

A Single-Amino-Acid Substitution in the Tv^b^{S1} Receptor Results in Decreased Susceptibility to Infection by Avian Sarcoma and Leukosis Virus Subgroups B and D and Resistance to Infection by Subgroup E In Vitro and In Vivo[∇]

Markéta Reinišová,¹§ Filip Šenigl,¹§ Xueqian Yin,² Jiří Plachý,¹ Josef Geryk,¹ Daniel Elleder,¹† Jan Svoboda,¹ Mark J. Federspiel,^{2*} and Jiří Hejnar^{1*}

Department of Cellular and Viral Genetics, Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Prague, Czech Republic,¹ and Department of Molecular Medicine, Mayo Clinic, Rochester, Minnesota²

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The avian sarcoma and leukosis virus (ASLV) family of retroviruses contains five highly related envelope subgroups (A to E) thought to have evolved from a common viral ancestor in the chicken population. Three genetic loci in chickens determine the susceptibility or resistance of cells to infection by the subgroup A to E ASLVs. Some inbred lines of chickens display phenotypes that are somewhere in between either efficiently susceptible or resistant to infection by specific subgroups of ASLV. The *tvb* gene encodes the receptor for subgroups B, D, and E ASLVs. The wild-type Tv^b^{S1} receptor confers susceptibility to subgroups B, D, and E ASLVs. In this study, the genetic defect that accounts for the altered susceptibility of an inbred chicken line, line M, to infection by ASLV(B), ASLV(D), and ASLV(E) was identified. The *tvb* gene in line M, *tvb*^{r2}, encodes a mutant Tv^b^{S1} receptor protein with a substitution of a serine for a cysteine at position 125 (C125S). Here, we show that the C125S substitution in Tv^b^{S1} significantly reduces the susceptibility of line M cells to infection by ASLV(B) and ASLV(D) and virtually eliminates susceptibility to ASLV(E) infection both in cultured cells and in the incidence and growth of avian sarcoma virus-induced sarcomas in chickens. The C125S substitution significantly reduces the binding affinity of the Tv^b^{S1} receptor for the subgroup B, D, and E ASLV envelope glycoproteins. These are the first results that demonstrate a possible role of the cysteine-rich domain 3 in the function of the Tv^b receptors.

Retroviruses cause serious diseases in animals and humans. The disease process begins with the virus infecting a cell(s), a process mediated by the interaction of the retroviral envelope glycoproteins with specific cell surface proteins that act as receptors (14, 24). A proper viral-glycoprotein–receptor interaction initiates conformational changes in the viral glycoproteins that ultimately result in the fusion of the viral and cellular membranes and entry of viral components (9). Despite the complexity and specificity of the viral-glycoprotein–receptor interaction required for virus entry, families of closely related retroviruses have evolved their glycoproteins to use different cellular proteins as receptors. Presumably, the presence of multiple viral subgroups that utilize different receptors is an advantage for viruses in overcoming host resistance. Resistance to retroviral infection occurs when the specific receptor protein is not available. Genetic alteration(s) can account for resistance, resulting in the complete lack of receptor protein expression or the expression of an aberrant protein not suitable

as a viral receptor. In addition, receptors can be saturated with viral glycoproteins expressed by the cell, physically blocking receptor accessibility, a phenomenon known as receptor interference (14, 24).

The avian sarcoma and leukosis virus (ASLV) family of retroviruses contains five highly related envelope subgroups (A to E) thought to have evolved in the chicken population from a common viral ancestor (4, 5, 24). Three genetic loci in chickens determine the susceptibility or resistance of cells to infection by the subgroup A to E ASLVs. Susceptibility to subgroup A ASLVs is determined by the *tva* locus; susceptibility to subgroup C ASLVs by the *tvb* locus; and susceptibility to the subgroup B, D, and E ASLVs by the *tvb* locus. The Tva proteins are related to the low-density lipoprotein receptor family (6, 25). The Tv^b proteins are related to the tumor necrosis factor receptor (TNFR) family (2, 3, 7). The Tvc proteins are most closely related to mammalian butyrophilins of the immunoglobulin superfamily (11). The normal functions and ligands of the Tva, Tv^b, and Tvc proteins in birds are unknown.

The genetic defects that account for resistance to infection by specific ASLVs, *tva*^r, *tvb*^r, and *tvc*^r alleles, have been identified in some lines of inbred chickens (10, 11, 16). The mutations found in the resistant alleles result in premature termination codons, or frameshifts, leading to severely truncated proteins or no receptor expression. In addition, single-amino-acid substitutions, often changing a cysteine residue, that result in a dramatic decrease in the affinity of the ASLV envelope glycoprotein interaction for the mutant receptor protein have been identified. An understanding of the variation in receptor

* Corresponding author. Mailing address for J. Hejnar: Institute of Molecular Genetics, Academy of Sciences of the Czech Republic, Vídeňská 1083, CZ-14220 Prague 4, Czech Republic. Phone: 420 296 443 443. Fax: 420 223 410 955. E-mail: hejnar@img.cas.cz. Mailing address for Mark J. Federspiel: Department of Molecular Medicine, Mayo Clinic, 200 First St., SW, Rochester, MN 55905. Phone: (507) 284-8895. Fax: (507) 266-2122. E-mail: federspiel.mark@mayo.edu.

† Present address: The Infectious Disease Laboratory, The Salk Institute, La Jolla, San Diego, CA 92037-1099.

§ M.R. and F.Š. contributed equally to this work.

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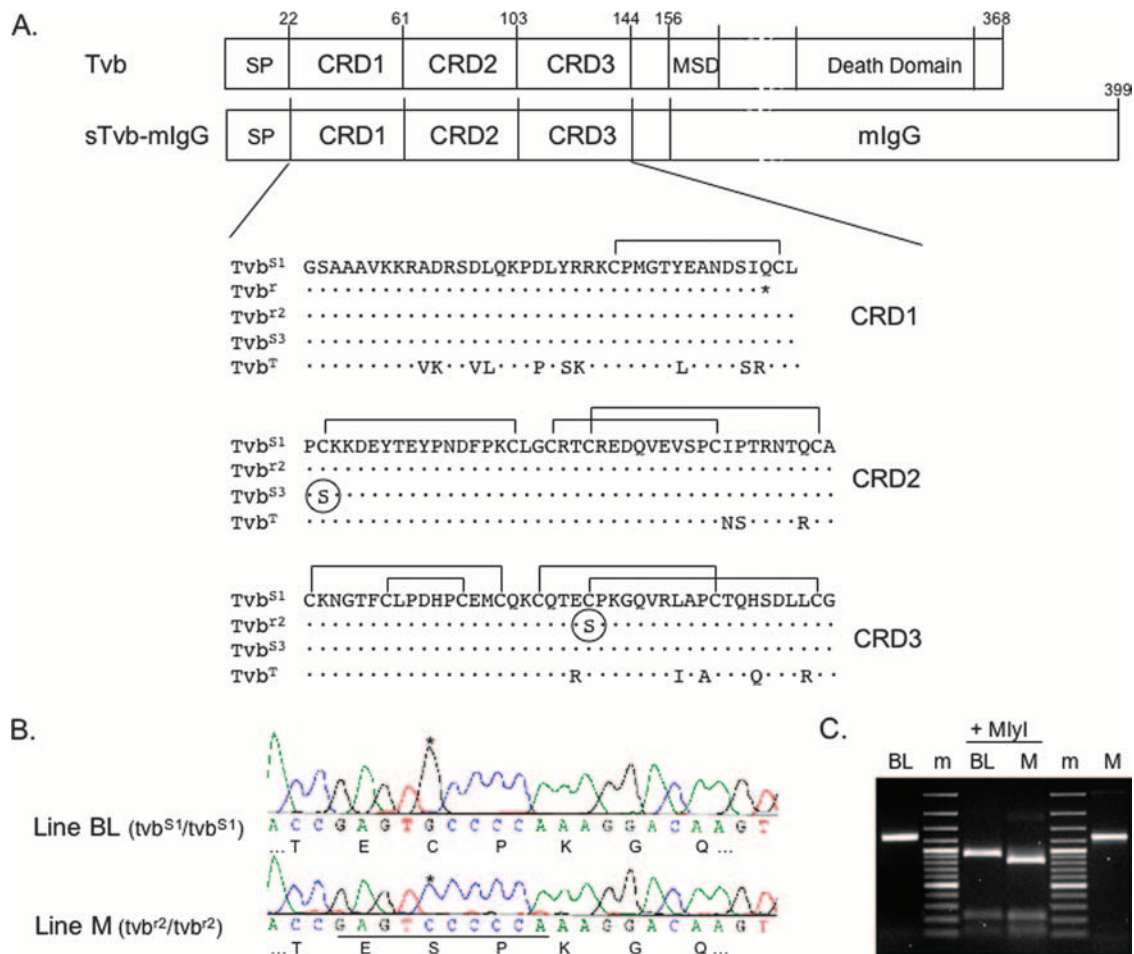


FIG. 1. The *tvb^{R2}* allele in chicken line M contains a C125S substitution in CRD3. (A) Schematic representations of the Tvb proteins and a soluble form of Tvb fused to an mIgG are shown (not drawn to scale). The numbering of the Tvb proteins includes the signal peptide (SP) for historical reasons. MSD, membrane-spanning domain. The deduced amino acid sequences of the CRDs of the known Tvb receptors, including the turkey ortholog, *Tvb^T*, are compared, with residues different from the *Tvb^{S1}* receptor indicated. The C125S substitution in *Tvb^{R2}* and the C62S substitution in *Tvb^{S3}* are circled. The putative disulfide bonds in the Tvb CRDs are indicated based on comparisons with the related TNFR proteins and the X-ray structure of the human DR5 TRAIL receptor protein. (B) Chromatograms of partial nucleotide and deduced amino acid sequences of CRD3s from chicken line M and outbred chicken line BL cDNAs. The G-to-C transversion is highlighted with an asterisk; the new MlyI site in line M is underlined. (C) A 1,259-bp *tvb* fragment was produced by reverse transcription-PCR using RNA from line BL and line M. After MlyI digestion, a 920-bp fragment was produced from the wild-type *tvb^{S1}* cDNA from line BL, while the additional MlyI site in the line M *tvb^{R2}* cDNA produced an 820-bp fragment. m, DNA marker 100-bp ladder.

proteins that can be encountered by ASLVs in chickens, including related receptor orthologs in other birds, provides valuable information on the evolutionary pressures on these viruses and enables the identification of domains of the receptor and envelope glycoprotein critical for an efficient virus-receptor interaction and subsequent virus entry.

The structures of TNFR-related proteins have shown that homologous proteins (e.g., the Tvb proteins) likely contain three cysteine-rich domains (CRDs) in the extracellular domain (Fig. 1A) (15). Two naturally occurring *tvb* susceptibility alleles have been identified in chickens; the *tvb^{S1}* allele confers susceptibility to subgroups B, D, and E ASLVs, while the *tvb^{S3}* allele confers susceptibility to only subgroup B and D ASLVs (3, 7). The substitution of a serine for a cysteine at residue 62 in the CRD2 domain of *Tvb^{S3}* distinguishes the *Tvb^{S1}* and *Tvb^{S3}* proteins. Presumably, this mutation alters the structure of CRD2, resulting in the loss of ASLV(E) binding and entry.

The major determinants of the Tvb receptor important for entry of ASLV(B), ASLV(D), and ASLV(E) were identified previously using a variety of mutational approaches (1, 17, 18). A 15-amino-acid peptide in the Tvb CRD1 domain appears to be sufficient for ASLV(B) and ASLV(D) binding and entry. In contrast, the main determinants of Tvb important for ASLV(E) binding and entry reside in the CRD2 domain. These studies did not demonstrate any significant role of the CRD3 domain in the binding and entry of ASLV(B), ASLV(D), or ASLV(E). A *tvb* allele, *tvb^R*, which contains a premature stop codon at residue 57 explaining the resistance to infection by all three subgroups (16), was identified and characterized from inbred line 7₂ chickens. Finally, a *tvb* homolog, *tvb^T*, that confers susceptibility only to ASLV(E) (2), has been identified in turkeys.

Some inbred lines of chickens display phenotypes that are somewhere in between completely susceptible and resistant to

infection by specific subgroups of ASLV. This altered susceptibility phenotype could be the result of mutations in the receptor that alter but do not eliminate the ability of ASLV to use the protein as a receptor. We hypothesized that such mutations, which reduce but do not eliminate the binding affinity of the mutant receptor for the viral glycoproteins, could explain this phenotype. In this study, the genetic defect that accounts for the altered susceptibility of an inbred chicken line, line M, to infection by ASLV(B), ASLV(D), and ASLV(E) was identified. The *tvb* gene in line M, *tvb*^{r2}, encodes a mutant Tvbs¹ receptor protein with a substitution of a serine for a cysteine at position 125 (C125S). These are the first results that demonstrate a possible role of CRD3 in the efficient functioning of the Tvbs receptors.

MATERIALS AND METHODS

Chicken lines. The inbred chicken lines M (Black Minorca), L15, CC.R1, and WA and the outbred chicken population Brown Leghorn (BL) have been maintained at the Institute of Molecular Genetics, Prague (20). Hens and cockerels were kept in individual cages (4,200 cm²) under standard conditions. Feed and water were provided ad libitum, and a light-dark photoperiod of 12 h-12 h was applied. Fertilized eggs were incubated at 38°C and 60% relative humidity in a forced-air incubator with a tilting motion every 2 h through a 90° angle. All experiments were performed in accordance with Czech legal requirements for animal handling.

Cell culture and virus propagation. Chicken embryo fibroblasts (CEFs) were prepared from 10-day-old embryos from line M, WA, and BL chickens as described previously (12). The DF-1 chicken fibroblast cell line (12a), the Rous sarcoma virus (RSV)-transformed 16Q quail cell line (19), and CEFs were grown in a mixture of two parts Dulbecco's modified Eagle's medium and one part F-12 medium supplemented with 5% calf serum, 5% fetal calf serum, 1% chicken serum, and penicillin/streptomycin (100 mg/ml each) in a 5% CO₂ atmosphere at 37°C. RCASBP(A)GFP, RCASBP(B)GFP, RCASBP(C)GFP, and RCASBP(D)GFP viruses (12, 13) were propagated by transfection of plasmid DNA containing the reporter vector into DF-1 cells, which are free of closely related endogenous retrovirus loci. The RCASBP(E)GFP virus was propagated in line L15 CEFs. Transfection was performed by applying a mixture of 10 µg plasmid DNA and 100 µl linear polyethylenimine (Polysciences; molecular weight, 25,000; 1 mg/ml; pH 7.5) in 12 ml serum-free culture medium on subconfluent DF-1 cells grown in 100 mm plates. Virus spread was observed as an increasing proportion of green fluorescent protein (GFP)-positive cells, and viral stocks were established from cell supernatants at day 4 or 5 after transfection. Viral stocks were cleared of debris by centrifugation at 2,000 × g for 10 min at 10°C and stored at -80°C.

The transforming subgroup A, B, C, D, and E ASLV specificities for in vivo sarcoma induction were produced by rescuing the replication-defective Bryan high-titer-RSV present in the 16Q cell line. DF-1 cells were infected with RCASBP(A)GFP, RCASBP(B)GFP, RCASBP(C)GFP, RCASBP(D)GFP, or RCASBP(E)GFP, and virus spread was monitored by fluorescence. After 4 days, the infected GFP-positive DF-1 cells were mixed with 16Q cells (19) and cultivated for a further 5 days. Viral stocks containing GFP reporter viruses, as well as transforming viruses of the same subgroup, were centrifuged at 2,000 × g for 10 min at 10°C and stored at -80°C. The titers of the transforming viruses were quantitated by an *src* focus assay on BL CEFs and reached titers of 10² to 10⁵ focus-forming unit (FFU) per ml.

Reverse transcription-PCR of the *tvb*^r allele from chicken line M. We prepared total RNA from blood collected from line M chickens using the RNeasy total RNA isolation system (Qiagen Inc.). Reverse transcription was carried out with 1 µg total RNA, Moloney murine leukemia virus reverse transcriptase (Promega), and oligo(dT)₁₅ primers (Promega). The cDNA resulting from this reaction was PCR amplified with primers TVB3 (5'-CAGACCTCCAGAAGCCA GAC-3') and TVB6 (5'-CGAGAGCACTGTCCACAGAGAT-3') with *Taq* polymerase (TaKaRa). The conditions for the amplification were as follows: 2 min at 94°C; 34 cycles of 15 s at 94°C, annealing for 40 s at 57°C, and 3 min at 72°C; and final extension for 10 min at 72°C. The final PCR product was treated with ExoSAP-IT (USB) and then sequenced using the BigDye Terminator v3.1 cycle-sequencing kit (PE Applied Biosystems). A 1,259-bp fragment of *tvb* was amplified from the cDNA using primers TVB5 (5'-TCCTAACTCGGTCCGAA TCC-3') and TVB6 and the Expand Long Template Polymerase (Roche). The

conditions for amplification were 2 min at 94°C and 35 cycles of 15 s at 94°C, annealing for 40 s at 56°C, and 90 s at 68°C; final extension was for 7 min at 68°C. The product was digested with MlyI, and the products were separated by agarose electrophoresis.

Virus entry analyzed by FACS. Line BL or M CEFs were seeded at 5 × 10⁵ per 60-mm plate and infected with RCASBP viruses the next day: 10⁵ infectious units were applied in 0.5 ml medium, and after 1 h, the volume of the medium was increased to 4 ml. The percentage of GFP-positive cells was quantitated by fluorescence-activated cell sorting (FACS) using an LSR II analyzer (Becton Dickinson) on days 1, 2, 3, and 6 postinfection. Each day, one half of the cell culture was used for FACS and the other half was passaged on a new dish. For FACS analysis, trypsinized cells were first washed in phosphate-buffered saline (PBS) and then analyzed.

DNA constructs. A gene encoding a soluble form of the chicken Tvbs¹C125S receptor (*stvbs*¹C125S-*mIgG*) was constructed as described previously for soluble forms of the chicken Tvbs³ receptor (*stvbs*³-*mIgG*) and Tvbs¹ receptor (*stvbs*¹-*mIgG*) (11). These genes encode the extracellular domain of the ASLV receptor fused to the constant region of a mouse immunoglobulin G (mIgG) heavy chain and are in the CLA12NCO adaptor plasmid (12). The *stvbs*¹C125S-*mIgG*, *stvbs*¹-*mIgG*, and *stvbs*³-*mIgG* gene cassettes were isolated as ClaI fragments and subcloned into the ClaI site of the RCASBP(A) vector. DF-1 cells were infected with each virus, and infected cell supernatants that contained either the Tvbs¹C125S-mIgG, Tvbs³-mIgG, or Tvbs¹-mIgG receptor proteins were collected. The supernatants were cleared by centrifugation at 2,000 × g for 10 min at 4°C and stored in aliquots at -80°C.

The recombinant, replication-competent ASLV vectors RCASBP(A)GFP, RCASBP(B)GFP, RCASBP(C)GFP, RCASBP(D)GFP, and RCASBP(E)GFP viruses containing the GFP gene have been described previously (12). The receptor subgroup of the viral envelope glycoprotein is denoted in parentheses [e.g., subgroup B by (B)].

Immunoprecipitations and Western immunoblot analysis. The Tvbs¹C125S-mIgG, Tvbs³-mIgG, and Tvbs¹-mIgG receptor proteins were immunoprecipitated separately from cell culture supernatants with anti-mIgG-agarose beads (Sigma) and analyzed by Western immunoblotting as previously described (11).

Binding affinity was analyzed by FACS. Line L15 CEFs and line L15 CEFs infected with either RCASBP(B), RCASBP(D), or RCASBP(E) were removed from culture with trypsin de Larco (Quality Biological, Inc.) and washed with PBS. The cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min and then washed with PBS. Approximately 1 × 10⁶ cells in PBS supplemented with 1% calf serum (PBS-CS) were incubated with supernatant containing one of the receptor-mIgG fusion proteins on ice for 30 min. The DF-1 cells were then washed with PBS-CS and incubated with either 5 µl of goat anti-mIgG (heavy plus light chains) linked to phycoerythrin or 5 µl of goat anti-rabbit IgG (rIgG) (heavy plus light chains) linked to phycoerythrin (Kirkegaard & Perry Laboratories, Gaithersburg, MD) in PBS-CS (1 ml total volume) on ice for 30 min. The cell-soluble receptor-IgG-phycoerythrin complexes were washed with PBS-CS, resuspended in 0.5 ml PBS-CS, and analyzed with a Becton Dickinson FACSCalibur using CELLQuest 3.1 software.

K_D calculations. The maximum possible fluorescence and apparent 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: $f(y) = M/[1 + e^{-r(\log y - \log K_d)}]$, where y is the mean fluorescence, M is the maximum fluorescence, r is the rate, x is the concentration of the receptor-mIgG or SU-rIgG fusion protein, and K_d is the dissociation constant, defined as the concentration of the receptor-mIgG or SU-rIgG fusion protein at half-maximal binding.

In vivo sarcoma induction and monitoring. Ten-day-old chickens from lines M, CC.R1, and L15 were inoculated with 10² or 10³ FFU of transforming virus rescued from 16Q cells in 0.1 ml of Iscove's Dulbecco's modified Eagle's medium subcutaneously into the pectoral muscle. The growth of sarcomas at the site of inoculation was monitored by calculating the area of prominent tumor. Transparent foil was placed on the tumor, the contours of the tumor were traced, and the area of the tumor was calculated in mm² (21). Birds bearing vast nonregressing tumors were sacrificed before they reached the terminal stage.

RESULTS

The *tvb* allele from the inbred chicken line M (*tvb*^{r2}) encodes a mutant Tvbs¹ receptor with the C125S substitution in CRD3. cDNAs were synthesized from mRNAs isolated from inbred chicken line M and line WA CEFs and used to PCR amplify

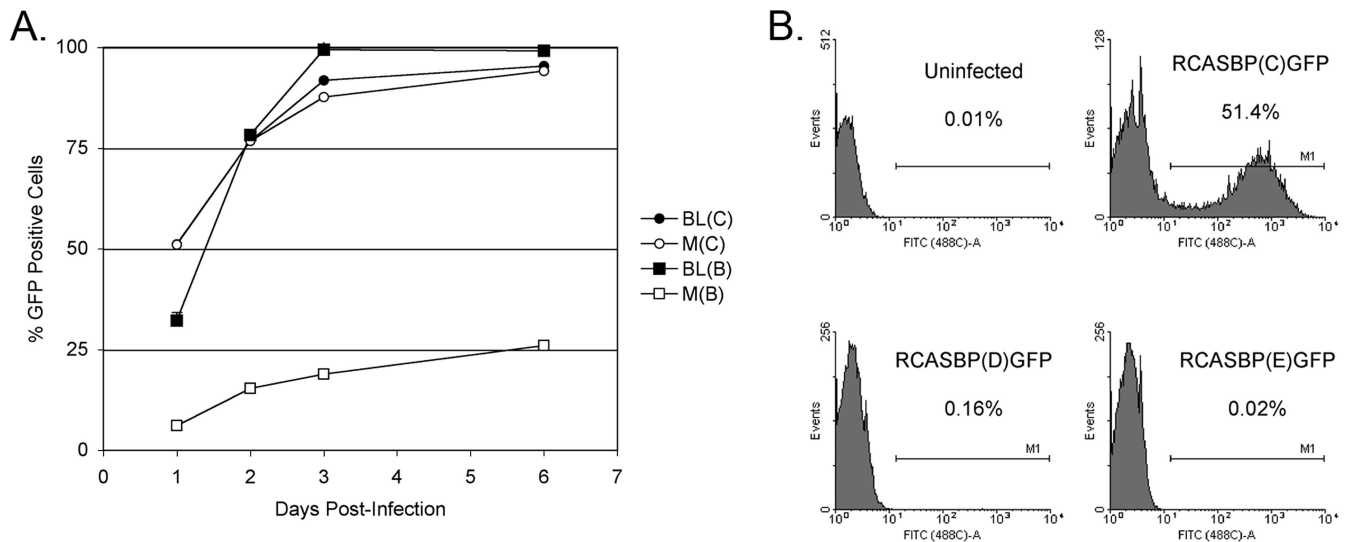


FIG. 2. The time course of infection of BL and line M (M) CEFs with ASLVs. CEFs were infected with replication-competent ASLVs encoding the GFP reporter proteins RCASBP(B)GFP, RCASBP(C)GFP, RCASBP(D)GFP, and RCASBP(E)GFP. (A) The proportion of GFP-positive cells was determined by FACS on the indicated day postinfection; the percentage of GFP-positive cells is indicated as a mean of three parallel dishes. (B) Line M CEFs were infected at an MOI of 0.1, and the percentage of GFP-positive cells was quantitated by FACS 1 day postinfection. In the histograms, the relative GFP fluorescence is plotted against the cell count and the percentage of GFP-positive cells is indicated as a mean of two parallel dishes.

tvb sequences and to clone the *tvb* cDNAs. We determined the nucleotide and deduced amino acid sequences of the coding regions of *tvb* receptor cDNA clones and compared the nucleotide sequences with the sequences of *tvb^{s1}* and *tvb^{s3}* genes (3, 7). The *tvb^{s1}* allele of line WA encodes the same single-nucleotide mutation in codon 58 (CAG to UAG) that results in a premature termination codon described previously for the chicken line 7_2 *tvb'* allele (16). However, the *tvb* gene in line M contains a different single-nucleotide mutation that changes the cysteine at position 125 to serine (UGC to UCC) in the Tvb^{S1} receptor protein (Fig. 1A) and is designated *tvb²*. This single-nucleotide change creates an additional MlyI recognition site in *tvb²* compared to *tvb^{s1}* (Fig. 1B). The presence of this polymorphic marker was further demonstrated by digestion of a 1,259-bp fragment of *tvb* cDNA amplified from line M RNA. The diagnostic 920-bp MlyI fragment was obtained using the control *tvb^{s1}* cDNA from line BL; a shortened 820-bp fragment was obtained after MlyI cleavage of *tvb²* cDNA (Fig. 1C).

The C125S substitution in the Tvb^{S1} receptor reduces the susceptibility of line M cells to subgroup B, D, and E ASLV infection. To determine the effect of the C125S substitution in the chicken Tvb^{S1} receptor on ASLV susceptibility, line M and BL CEFs were infected at a multiplicity of infection (MOI) of 0.1 with RCASBP(GFP) reporter virus, a replication-competent ASLV vector encoding GFP, and the time course of infection was followed by quantitating the percentage of green fluorescent cells by flow cytometry. Line BL CEFs are susceptible to subgroup A, B, C, and D ASLVs and were used as a positive control. As expected, both line M and line BL CEFs were efficiently infected by RCASBP(C)GFP, as shown in Fig. 2A, with almost one-half of the cells infected at 1 day postinfection and virtually all cells infected by day 3. A very different result was obtained when the CEFs were infected with RCASBP(B)-

GFP. As expected, line BL CEFs were efficiently infected with RCASBP(B)GFP at a rate similar to that of RCASBP(C)GFP infection (Fig. 2A). However, RCASBP(B)-GFP infected line M CEFs much less efficiently, with only 6.6% of the cells infected at day 1 (5- to 10-fold lower than line BL), and virus spread through the cells very slowly, with only ~25% of the cell population infected by day 6. In a separate experiment, line M CEFs were infected with RCASBP(C)GFP, RCASBP(D)GFP, or RCASBP(E)GFP at 0.1 MOI, and the infected cells were quantitated the next day (Fig. 2B). As in the first experiment, ~50% of the line M CEFs were infected with the control RCASBP(C)GFP virus, but only 0.16% of the line M CEFs were infected with RCASBP(D)GFP and virtually no line M CEFs were infected with RCASBP(E)GFP. These data clearly demonstrate, at least in cultured cells, that the C125S substitution in the Tvb^{S1} receptor resulted in lower susceptibility of line M CEFs to ASLV(B) infection, including a significant decrease in the rate of virus spread, perhaps an even lower level of susceptibility to ASLV(D) infection and virus spread, and almost complete resistance to infection by ASLV(E).

The C125S substitution in Tvb^{S1} lowers the binding affinity for the ASLV envelope glycoproteins. Soluble forms of the Tvb receptors fused to an mIgG domain (Fig. 1A) were used to estimate by FACS the binding affinities of the Tvb^{S1}, Tvb^{S1C125S}, and Tvb^{S3} receptors for ASLV envelope glycoproteins expressed on the surfaces of infected line L15 CEFs, as described previously (11). The integrity of the soluble Tvb (sTvb)-mIgG proteins was verified by immunoprecipitation and Western immunoblot analysis (Fig. 3A). The concentration of each protein stock was determined by an enzyme-linked immunosorbent assay for mIgG as described previously (10). The sTvb^{S1}-mIgG protein bound the ASLV(B)-infected cells (Fig. 3B), the ASLV(D)-infected cells (Fig. 3C), and the ASLV(E)-infected cells (Fig. 3D) with subnanomolar affinities

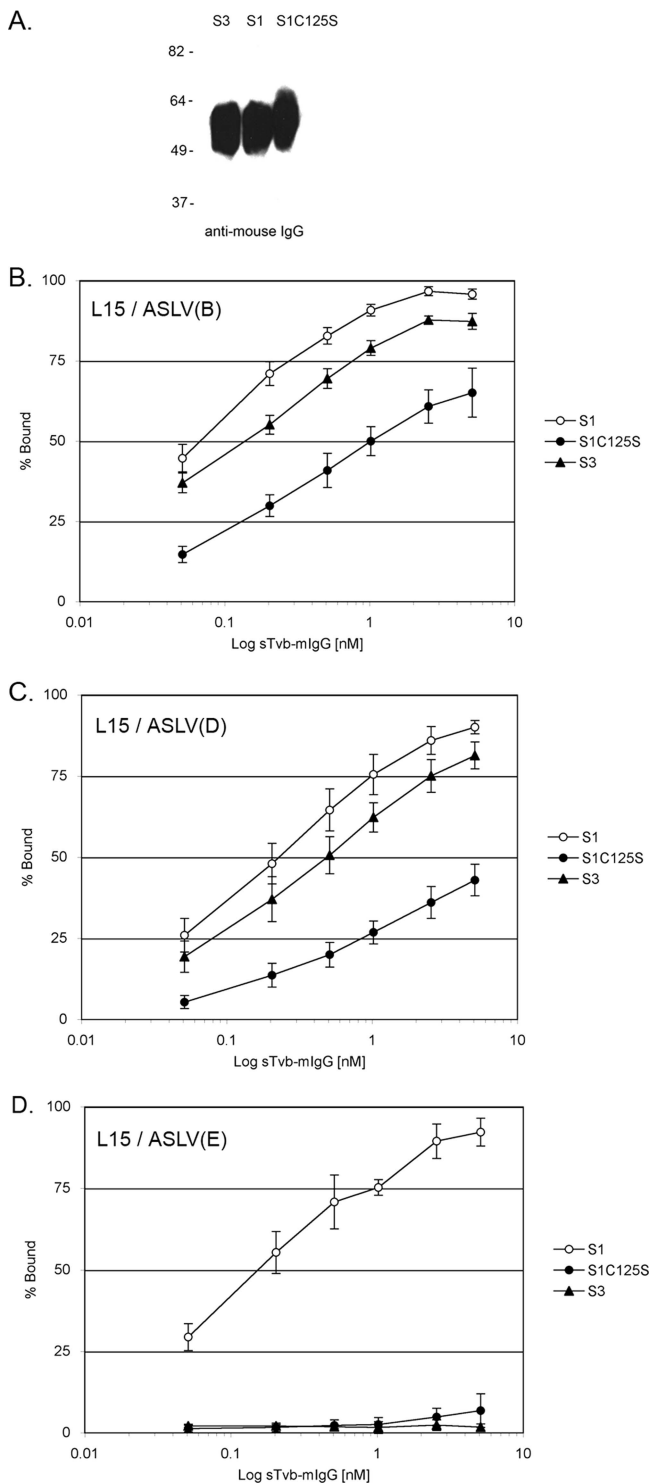


FIG. 3. Binding affinities of ASLV envelope glycoproteins for Tvb receptors. (A) Western immunoblot analysis of the soluble forms of the Tvb proteins, sTvb^{S3}-mIgG (S3), sTvb^{S1}-mIgG (S1), and sTvb^{S1C125S} (S1C125S), immunoprecipitated with anti-mIgG agarose beads, denatured and separated by sodium dodecyl sulfate-12% polyacrylamide gel electrophoresis, and transferred to nitrocellulose. The mIgG-tagged proteins were probed with anti-mIgG-conjugated to horseradish peroxidase and visualized by chemiluminescence. Molecular masses (in kilodaltons) are given on the left. (B to D) Line L15 CEFs chronically infected with ASLV(B) (B), ASLV(D) (C), or ASLV(E) (D) were fixed with paraformaldehyde and incubated with

TABLE 1. Estimated binding affinities of soluble forms of the Tvb receptors for ASLV envelope glycoproteins expressed on the surfaces of L15 CEFs

Cells	Apparent K_D (nM) ^a		
	sTvb ^{S1} -mIgG	sTvb ^{S1C125S} -mIgG	sTvb ^{S3} -mIgG
L15/ASLV(B)	0.07 ± 0.01	1.27 ± 0.65	0.15 ± 0.03
L15/ASLV(D)	0.23 ± 0.08	8.77 ± 3.17	0.50 ± 0.14
L15/ASLV(E)	0.17 ± 0.05	>>1,000 ^b	NDB ^c

^a Apparent K_D values were estimated by fitting data via nonlinear least squares to log logistic growth curve function as described in Materials and Methods. Each result is the average and standard deviation from three experiments.

^b Binding of the sTvb^{S1C125S}-mIgG receptor was detectable but not sufficient to calculate a reliable apparent K_D (Fig. 3).

^c NDB, no detectable binding.

(Table 1). As expected, the sTvb^{S3}-mIgG protein bound subgroup B and D glycoproteins with affinities similar to that of sTvb^{S1}-mIgG but did not bind subgroup E glycoproteins at a detectable level. Binding of the sTvb^{S1C125S}-mIgG protein to all three ASLV glycoproteins could be detected, but with significantly lower affinities than for sTvb^{S1}-mIgG: ASLV(B) at 10- to 25-fold lower affinity, ASLV(D) at 25- to 50-fold lower affinity, and ASLV(E) at barely detectable levels. The reduction in the binding affinity of sTvb^{S1C125S}-mIgG correlates with the reduced infectivity of subgroups B, D, and E ASLVs in line M CEFs (Fig. 2).

Compared to chickens with a wild-type Tvb^{S1} receptor, the incidence and growth of sarcomas induced by subgroups B, D, and E ASVs were significantly altered in line M chickens. In order to see the in vivo effects of the C125S substitution in the Tvb^{S1} receptor, line M chickens were infected with transforming viruses and the formation and growth rates of the induced sarcomas were measured. ASLVs containing the *src* oncogene (avian sarcoma virus [ASV]) were produced by the rescue of *env*-defective Bryan high-titer-RSV from 16Q cells (19) by infection with the appropriate envelope subgroup RCASB-PGFP virus. The titer of the rescued transforming virus stocks was quantitated by in vitro focus assay. Ten-day-old line M chicks and age-matched controls of line CC.R1 and line L15 were challenged with 1,000 FFU or 100 FFU of the ASV stock injected into the pectoral muscle. Line CC.R1 is susceptible to subgroup B, C, and D ASLV infection. Line L15 is susceptible to subgroup A, B, D, and E ASLVs—a rare inbred chicken line susceptible to subgroup E viruses (8). The incidence and growth rates of sarcomas induced at the site of virus inoculation were quantitated.

As expected, line CC.R1 was susceptible to sarcoma formation induced by ASV(C), ASV(B), and ASV(D) infection while line M was susceptible to sarcoma formation induced by ASV(A) and ASV(C) infection. In these cases, the tumor in-

different amounts of soluble receptor. The viral glycoprotein-soluble-receptor complexes were bound to goat-anti-mIgG linked to phycoerythrin. The amount of phycoerythrin bound to the cells was quantitated by FACS, and the maximum fluorescence was estimated (see Materials and Methods). The data were plotted as percent maximum fluorescence. The values shown are the averages and standard deviations from four experiments.

cidence was 100% in both lines, with similar tumor growth rates, depending on the initial dose of ASV (Fig. 4A, B, and C). Animals that received the 1,000-FFU dose were terminated due to tumor burden by day 18 and those that received the 100-FFU dose by days 23 to 28. Very different tumor incidence and progression were observed with ASV(B) and ASV(D) infections of line M chicks. The 1,000-FFU ASV(B) dose induced sarcomas in 100% of the infected line M animals, but the tumors grew at significantly lower rates than in line CC.R1, with all animals requiring termination by day 28 (Fig. 4B). The 100-FFU ASV(B) dose induced sarcomas in only 45% of the infected line M animals (four of nine chicks), and the tumors that did form grew very slowly. A similar pattern was observed with ASV(D) infection of line M. The 1,000-FFU ASV(D) dose induced tumors in 100% of the infected animals, but the tumors grew at an even lower rate (Fig. 4C) than ASV(B)-induced tumors (Fig. 4B). The 100-FFU ASV(D) dose induced sarcomas in only 45% of the infected animals (four of nine chicks), and the tumors that did form grew even more slowly. Finally, ASV(E) induced sarcomas in 100% of the infected line L15 animals, and the tumor growth rate was dependent on the virus dose (Fig. 4D). However, even at the 1,000-FFU ASV(E) dose, no infected line M animals (zero of eight chicks) produced detectable tumors even after 100 days postinfection. Assuming that the continual cell-to-cell spread of a transforming virus by reinfection is the major factor facilitating the progression of ASV-induced sarcomas (22), we conclude that the C125S substitution in the Tv^b^{S1} receptor is responsible for the reduced susceptibility of line M to ASLV(B) and ASLV(D) infection and the complete resistance to ASLV(E) infection *in vivo*.

DISCUSSION

In this study, we describe the identification of the molecular defect in the *tvb* gene of inbred line M chickens, the C125S substitution in the Tv^b^{S1} receptor, that significantly decreases the sensitivity of these birds to infection by ASLV(B) and ASLV(D) and completely abrogates their sensitivity to ASLV(E) infection. This single-amino-acid substitution in the Tv^b^{R2} receptor in the inbred line M chickens significantly reduces the binding affinity of the Tv^b^{S1} receptor for the ASLV glycoproteins and explains the decreased susceptibility to the ASLV(B) and ASLV(D) infection and resistance to ASLV(E) infection. This is the first reported example of a mutation in the ASLV receptors that results in a quantitative effect on ASLV susceptibility and pathogenesis. Various levels of resistance to human immunodeficiency virus type 1 infection have been reported that are explained by polymorphisms in the CD4 receptor and/or the viral coreceptors that result in a significant reduction in the efficiency of the human immunodeficiency virus type 1 glycoprotein interaction with the mutant receptor (23). Therefore, one mechanism whereby this initial interaction between the receptor and virus required for entry can cause selective pressure on the virus population is the interaction of the viral glycoproteins with variant receptors, resulting in quantitative differences in host sensitivity. The altered susceptibility of line M for infection by ASLV(B), ASLV(D), and ASLV(E) was observed both in cultured CEFs and in chicks challenged with ASV.

Originally, the major determinants of the interaction of the ASLV(B), ASLV(D), and ASLV(E) glycoproteins with the Tv^b receptors were thought to be independent of CRD3. Indeed, mutant receptors with CRD3 completely deleted were constructed in previous studies and appeared to bind the viral glycoproteins and to function as receptors for these three ASLV subgroups as efficiently as wild-type Tv^b (Fig. 5). A 15-amino-acid domain in Tv^b CRD1, residues 32 to 46, has been reported to contain the interaction determinants for subgroup B and D ASLVs: viral entry can be mediated using only this domain as a synthesized peptide (18). Residues L36, Q37, L41, and Y42 in this domain are critical for ASLV(B) and ASLV(D) binding and entry (18). The disulfide bond pattern of CRD1 did not appear to be important for Tv^b to function as a receptor for ASLV(B) and ASLV(D) in these studies. From the sequence alignment of the Tv^b receptor with several TNFR family members, and based on the X-ray structure of the DR5 TRAIL receptor, the Tv^b₃₂₋₄₆ domain is predicted to be in the nonstructured N terminus of the CRD1 domain, perhaps explaining the retention of receptor function without a particular pattern of disulfide bonds in CRD1.

In contrast to ASLV(B) and ASLV(D), both CRD1 and CRD2 of Tv^b are required for efficient ASLV(E) binding and entry (17). The structural integrity of the protein, including the disulfide bonds and residues Y67, N72, and D73 in CRD2, are critical for proper receptor function. Modeling Tv^b on the X-ray structure of DR5 TRAIL receptor predicts that these critical residues reside in a highly structured region of the protein. The Tv^b^{S3} protein contains the C62S substitution, which presumably alters the structure of the critical CRD2, eliminating binding of the ASLV(E) glycoproteins and virus entry but having no detectable effect on ASLV(B) and ASLV(D) binding and entry (Fig. 5). It has also been suggested that the Tv^b^{S1} receptor protein may exist in at least two forms that result from alternate patterns of protein folding and disulfide bonds: type 1 confers susceptibility to subgroup B, D, and E ASLVs; type 2 confers susceptibility to only subgroup B and D ASLVs (1).

The C125S substitution likely alters the folding and final structure of at least CRD3 in the Tv^b^{R2} protein, since the C125 residue in Tv^b is predicted to exist as a disulfide bond with C143 in CRD3 (Fig. 5, Tv^b^{R2} A). Published studies have demonstrated that Tv^b receptors with the CRD3 domain deleted (Tv^bΔCRD3) appear to function as efficiently as a wild-type Tv^b^{S1} receptor (17). Therefore, a simple model requiring the interaction of the viral glycoproteins with two domains for efficient virus binding and entry, ASLV(B) and ASLV(D) with the Tv^b₃₂₋₄₆ domain and CRD3 and ASLV(E) with CRD2 and CRD3, does not seem likely. The Tv^b₃₂₋₄₆ domain expressed in a heterologous protein can also act as an ASLV(B) and ASLV(D) receptor (18). Invariably, experiments that study the functions of different domains and/or mutations in receptor function express the proteins at very high levels compared to the levels found naturally in chicken cells. In an earlier study, we reported that the *tva*^r allele found in line C chickens contains a cysteine-to-tryptophan substitution that significantly reduces the binding affinity of ASLV(A) glycoproteins for the Tva^R receptor (10). However, the ectopic expression of the Tva^R receptor at high levels in resistant cells conferred susceptibility to ASLV(A) infection at levels similar to wild-type

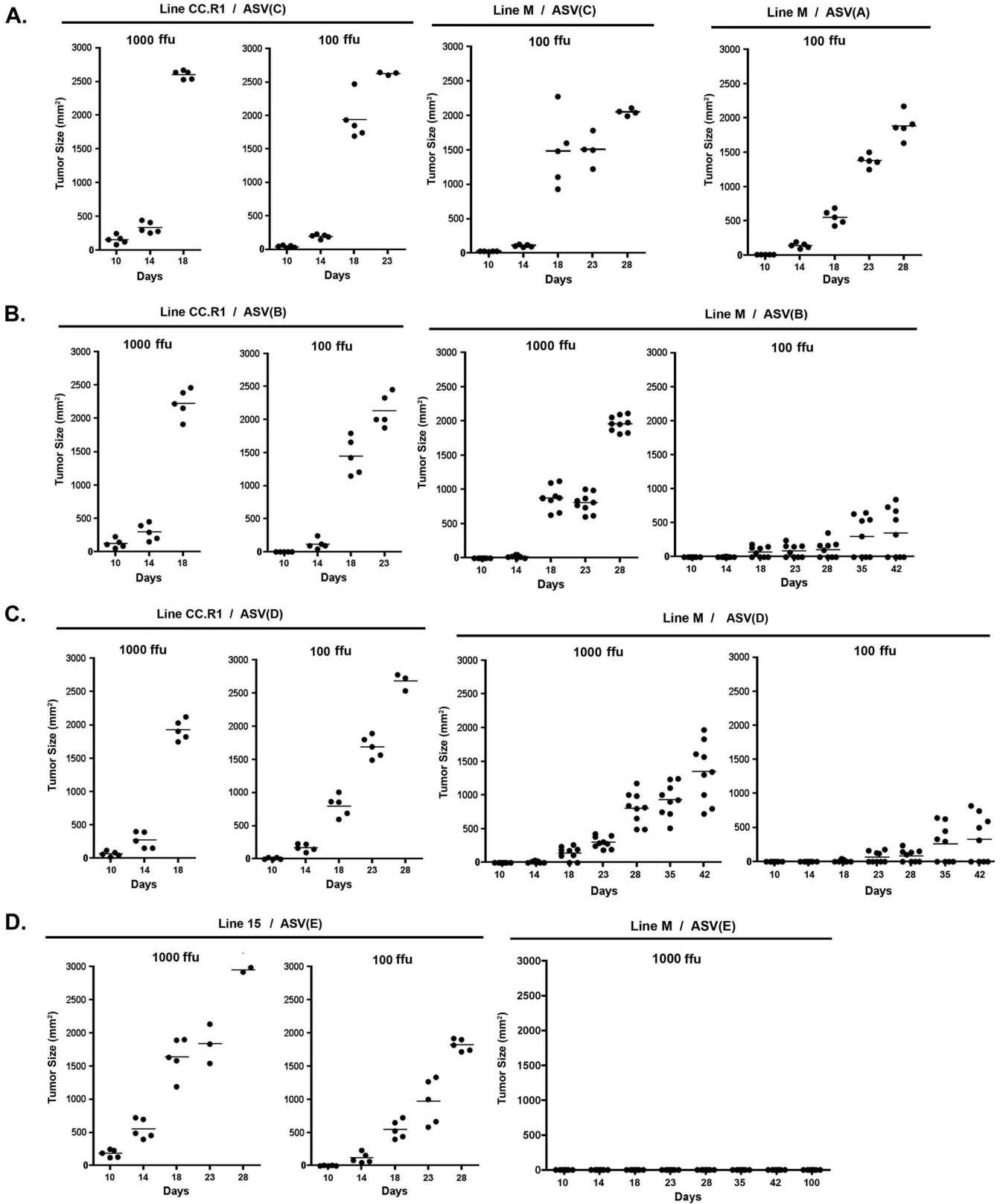


FIG. 4. The growth of sarcomas induced after infection with 1,000 FFU or 100 FFU of ASV in line CC.R1, line L15, or line M 10-day-old chicks. The sizes of tumors induced in chicks infected with ASV(A) or ASV(C) (A), ASV(B) (B), ASV(D) (C), or ASV(E) (D) were measured on the days indicated. The tumor size in each bird is indicated by an individual dot, with the average tumor size for the group shown with a horizontal bar.

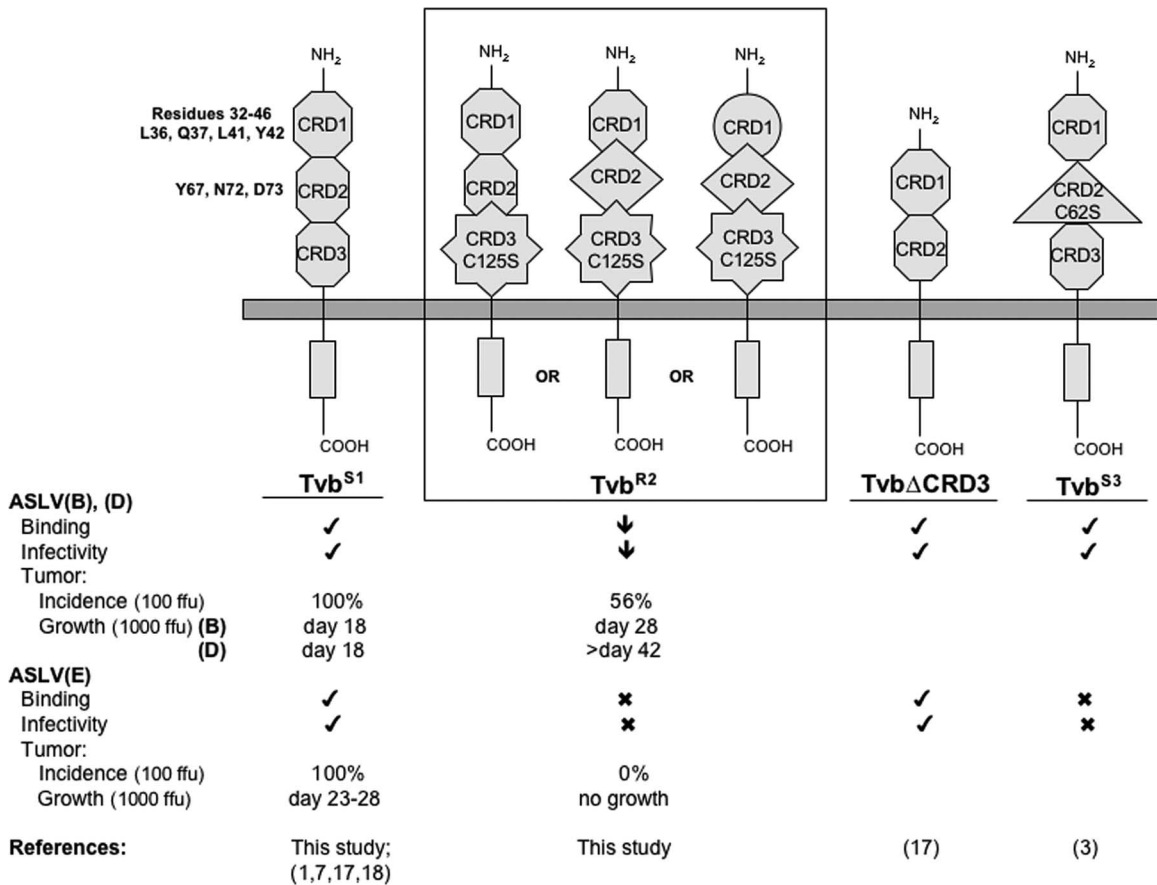


FIG. 5. Schematic representations of membrane-bound forms of the known and functional chicken *Tvb* proteins. Hypothetical models are shown for *Tvb^{S1}*, *Tvb^{R2}* (A, B, and C), the published *Tvb^{S1}* with CRD3 deleted (*Tvb Δ CRD3*), and *Tvb^{S3}*. *Tvb^{S1}* depicts the wild-type structure of the CRDs (hexagons). The different shapes of the CRDs denote altered structures induced by either the C125S substitution in *Tvb^{R2}* or the C62S substitution in *Tvb^{S3}*. The abilities of the proteins to function as ASLV(B), ASLV(D), and ASLV(E) receptors are indicated. Check mark, wild-type activity; ↓, significantly reduced activity; X, little or no detectable activity. Tumor incidence (the percentages of chickens infected with 100 FFU of ASVs that formed tumors) and growth (the day when all chickens were sacrificed due to tumor burden from infection with 1,000 FFU ASVs) were summarized from Fig. 4.

Tva. The true resistant phenotype of *Tva^R* became apparent experimentally only when very low levels of the receptors were expressed: wild-type *Tva* still conferred a high level of susceptibility, while the *Tva^R* protein was >1,000-fold less efficient as an ASLV(A) receptor. It may be that when the *Tvb* proteins are expressed at wild-type levels, mutant proteins with only the *Tvb₃₂₋₄₆* domain or just CRD1 and CRD2 may still function but at a lower efficiency than receptors that also contain CRD3. The *Tvb* receptors expressed at levels found on chicken cells may require the interaction of the ASLV glycoproteins with the known domains and CRD3 on the receptor for optimal function.

Alternatively, since the *Tvb* proteins may naturally exist in several forms, the C125S mutation may result in structural alterations in other regions of the protein. Given the existence of two types of *Tvb^{S1}* receptors with altered disulfide bonds and the *Tvb^{S3}* receptor, both with putative alterations in the CRD2 domain that eliminate ASLV(E) binding and entry, it seems obvious to propose that the C125S substitution may also cause alterations in the CRD2 domain structure. Another possible model of the *Tvb^{R2}* protein to explain the altered ASLV

susceptibility phenotype would have an altered CRD3 structure interfering with and/or altering the structure of CRD2, thereby blocking the use of the protein as an ASLV(E) receptor. However, in contrast to the phenotype of the *Tvb^{S3}* receptor, the binding and entry of ASLV(B) and ASLV(D) are also affected in the *Tvb^{R2}* receptor. Therefore, if C125S alters both CRD3 and CRD2, the structure of CRD2 in *Tvb^{R2}* would likely be different than *Tvb^{S3}* and might inhibit the interaction of ASLV(B) and ASLV(D) with CRD1 (Fig. 5, *Tvb^{R2}* B). Finally, the C125S substitution could result in an alteration in the structures of all three CRDs (Fig. 5, *Tvb^{R2}* C), eliminating the use of the protein as an ASLV(E) receptor and significantly reducing, but not eliminating, the binding and entry of ASLV(B) and ASLV(D). Other combinations of these and other models could account for the phenotype of the *Tvb^{R2}* receptor, including the altered structure of CRD3 resulting from the C125S substitution altering the presentation of intact CRD1 and CRD2 on the membrane so that the virus is sterically blocked from effective interactions.

The normal functions of the *Tvb* proteins in chickens are unknown and can be estimated only from their similarity to the

TNFR family of proteins. Until we identify these functions, the effect of the C125S substitution on the normal activity of this protein cannot be evaluated. The ASLV glycoproteins may interact with regions of the receptor that do not compete with the interaction of normal ligand(s) of the protein, providing possible scenarios in which the protein can still act as a receptor but loses normal function, and vice versa. Subgroup E ASLVs are endogenous to the germ lines of almost all chickens. To control the spread and accumulation of endogenous ASLV proviruses and possible pathogenesis, polymorphisms in the ASLV receptors that altered the binding of the ASLV glycoproteins while retaining normal function might have offered a positive selective advantage in the chicken population. Selection of mutant Tv_b proteins that have lost the ability to function as an ASLV(E) receptor while possibly retaining the “normal” Tv_b function may have provided the greatest selective advantage, resulting in fixing of the *tvb*^{s3} and *tvb*^{r2} loci in the germ lines of certain lines of inbred chickens.

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