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Analysis of the Subgroup A Avian Sarcoma and Leukosis Virus Receptor: the 40-Residue, Cysteine-Rich, Low-Density Lipoprotein Receptor Repeat Motif of Tva Is Sufficient To Mediate Viral Entry

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The genes encoding the receptor for subgroup A Rous sarcoma viruses (*tva*) were recently cloned from both chicken and quail cells (P. Bates, J. A. T. Young, and H. E. Varmus, Cell 74:1043–1051, 1993; J. A. T. Young, P. Bates, and H. E. Varmus, J. Virol. 67:1811–1816, 1993). Previous work suggested that only the extracellular domain of Tva interacts with the virus (P. Bates, J. A. T. Young, and H. E. Varmus, Cell 74:1043–1051, 1993). Tva is a small membrane-associated protein containing in its extracellular domain a 40-amino-acid region which is closely related to the low-density lipoprotein receptor (LDLR) repeat motif. To determine the region of the Tva extracellular domain responsible for viral receptor function, we created chimeric proteins containing various regions of the Tva extracellular domain fused with a murine CD8 membrane anchor. Analysis of these proteins demonstrates that any chimera containing the Tva LDLR repeat motif can specifically bind the envelope protein of subgroup A avian sarcoma and leukosis viruses. Furthermore, NIH 3T3 cell lines expressing these chimeric proteins were efficiently infected by subgroup A avian sarcoma and leukosis virus vectors. Our results demonstrate that the 40-residue-long LDLR repeat motif of Tva is responsible for viral receptor function.

Viruses bind to specific receptors on the surface of the host cell to initiate infection. The genes encoding the cellular receptor for subgroup A avian sarcoma and leukosis viruses (ASLV-A) have been cloned from chicken and quail cells (2, 26). The chicken gene has recently been genetically mapped to the locus which determines susceptibility to subgroup A viruses (*tva*); thus, the receptor protein will be referred to as Tva (1). Two alternatively spliced transcripts encoding functional receptors were derived from the quail gene by utilizing an exon trapping protocol (2). The extensively modified proteins encoded by these transcripts are tethered to the membrane by different anchors (22). One ASLV-A receptor contains a transmembrane domain, while the other is glycosyl phosphatidylinositol linked. These proteins share 73 amino acids in the extracellular region, suggesting that residues required for receptor function are localized to these 73 residues. In addition, secreted, recombinant forms of the receptor containing only the extracellular domain of the ASLV-A receptor, produced either in quail cells or from insect cells, specifically bind to subgroup A envelope protein and block infection by subgroup A viruses (5, 9), confirming that the sites of interaction with ASLV-A lie entirely within the extracellular domain.

The extracellular domain of the ASLV-A receptor contains three potential sites for modification by N-linked sugars and numerous potential O-glycosylation sites. Analysis of the receptor protein suggests that extensive posttranslational modification occurs (22). Within the extracellular domain is a single copy of a cysteine-rich motif found repeated seven times in the low-density lipoprotein receptor (LDLR). In the LDLR, each of these repeats is 40 to 50 residues in length and contains six highly conserved cysteine residues (10, 25). These repeats form the ligand binding domain of LDLR and determine the specificity of the LDLR interaction with lipoproteins (7, 18). The LDLR-like repeat motif defines a family of proteins, and in many family members the LDLR-like repeats are believed to mediate extracellular protein-protein interactions (13, 19, 21). In the present study, we analyzed the features of the ASLV-A receptor which are required for envelope binding and viral entry. We found no specific requirement for the extensive glycosylation of Tva for ASLV-A receptor function. Furthermore, the LDLR repeat motif region of Tva is sufficient for efficient virus receptor function.

MATERIALS AND METHODS

Cells and viruses. Chicken embryo fibroblasts (CEFs [line 0 C/E]) were maintained in M199 medium supplemented with 10% tryptose phosphate broth, 5% fetal calf serum, and 1% chick serum. The NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% bovine calf serum. RCAS(A)HAP virus vector was provided by Stephen Hughes (National Institutes of Health). RCAS(A)HAP virus stocks were produced by transfecting CEFs with the vector DNA and then allowing the virus to spread for 4 days before being harvested. NIH 3T3 cells expressing Rous sarcoma virus subgroup A or C envelope protein were obtained from Judith White (University of Virginia).

Construction of Tva/CD8 chimeras. Different quail Tva/CD8 chimeras, as shown in Fig. 1B, were constructed by a PCR-based overlap extension technique (11) with the following oligonucleotide primers: OS6, 5′-GAAGAGATCTCTGAAGT CATG-3′; OS15, 5′-GATTTAGGTGACACTATAG-3′; OS24, 5′-CTGAAGCTT GCATGCCTG-3′; OS26, 5′-TTAATTCAGATCTGGTACC-3′; OS37, 5′-CTG CTGCTGCCCGGTAACGTGACCGGTCGTTGCCCCCCCGGGCAGTTCC GCTGC-3′; OS38, 5′-GCAGCGGAACTGCCCGGGGGGGCAACGACCGGTC ACGTTACCGGGCAGCAGCAG-3′; OS39, 5′-ACCGGTAACGGGGCCGGTA ACGGTGCTTTGGCCCGTTGCCCCCCC-3′; OS40, 5′-GGGGGGGCAACG GGCCAAAGCACCGGTACCGGCCCCGTTACCGGT-3′; OS45, 5′-GGGACC AGCGCGACCCCCATCAGCAACTCGGTGATG-3′; OS46, 5′-CATCACCGA GTTGCTGATGGGGGTCGCGCTGGTCCC-3′; OS47, 5′-GCCAGGAATCAC GGCCGCATCAGCAACTCGGTGATG-3′; OS48, 5′-CATCACCGAGTTGCTG ATGCGGCCGTGATTCCTGGC-3′; OS71, 5′-AACGGTTCTTTGTCCCGTGG

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GACCAGCGCGACCCCC-3′; OS72, 5′-GGGGGTCGCGCTGGTCCCACGG GACAAAGAACCGTT-3′; OS73, 5′-GGGACCAGCGCGACCCCCTTGGACT TCGCCAGTGATATTTAC-3′; and OS74, 5′-GTAAATATCACTGGCGAAGT CCAAGGGGGTCGCGCTGGTCCC-3′.

(i) Tva 1–83/CD8. The Tva 1–83 coding region was amplified by OS26 and OS48 and the mouse CD8 tail region was amplified by OS6 and OS47 with Tva_{800} and mouse CD8 tail DNAs as templates, respectively. The two DNA fragments were then annealed to act as a template to generate the entire Tva 1–83/CD8 chimera by OS6 and OS26.

(ii) Tva 1–56/CD8. The coding region of Tva from residue 1 to residue 56 was amplified by OS26 and OS46 and the mouse CD8 tail region was amplified by OS6 and OS47 with Tva_{800} and mouse CD8 tail DNAs as templates, respectively. The Tva 1–56/CD8 chimera was generated by OS6 and OS26 with two annealed DNA fragments as template.

(iii) Tva 1–56 $S_{3,7,9}$A/CD8. To generate Tva 1–56 $S_{3,7,9}$A/CD8, first, three codons for serine were mutated to that of alanine at amino acid positions 3, 7, and 9 on the Tva_{950} backbone DNA with OS15, OS39, OS24, and OS40. The mutant Tva_{950} DNA was then used to generate Tva 1–56 $S_{3,7,9}$A by OS26 and OS46. CD8 tail DNA (amplified by OS6 and OS45) was annealed with Tva 1–56 $S_{3,7,9}$A/CD8 DNA to generate a Tva 1–56 $S_{3,7,9}$A/CD8 chimera by OS6 and OS26.

(iv) Tva 10–56/CD8. Tva 10–56 and mouse CD8 tail regions were amplified by OS26 and OS38 and OS6 and OS37, respectively, with Tva 1–56/CD8 as a template. The two DNA fragments were then annealed as a template to amplify the entire Tva 10–56/CD8 chimera by OS6 and OS26.

(v) Tva 1–83ΔLDLR/CD8. Tva 1–83/CD8 DNA was used as a template to amplify Tva 1–83ΔLDLR and CD8 tail regions by OS26 and OS72 and OS6 and OS71, respectively. Tva 1–83ΔLDLR/CD8 was then generated by OS6 and OS26 with the two annealed DNA fragments as a template.

(vi) Tva 10–56/CD8Δhinge. Tva 10–56 and CD8Δhinge were generated by OS26 and OS74 and OS6 and OS73, respectively, with Tva 10–56/CD8 as a template. The Tva 10–56/CD8Δhinge DNA was amplified by OS6 and OS26 with the two annealed DNA fragments as template. In this construct, the cysteine residue of the CD8 tail proximal to the CD8 transmembrane region was mutated to a serine.

Each of these Tva/CD8 chimeric DNAs was digested with the restriction endonucleases *Kpn*I and *Bgl*II and cloned into the *Bam*HI- and *Kpn*I-cut expression vector pCB6. DNA from each Tva/CD8 construct was sequenced to confirm that no additional mutation was introduced by PCR.

NIH 3T3 cells were transfected with plasmid DNA by calcium phosphate precipitation, and clones resistant to G418 were selected in medium containing 300 μg of G418 per ml. Clones expressing high levels of Tva/CD8 chimeric proteins were identified by Western blotting (immunoblotting) with anti-Tva antibody (described below), and the clones were propagated.

Cell lysate preparation and Western blotting. NIH 3T3 cells expressing Tva/CD8 chimeric proteins were seeded on 100-mm-diameter plastic plates. The cells were induced with 10 mM sodium butyrate when they were 70% confluent. After overnight induction, the cell monolayer was washed twice with 1× phosphate-buffered saline (PBS), and then the cells were lysed with Triton lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris [pH 8.0], 5 mM EDTA) containing protease inhibitors (2 mM phenylmethylsulfonyl fluoride 10 μg of pepstatin A per ml, 10 μg of leupeptin per ml, 10 μg of aprotinin per ml) on ice for 30 min. The cell debris was pelleted, and the supernatant was saved. A portion (10 to 50 μl) of the supernatant was mixed with sodium dodecyl sulfate (SDS) sample buffer and boiled for 3 min before being subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE [12.5% polyacrylamide]) and Western transfer to polyvinylidene difluoride in the absence of SDS. After being blocked with 1% bovine serum albumin (BSA) in TTBS (20 mM Tris [pH 7.5], 500 mM NaCl, 0.1% Tween 20) for 30 min at room temperature, the blots were sequentially incubated with polyclonal anti-glutathione *S*-transferase–Tva antiserum (2) at a 1:2,000 dilution for 1 h and then with peroxidase-conjugated goat anti-rabbit antibody at a 1:20,000 dilution for 20 min, followed by ECL chemiluminescence detection (Amersham, Arlington Heights, Ill.).

Biotinylation of surface Tva/CD8 chimeric proteins. Biotinylation of surface Tva/CD8 chimeric proteins was performed according to a previously published protocol (9) with minor modification. Briefly, NIH 3T3 cells expressing Tva/CD8 chimeric proteins were induced with 10 mM sodium butyrate when they were 70% confluent on 100-mm-diameter plates. After overnight induction, the monolayer was washed twice with 1× PBS, and the cells were then released from the dish with 10 ml of PBS containing 0.5 mM EDTA and EGTA. Three milliliters of biotin buffer containing NHS-LC-biotin (1 mg/ml [Pierce]) was added to each sample, and biotinylation proceeded for 45 min on ice. The reaction was quenched with BSA (final concentration, 0.5 mg/ml) in the presence of 100 mM glycine. The cell pellets were washed twice with biotin buffer containing 20 mM glycine. Cells were lysed with Triton lysis buffer containing protease inhibitors as described above.

Biotinylated proteins were precipitated with the following protocol. An aliquot of each cell lysate was incubated with 30 μl of streptavidin-agarose (Sigma, St. Louis, Mo.) on ice for 30 min, and the pellet was washed four times with Triton lysis buffer. The pellet was then resuspended in Triton lysis buffer with protein loading buffer. The samples were heated for 10 min at 95°C before SDS-PAGE. Western blotting was performed as described above.

Binding of Tva/CD8 chimeric proteins to subgroup A envelope protein. NIH 3T3 cells expressing ASLV-A or subgroup C envelope protein were induced with 10 mM sodium butyrate, biotinylated, and lysed according to the protocols described above. Each lysate was incubated with cell lysates of NIH 3T3 cells expressing Tva/CD8 chimeric proteins for 30 min on ice with constant shaking. Thirty microliters of streptavidin-agarose was then added to each sample, and each mixture was incubated for 30 min on ice with shaking. The streptavidin-agarose beads were pelleted by microcentrifugation and washed four times with Triton lysis buffer. Each pellet was resuspended in Triton lysis buffer and SDS sample buffer and analyzed by SDS-PAGE and Western blotting with anti-Tva antisera.

Infection assay by in situ AP activity. The viral stock of RCAS(A)HAP was produced by transfection of RCAS(A)HAP vector DNA into line 0 chicken CEFs as has been described previously for other RCAS vectors (26). This virus expresses the subgroup A envelope and carries an alkaline phosphatase (AP) marker gene which allows histochemical staining of infected cells. The titer of virus on line 0 C/O CEFs was determined by in situ AP staining of cells 24 h postinfection as follows. The cell monolayer was washed twice with 1× PBS, fixed with 4% paraformaldehyde (3 min at room temperature). Cells were washed once with PBS and then stained for AP activity with a solution containing 50 mM Tris (pH 9.0), 1 mg of fast red TR salt per ml (Sigma), 0.5 mg of naphthol AS-BI phosphate per ml (Sigma), and 1% *N*,*N*-dimethylformamide for 30 min at 37°C. After staining, the monolayer was washed twice with PBS and fixed again in 4% paraformaldehyde. AP-positive cells were scored by microscopic examination.

Assays to determine the function of the receptor in mammalian cells were done as follows. NIH 3T3 cells expressing Tva/CD8 chimeric proteins were seeded on six-well dishes. Approximately 10^3 infectious units of RCAS(A)HAP viral stock was added to the monolayer cells when cell density reached 20 to 30% confluence. Forty-eight hours after infection, the cell monolayer was stained for AP activity as described above. For receptor analysis in transiently expressing cells, 293T cells were seeded at 1.6×10^6 cells per 60-mm-diameter dish. The next day, 3 μg of pCB6 plasmid DNA carrying either Tva_{950} or the Tva/CD8 chimeras was mixed with 15 μl of Lipofectamine (Gibco/BRL) and transfected according to the manufacturer's instructions. Twenty-four hours posttransfection, the plates were divided into two six-well dishes. One plate was used for infection with RCAS(A)HAP as described above, and the other plate was used for analysis of Tva protein expression.

RESULTS

Construction of Tva/CD8 chimeras. Previously, two forms of Tva derived by differential splicing (Tva_{950} and Tva_{800}) were shown to function as the ASLV-A receptor (2). Tva_{950} has a typical transmembrane domain, while Tva_{800} associates with membranes via a glycosyl phosphatidylinositol linkage (22). Both forms contain identical extracellular regions extending through residue 73, where Tva_{800} attaches to a glycosyl phosphatidylinositol anchor. Tva_{950} contains an additional 10 residues before the start of its transmembrane domain. This suggests that 73 residues of Tva are sufficient for receptor function. The amino acid sequence of the Tva ectodomain of Tva_{950} is shown in Fig. 1A. Contained within this domain is a region highly homologous to the LDLR (Tva residues 11 to 50), three potential sites of N-linked glycosylation, numerous sites for potential O-linked modification (amino acids 52 to 70), and sites for potential glucosaminoglycan addition. Both Tva_{950} and Tva_{800} are extensively modified to a heterogeneous array of products, containing N- and O-linked sugars, ranging in size from approximately 29 to 45 kDa (22).

To delineate which of the sequences and/or modifications of Tva are required for receptor function, we constructed a series of chimeras in which various portions of the quail Tva ectodomain were appended to a heterologous membrane anchor. The entire 83-amino-acid extracellular domain of Tva_{950} was fused to 91 residues of a mouse CD8 membrane anchor, including the extracellular hinge, transmembrane domain, and cytoplasmic tail, generating clone 1–83 (Fig. 1B). A second chimera was designed to focus on the LDLR repeat motif region and contains only the 56 amino-terminal residues of Tva, 1–56. Additional constructs contain mutations and deletions of the sequences in Tva which are sites of potential modification. The 1–56 $S_{3,7,9}$A construct includes residues 1 to 56 of Tva but contains mutations in three serine residues which abolish po-

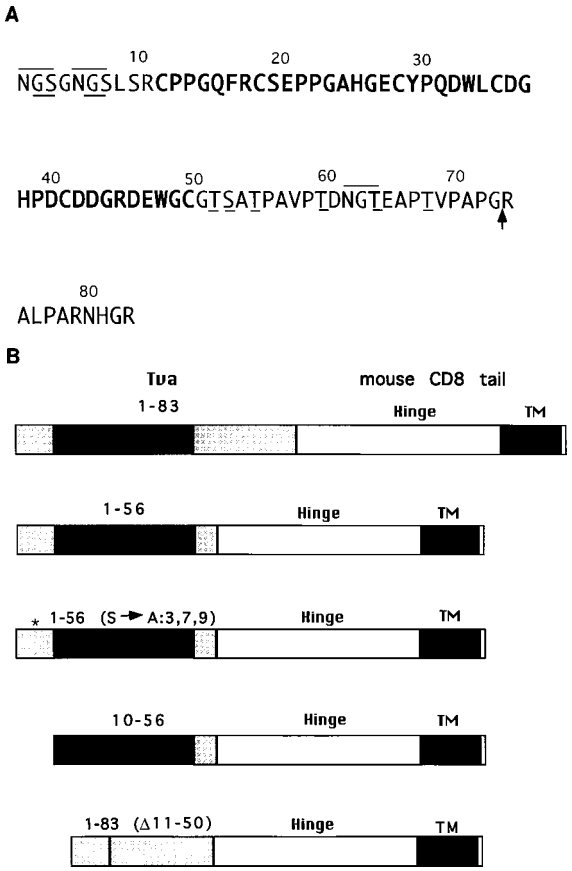

FIG. 1. (A) Amino acid sequence of the ectodomain of quail Tva (residues 1 to 83). Residues 11 to 50 (boldface) are the LDLR homology motif. Sites of potential carbohydrate modification are highlighted: potential N-linked sites are overlined, while O-linked and proteoglycan addition sites are underlined. The arrow indicates the predicted glycosyl phosphatidylinositol linkage site in Tva_{800}. (B) Quail Tva-mouse CD8 tail chimeric constructs (Tva/CD8). Various regions of the Tva ectodomain (as indicated by the numbers) were fused to a murine CD8 membrane anchor, which includes an extracellular hinge (63 amino acids) and a transmembrane domain, and a short cytoplasmic tail, by a PCR-based technique as described in Materials and Methods. Amino acids 1 to 10 and 51 to 83 of Tva are shaded gray, while the LDLR homology motif (residues 11 to 50) is in black. The mouse CD8 transmembrane (TM) domain is black, and the CD8 hinge region is white. * 1–56 (S→A:3,7,9), Tva 1–56 $S_{3,7,9}A$; 1-83 (Δ11-50), ΔLDLR.

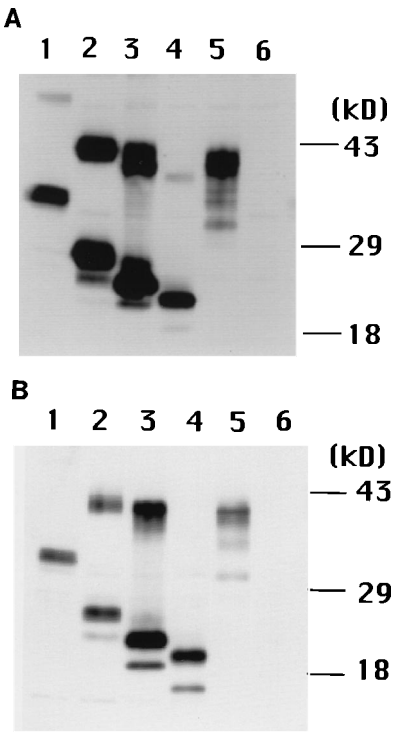

FIG. 2. Chimeric Tva/CD8 protein expression in NIH 3T3 cells. (A) Total cell lysates were prepared from NIH 3T3 cells stably expressing Tva/CD8 chimeric proteins. Equal amounts of protein from each cell lysate were used for Western blotting after SDS-PAGE, with a polyclonal anti-Tva serum as the primary antibody. Lanes: 1, Tva 1–83/CD8; 2, Tva 1–56/CD8; 3, Tva 1–56 $S_{3,7,9}A$/CD8; 4, Tva 10–56/CD8; 5, wild-type Tva (quail processed receptor gene [pg 950]) as a positive control; 6, pCB6 alone (the expression vector used in this study) as a negative control. Migration of molecular mass markers is shown on the right in kilodaltons. (B) Surface expression of the Tva/CD8 chimeric proteins in NIH 3T3 cells. NIH 3T3 cells stably expressing Tva/CD8 chimeric proteins were labeled with the membrane-impermeant reagent NHS-LC-biotin, and the biotinylated surface proteins were precipitated by streptavidin-agarose and analyzed by blotting as described in Materials and Methods. Lanes: 1, Tva 1–83/CD8; 2, Tva 1–56/CD8; 3, Tva 1–56 $S_{3,7,9}A$/CD8; 4, Tva 10–56/CD8; 5, wild-type Tva (pg 950); lane 6, pCB6 alone.

tential N-linked glycosylation and proteoglycan addition sites upstream of the LDLR repeat motif. Construct 10–56, containing only 47 amino acids of Tva, has had the entire region amino terminal to the LDLR repeat motif deleted. To analyze whether sequences outside the LDLR repeat motif region contributed to receptor function, amino acids 11 to 50, encompassing the entire LDLR repeat motif region, were deleted (ΔLDLR). Each of these constructs was generated by overlap extension PCR and cloned into the expression vector pCB6.

Expression of chimeric proteins in NIH 3T3 cells. Cell lines expressing the Tva/CD8 chimeric proteins depicted in Fig. 1B were generated by transfecting NIH 3T3 cells with plasmid pCB6 carrying the various chimeric constructs. Individual G418-resistant cell clones were isolated, and expression of the constructs was determined by Western blot analysis with an anti-Tva antibody. All of the chimeric proteins which contained the LDLR motif were efficiently expressed in NIH 3T3 cells compared with the wild-type transmembrane form of Tva, Tva_{950} (Fig. 2A). In contrast, no stable lines expressing the ΔLDLR protein could be obtained. In addition, production of the ΔLDLR chimeric protein was not detected after transient transfection of human 293T cells, suggesting that this protein was unstable, which precluded further analysis of this chimera. Four NIH 3T3 cell lines expressing the 1–83, 1–56, 1–56 $S_{3,7,9}A$, and 10–56 chimeras, with expression levels comparable to the Tva_{950} line, were analyzed further.

Wild-type Tva_{950} migrates on SDS-PAGE as a heterogeneous collection of bands ranging in size from 29 to 43 kDa (Fig. 2A, lane 5) because of extensive posttranslational modification (22). Similarly, each Tva/CD8 chimera migrated as multiple species, generally two or three major forms, presumably reflecting different levels of modification (Fig. 2A, lanes 1 to 4). The general migration patterns of the chimeric proteins were consistent with the predicted relative size of each chimera, that is (from slow to fast) 1–83<1–56<1–56 $S_{3,7,9}A$<10–56. Also, there appeared to be some variation in the efficiency of processing. For example, the cell lines expressing the 1–83 and the 10–56 proteins (Fig. 2A, lanes 1 and 4) contained much less of the slower migrating species of the Tva/CD8 protein than cells expressing either wild-type Tva or the 1–56 and 1–56 $S_{3,7,9}A$ chimeras. The diminished modification of the 1–83 chimera is surprising because it contains all of the modification

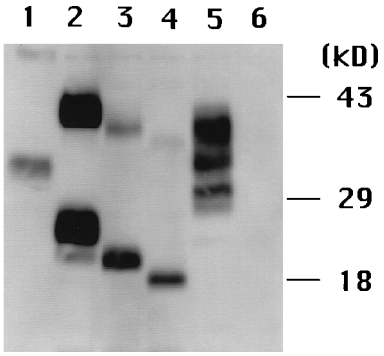

FIG. 3. Binding of Tva/CD8 chimeric proteins to ASLV-A envelope protein. NIH 3T3 cells expressing subgroup A envelope protein were surface labeled with NHS-LC-biotin. The cell lysate was incubated with each cell lysate prepared from NIH 3T3 cells expressing Tva/CD8 chimeric proteins, and the biotinylated proteins were precipitated by streptavidin-agarose. Tva/CD8 chimeric proteins were detected by Western blotting with polyclonal anti-Tva serum as the primary antibody. Lanes: 1, Tva 1–83/CD8; 2, Tva 1–56/CD8; 3, Tva 1–56 $S_{3,7,9}$A/CD8; 4, Tva 10–56/CD8; 5, wild-type Tva (quail processed receptor gene [pg 950]); 6, pCB6 alone. Migration of molecular mass markers is indicated on the right.

sites of Tva plus the additional N and O glycosylation sites contained in the CD8 hinge region. Lower levels of the slowly migrating forms in cell lysates suggest that the 1–83 and 10–56 proteins are modified less efficiently and thus may be processed differently from the other chimeras. However, the nature of this difference has not been determined.

The ability of the chimeric Tva proteins to be presented on the surface of mouse cells was determined by biotinylation of surface proteins by using a membrane-impermeable compound. After biotinylation, surface proteins were collected on streptavidin-agarose beads and analyzed by Western blotting with anti-Tva antibody. Each of the chimeric proteins was expressed on the cell surface at levels roughly equivalent to Tva_{950} (Fig. 2B). Furthermore, all forms of the chimeric proteins detected by Western blotting appeared to be expressed on the surface (compare Fig. 2A and B).

Envelope binding by the chimeric receptor proteins. We next sought to determine if the regions of the receptor contained in each of the chimeric proteins were sufficient to bind the viral envelope glycoprotein. To assay binding, lysate from biotin-labeled cells expressing subgroup A envelope (EnvA) was mixed with lysates from cells expressing the various Tva/CD8 chimeric proteins. After incubation, the envelope proteins were collected on streptavidin-agarose beads. Envelope-bound Tva proteins were detected after SDS-PAGE by Western blotting with anti-Tva antibody. All of the Tva/CD8 chimeras bound to subgroup A envelope protein (Fig. 3). Also, the modified forms of the chimeric proteins and of wild-type Tva were capable of binding to EnvA (compare Fig. 2A with Fig. 3). Even the protein comprising just 47 residues of Tva, 10–56, bound efficiently to EnvA (Fig. 3, lane 4). The binding specificity of the chimeric proteins for EnvA was confirmed in control experiments with the subgroup C envelope protein. Previous experiments demonstrated that Tva_{950} does not bind to EnvC (9). Like Tva_{950}, none of the Tva/CD8 chimeras bound to EnvC (data not shown). Thus, chimeric proteins containing the cysteine-rich motif of Tva retain both the binding and specificity properties of wild-type Tva.

Infection of 3T3 cells expressing chimeric Tva proteins by ASLV vectors. Mammalian cells which are resistant to infection by ASLV-A viruses because they lack a functional receptor are rendered susceptible by introduction of *tva* (2, 26). The ability of each of the chimeric receptor proteins to mediate subgroup A virus entry was assayed by infecting the stable NIH 3T3 cells expressing Tva/CD8 proteins with the virus RCAS(A) HAP. This virus expresses the subgroup A envelope and carries an AP marker gene which allows histochemical staining of infected cells. After infection at low multiplicity with RCAS(A) HAP virus, numerous foci of infected cells were readily detected by AP staining (Fig. 4). NIH 3T3 cell lines expressing any of the four CD8/Tva chimeric proteins were infected as efficiently as NIH 3T3 cells expressing wild-type Tva_{950}. Thus, these experiments demonstrate that the minimal region of Tva required for efficient viral receptor function is a 47-residue region containing the LDLR repeat motif.

Specific modification of Tva is not essential for receptor function. Tva is normally highly modified by N and O glycosylation (22). The hinge region of mouse CD8 tail also contains numerous carbohydrate addition sites which are modified in CD8 and appear to be utilized in the chimeric proteins (Fig. 2, lanes 3 and 4). Thus, we were concerned about the possibility that these modifications may be contributing to the receptor function seen above. To confirm that the LDLR motif in Tva was indeed sufficient to mediate entry of ASLV-A viruses, we constructed a chimera in which residues 10 to 56 of Tva had been appended to a minimal extracellular domain and membrane anchor. This construct, 10–56Δhinge, removes the hinge region of CD8 and fuses residues 10 to 56 of Tva within 6 amino acids of the membrane-spanning domain of CD8, producing a mature protein with a length of only 81 amino acids (Fig. 5A). 10–56Δhinge removes all of the modification sites found in the ectodomain of CD8. The only potential modification sites are a single threonine and two serine residues in Tva and a serine replacing a cysteine in the CD8 ectodomain.

A stable cell line expressing the 10–56Δhinge protein was isolated and analyzed. Unlike Tva or the other Tva/CD8 proteins, which upon gel analysis appear as multiple heterogeneous bands, the 10–56Δhinge protein migrated as a single, discrete species approximately 14 kDa in size upon Western blotting of SDS gels (Fig. 5B, lane 1). This suggests that the 10–56Δhinge protein is not significantly modified. However, the apparent molecular mass of 14 kDa seen for 10–56Δhinge is larger than the 8.9 kDa predicted for a protein with a length of 81 amino acids. This discrepancy in size does not necessarily imply that the 10–56Δhinge protein is modified, because all of the proteins analyzed to date which contain the LDLR repeat motif of Tva migrate with a significantly larger than predicted apparent molecular mass in SDS gels, including Tva synthesized in vitro with rabbit reticulocyte lysates (22a). The level of expression of 10–56Δhinge appears to be much lower than that of any of the other Tva chimeric proteins (Fig. 5, compare lanes 1 and 2). Lower levels of 10–56Δhinge were consistently found, both in stable lines and in transiently transfected cells (data not shown). This may reflect the fact that folding of the protein or stability on the cell surface requires some glycosylation, without which the protein is turned over rapidly.

The ability of 10–56Δhinge protein to function as a subgroup A receptor was assayed by infection with RCAS(A)HAP. Foci of cells expressing the AP marker were readily found after infection of 10–56Δhinge cell lines (Fig. 5C). However, the cell line expressing 10–56Δhinge was significantly less susceptible to infection by ASLV-A vectors than cells expressing the other Tva chimeras or wild-type Tva. Similar results showing low levels of expression and infectivity with 10–56Δhinge were obtained when 293T cells transfected with pCB6 vectors expressing the various Tva/CD8 constructs were used in transient expression experiments. Briefly, 32 h posttransfection, the cells were infected with 10^3 infectious units of RCAS(A)HAP. After 48 h of infection, the cells were stained for AP activity. An

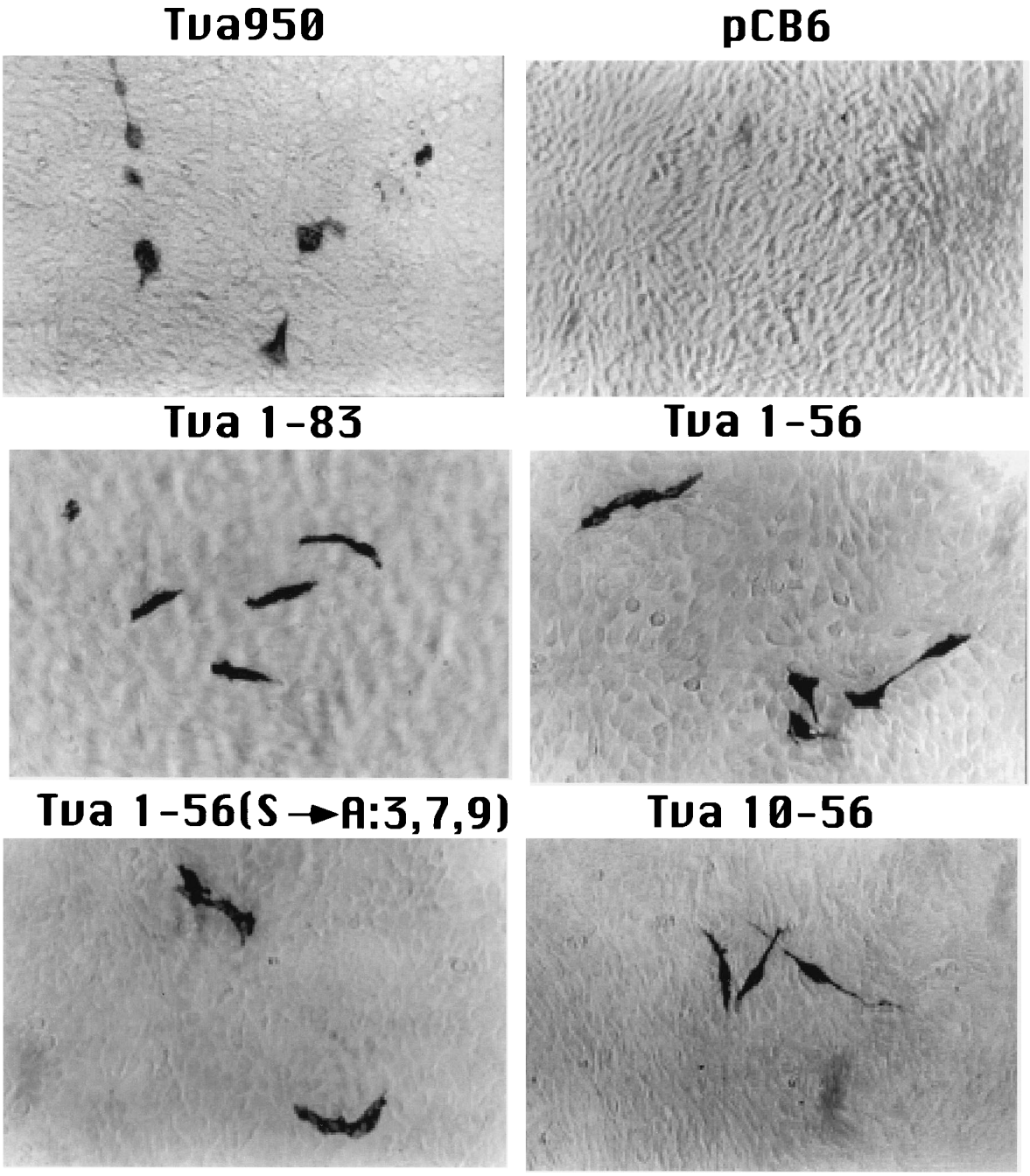

FIG. 4. Infection of NIH 3T3 cells stably expressing Tva/CD8 chimeric proteins by RCAS(A)HAP viruses. NIH 3T3 cells stably expressing Tva/CD8 chimeric proteins were infected with RCAS(A)HAP viruses for 48 h before in situ alkaline phosphatase activity was assayed. The darkly stained cells are AP positive. Magnification, ×200. Tva 1–56(S→A:3,7,9), Tva 1–56 $S_{3,7,9}A$.

average of 10 fields of infected cells was then counted (magnification, ×200) and compared to give a quantitative analysis of receptor function. The numbers of AP-positive cells per field were 20 for Tva_{950}, 31 for 1–56, 57 for 10–56, and 3 for 1–56Δhinge. Note that the cells expressing 10–56Δhinge were approximately 6- to 20-fold less susceptible to RCAS(A)HAP vectors than cells expressing the other chimeras or wild-type Tva. Perhaps the reduced viral receptor function is caused by the lower level of 10–56Δhinge protein on the target cells; however, avian or murine cells expressing undetectable levels of wild-type Tva are efficient targets for ASLV-A, suggesting that this is not the case. Alternatively, the Tva sequences in 10–56Δhinge are within six residues of the transmembrane domain and may be less accessible to the virus because of their proximity to the cell membrane. In spite of the diminished receptor function of this chimera, these experiments demonstrate that an 81-residue-long protein containing the LDLR repeat motif of Tva (residues 10 to 56) and a minimal heterologous transmembrane anchor can mediate ASLV-A infection. Therefore, the extensive modification normally seen for

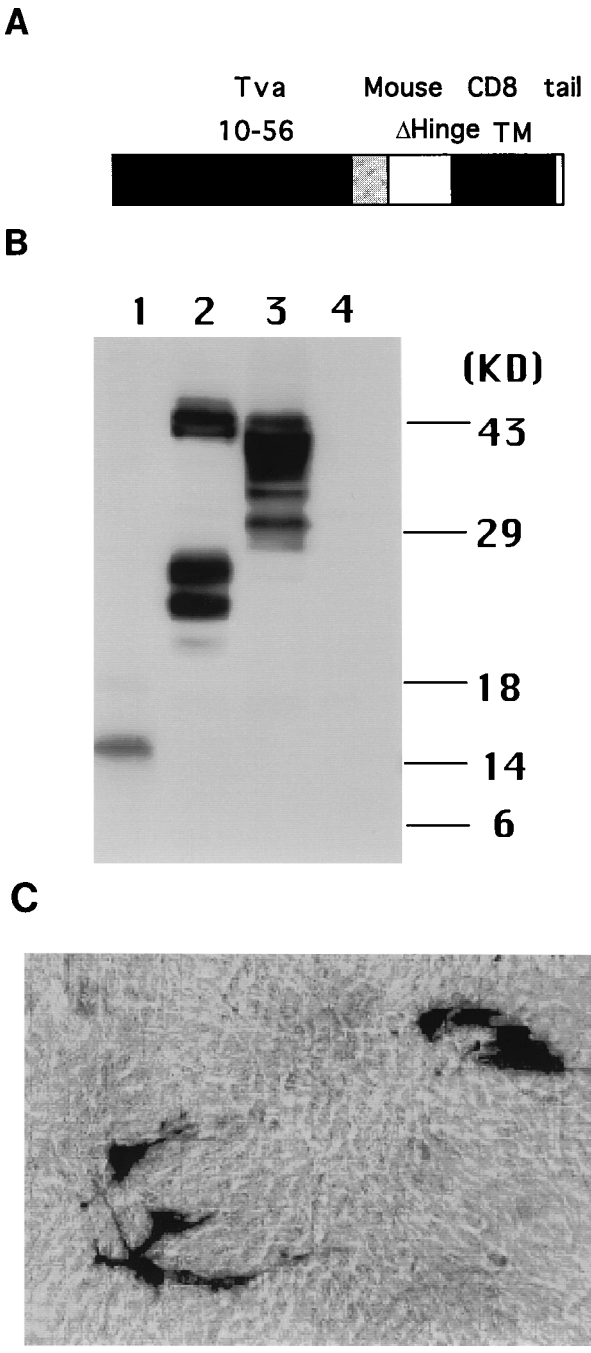

FIG. 5. (A) Construction of 10–56Δhinge. The entire hinge region of mouse CD8 tail, except six amino acids proximal to the transmembrane (TM) domain was deleted from Tva 10–56/CD8 tail (compare with Fig. 1B). (B) Tva 10–56Δhinge chimeric protein expression in NIH 3T3 cells. Total cell lysate from NIH 3T3 cells stably expressing Tva 10–56Δhinge was prepared, and Western blotting was performed as described in Fig. 2. Lanes: 1, 10–56Δhinge; 2, 1–56/CD8; 3, wild-type Tva (pg 950); 4, pCB6 alone. (C) Infection of NIH 3T3 cells stably expressing 10–56Δhinge chimeric protein by RCAS(A)HAP virus as described in the legend to Figure 4. Magnification, ×156.

Tva is not essential for virus receptor function, and the LDLR motif is sufficient for ASLV-A receptor function.

Comparison of chicken and quail LDLR motifs. Chimeras expressing residues 1 to 56 of the chicken Tva protein fused to CD8 (analogous to the quail 1–56 construct in Fig. 1) were expressed in NIH 3T3 cells, and stable lines were selected. Susceptibility of the cell lines expressing chick Tva 1–56/CD8 proteins to ASLV-A was tested by infection with RCAS(A) HAP. The NIH 3T3 cells expressing the chick Tva/CD8 chimeric protein were efficiently infected by RCAS(A)HAP, as demonstrated by AP staining (data not shown). Thus, the LDLR motif of chicken Tva is also sufficient to mediate entry of ASLV-A. Comparison of the sequences of the chick and quail Tva proteins in the LDLR repeat motif region required for receptor function (residues 11 to 56 of quail Tva) reveals that the protein sequence is extremely conserved from residue 28 through residue 51 (Fig. 6). Additional conserved residues are found at the amino terminus of the LDLR-like motif (amino acids 10 to 20). The 24 residues in the most conserved region represent roughly the carboxyl-terminal half of the LDLR repeat motif in Tva (from cysteine 4 to cysteine 6 in the repeat). Interestingly, the carboxy-terminal portion of the repeats in LDLR appears to be important in apolipoprotein binding (7, 18).

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Quail   RCPPGQFRCSEPPGAHGECYPQDWLCDGHPDCDDGRDEWGCGTSATP
Chicken QCSPGQFHCSEPRDPQTDCYPLEWLCDGHPDCDDGRDEWGCGRGGSP
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FIG. 6. Amino acid alignment of residues 10 to 56 of quail and chicken Tva. The region between cysteines 10 and 50 comprises the LDLR-like motif. Identical residues are in boldface.

DISCUSSION

In this study, we have used a series of chimeric proteins to map the region of the ASLV-A receptor sufficient to mediate viral entry. We demonstrate that a 40-residue-long LDLR repeat motif, which is localized near the amino terminus of wild-type Tva, is sufficient to mediate both specific binding of envelope protein and entry by ASLV-A. After transfection with any of the chimeras containing the Tva LDLR motif, mammalian cells become susceptible to infection with ASLV-A vectors. Although wild-type Tva is heavily modified by N and O glycosylation, our results indicate that there is no specific requirement for modification, since constructs in which the Tva carbohydrate addition sites are removed (10–56 and 10–56Δhinge) are still functional.

Cysteine-rich motifs homologous to LDLR repeats are found in a number of extracellular proteins and are repeated from 1 (Tva, C6, C7, C8, and C9 terminal complement complex proteins) to 37 times (LDLR-related protein) in these proteins. In LDLR, the seven cysteine-rich repeats form the ligand binding domain (10, 25). Each of the repeats is thought to fold independently, and different repeats or combinations of repeats interact with various ligands to determine receptor specificity. Supporting this hypothesis, mutations within the seven LDLR repeats or deletions of entire repeat units differentially affect the binding of LDLR to apolipoprotein B and apolipoprotein E (7, 12, 18). The cysteine-rich repeats of other members of the LDLR family are also thought to be important for extracellular protein-protein interactions (13, 15, 19, 21, 23). Our results demonstrate that the 40-residue, cysteine-rich motif of Tva binds to ASLV-A envelope protein. This is, to our knowledge, the first demonstration that a single, isolated LDLR repeat motif is sufficient to mediate protein-protein interactions.

It is interesting that the other members of LDLR family appear to be involved in viral replication. LDLR and LDLR-related protein have been identified as receptors for the minor-group human rhinoviruses, HRV2 (14). Also, it was recently demonstrated that a soluble form of LDLR containing only the

cysteine-rich repeat region of LDLR can inhibit vesicular stomatitis virus infection (8). Together with the data presented here, these observations suggest the cysteine-rich motif of LDLR family members may be utilized in other virus-host interactions.

Conservation of the LDLR repeat motif sequences in the Tva proteins from chicken and quail cells in the region encompassing cysteines 4 through 6 suggests that this region may contain the amino acid determinants for envelope binding (Fig. 6). Supporting this hypothesis, Tva mutants containing deletions which remove the amino-terminal portion of the LDLR-like motif (residues 10 to 27 of Tva) retain receptor function (3, 20). Analysis of mutations in the cysteine-rich repeats in LDLR suggests that sequences in the carboxy-terminal half of the repeats of LDLR are involved in ligand binding. In addition, the numerous acidic residues in this region of LDLR appear to be critical for binding (7, 18). Thus, similar regions of the motifs of Tva and LDLR appear to be important for ligand binding. The overlap in functional requirements between Tva and LDLR may reflect some conserved features of the binding interactions of LDLR-apolipoprotein and Tva-EnvA or may be determined by the structure of the LDLR repeat motif.

We have previously suggested a functional analogy between apolipoproteins and EnvA in binding to their receptors via an LDLR cysteine-rich repeat motif (2). The data presented in this study, demonstrating that the Tva LDLR repeat motif binds to EnvA, strengthen this argument. There is no primary sequence homology between EnvA and any of the known ligands for the LDLR family. However, within the region of the ASLV envelope which determines receptor specificity of the virus, EnvA contains clusters of basic residues which are absent in the other ASLV subgroups (4, 6). Clustered, positively charged residues are strongly implicated as the receptor binding sites in apolipoprotein B and apolipoprotein E (16, 17, 24). Whether the basic residues in EnvA are required for Tva binding can now be directly addressed. In addition, the small size of the LDLR repeat motif in Tva makes the ASLV system ideal for analysis of the structural and functional determinants of a virus receptor.

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