significantly extends the host range. We had previously shown that the EK, YN, and YNEK mutant viruses had patterns of receptor interference in chicken DF-1 cells previously infected by subgroup A, B, C, and J ASLVs similar to those of cells infected with wild-type ASLV(A), except that YN and YNEK mutant viruses were ~10-fold less efficient at infecting DF-1 cells previously infected with subgroup B and C ASLVs (22). In addition, we showed that the EK, YN, and YNEK mutant viruses infected cells (quail and NIH 3T3 cells) expressing the quail TVA receptor in direct relation to their binding affinity for quail sTVA-mIgG (22). Since we had observed that other ASLV(A) mutants genetically selected to evade an sTVA-mIgG inhibitor also had an altered host range (37) and another group had reported that mutant subgroup B ASLVs could be genetically selected for a broadened host range (38), we determined the ability of the wild-type (A)AP and EK(A)AP, YN(A)AP, and YNEK(A)AP mutant viruses to infect a variety of mammalian cell lines (Fig. 7). DF-1 cells expressing wild-type chicken TVA were the positive controls. The titers of (A)AP virus stocks obtained using mammalian cells engineered to express the TVA receptors can reach 10^4 inclusion-forming units (ifu)/ml; these titers are always lower by several logs than those for DF-1 cells. Even quail cells are less efficiently infected by ASLV(A)s than chicken cells (22). The Y142N mutation significantly altered the host range of a subgroup A ASLV, improving the infectivity of several mammalian cell lines by 2 to 3 log units (Fig. 7). The E149K mutation had no effect on the ASLV(A) host range. The host range of the YNEK mutant virus altered by the Y142N mutation, combined with the effects of the combined mutations on the binding affinity and efficient use of the quail TVA receptor for infection,

TABLE 5 Estimated binding affinities of quail and chicken TVA immunoadhesin chimeras for wild-type and mutant EnvA glycoproteins^e

	DF-1 cells with WTA ^f		DF-1 cells with EK mutant glycoprotein		DF-1 cells with YN mutant glycoprotein		DF-1 cells with YNEK mutant glycoprotein	
	Apparent K_d (nM) ^a	% maximum binding ^b	Apparent K_d (nM)	% maximum binding	Apparent K_d (nM)	% maximum binding	Apparent K_d (nM)	% maximum binding
sTVA-mIgG								
wtCK ^c	0.133 ± 0.058		ND ^g		0.135 ± 0.045		0.215 ± 0.097	
CK-L31A ^c	0.081 ± 0.032		ND		54.8 ± 38.0		153.3 ± 145.7	
CK-L34A ^c	0.323 ± 0.172		ND		>1,000	18.6 ± 6.1	>1,000	18.1 ± 8.2
CK-F16A ^c	0.146 ± 0.067		ND		47.4 ± 18.7		>1,000	20.1 ± 1.6
CK-F16A- L31A- L34A ^c	>>1,000	7.6 ± 4.1	ND		>1,000	10.0 ± 7.2	>1,000	7.6 ± 3.8
wtCK K_d	0.051 ± 0.004		0.190 ± 0.026		0.415 ± 0.145		0.470 ± 0.107	
CK-F16A- L31A ^d	251.9 ± 134.1	13.6 ± 5.8	Undetectable		Undetectable		Undetectable	
CK-F16A- L34A ^d	Undetectable		Undetectable		Undetectable		Undetectable	
CK-L31A- L34A ^d	62.5 ± 18.8	22.4 ± 1.8	Undetectable		Undetectable		Undetectable	

^a The apparent K_d values were estimated by fitting the data via the nonlinear least-squares method to a logistic growth curve function, as described in Materials and Methods. Each result is the average and standard deviation from 3 experiments.

^b If the apparent K_d values were estimated from binding curves that did not average at least 50% maximum total binding, the average percent maximum binding and standard deviation obtained are shown.

^c The maximum concentration of sTVA-mIgG proteins was 33.9 nM.

^d The maximum concentration of sTVA-mIgG proteins was 11 nM.

^e See Fig. 6.

^f WTA, wild-type EnvA glycoprotein.

^g ND not done.

FIG 7 Host range analysis of ASLV(A)s. Cultures of chicken DF-1 cells, human embryonic kidney 293T cells (293T), human embryonic kidney 293 cells (293), African green monkey Vero cells (Vero), canine osteosarcoma D17 cells (D17), Chinese hamster ovary cells (CHO), and mouse 3T3 fibroblasts (3T3) were infected with 10-fold serial dilutions of the ASLV(A) stocks with wild-type [(A)AP] or mutant [EK(A)AP, YN(A)AP, YNEK(A)AP] envelope glycoproteins, and the titers were determined by AP assay. The results are the averages of three different experiments. Error bars show standard deviations.

may explain the difficulty in recovering all of the possible infectivity of YNEK mutant viruses when quail chicken TVA chimeras are used.

Conclusions. The selection of ASLV(A) with mutations in the envelope glycoproteins that can distinguish the different TVA receptor homologs has allowed an in-depth analysis of the complex, multiple-domain interaction that drives ASLV entry. Previous work by multiple groups characterized the importance of the LDL-A region of TVA for use as an ASLV(A) receptor but focused on the region conserved in the quail and chicken TVA homologs, identifying the tryptophan at position 48 to be critical for function (23–27). The selected ASLV(A) mutants, especially the YN and YNEK mutants with mutations in the hr1 region of the viral glycoprotein, allowed the characterization of the divergent regions of the TVA receptors for binding with the viral glycoproteins and the efficiency of infection while maintaining the conserved region that includes W48. The experimental system described here was found to be powerful, in that wild-type levels of binding affinity for the chicken TVA receptor and wild-type levels of infectivity of viruses with the mutant glycoproteins were retained and up to a 3-log-unit reduction in binding affinity for the quail TVA receptor and the infectivity of the same viruses was achieved.

It was very surprising that the Y142N mutation significantly expanded the host range of the mutant virus to mammalian cells but still allowed the virus to retain the exquisite binding affinity for the chicken TVA receptor. The subgroup A to E ASLVs most likely evolved to use different cell surface proteins as receptors due to genetic and/or receptor interference selection, most likely in multiple evolutionary steps. From this and other studies using various ASLVs, the initial mutations that arise in genetic selection schemes begin by broadening ASLV receptor usage, as with the YN mutant. Therefore, a broader receptor usage would appear to be favored for ASLV, but in the past, these viruses acquired additional mutations to become very selective for binding to a primary receptor, type such as TVA. A broadened receptor usage may then be a disadvantage over time and perhaps causes high levels of cell fusion and cytotoxicity because of interaction with many cell sur-

face proteins and/or may be an immunologic disadvantage because it is too easily neutralized.

This study has demonstrated that the amino acid at residue 31, Q31 or L31, in the quail and chicken TVA LDL-A domains is the major determinant of the binding affinity of an sTVA-mIgG immunoadhesin for the EK, YN, and YNEK mutant EnvA glycoproteins. While an increase in the binding affinity of chimeric TVA receptors for the mutant envelope glycoproteins almost always correlated with an improvement of the infectivity of the mutant virus, we did observe chimeric sTVA-mIgGs that recovered what appeared to be 100% of the binding affinity for the YNEK mutant glycoproteins but did not recover all of the infectivity in our assay. In quail TVA, specific amino acids at residues 10 and 14 in combination with residue 31 were required to recover the maximum levels of infectivity of the YNEK mutant but did not increase the apparent binding affinity, at least by our sTVA-mIgG FACS assay. These results could indicate a limitation with the sTVA-mIgG FACS binding assay, since the mIgG domains are likely dimers and may overestimate the affinity, and/or the lower infectivity could indicate a lower rate of receptor triggering of the glycoproteins required for fusion of the viral and cellular membranes and virus entry. Unfortunately, residue 10 was not included in the Agard 42-residue quail TVA protein (TVA-42) structure used for the NMR analysis, so there is no structural information. However, residue 14 has been highlighted in the TVA structures and models, demonstrating this residue is in close proximity to residue 31 that is critical for efficient EnvA binding and ASLV infection.

We believe that the results from this study support the quail sTVA solution structure of the Agard group (29). We envisage the quail TVA LDL-A structure to be an open left hand with the palm up and with the divergent residues 22 to 27 being the thumb (Fig. 5C). We propose that the major binding interactions of the ASLV(A) envelope glycoproteins lie along the palm in the structure from W48 through the G14, F16, Q31, and L34 domain. This model would explain why the substitution of chicken TVA residues 22 to 27 that model as protruding into this valley in the palm region would reduce the binding affinity. As the data demonstrate, though, the interactions between the TVA receptor and the ASLV(A) glycoproteins are complex, with multiple determinants being required for maximum binding and efficient entry.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI48682 and the Mayo Foundation.

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