

A Soluble Form of a Receptor for Subgroup A Avian Leukosis and Sarcoma Viruses (ALSV-A) Blocks Infection and Binds Directly to ALSV-A

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A receptor that confers susceptibility to infection by subgroup A avian leukosis and sarcoma viruses (ALSV-A) has been described (P. Bates, J. A. T. Young, and H. E. Varmus, Cell 74:1043–1051, 1993). A soluble form of the receptor was generated to determine whether this protein interacts directly with virus particles in the absence of other cell surface factors. The soluble protein comprised the extracellular region of the ALSV-A receptor fused to an antibody epitope tag and six histidine residues. Preincubating this protein with virus led to an efficient block to infection of avian cells by ALSV-A but had no effect on infection by ALSV-B, ALSV-C, or ALSV-D. Furthermore, an antibody directed against the introduced epitope tag immunoprecipitated ALSV-A particles bound to the soluble receptor. In contrast, other ALSV subgroups were not immunoprecipitated by this procedure. These data demonstrate that the cloned receptor interacts directly with ALSV-A and discriminates between different ALSV subgroups at the level of virus binding.

Avian leukosis and sarcoma virus (ALSV)-receptor interactions are a useful model system for understanding the early events in the retrovirus replication cycle. Like most retroviruses, ALSV infects cells by a pH-independent mechanism which is consistent with direct fusion between viral and host cell surface membranes (11, 17). Also, there is a family of related subgroups of ALSVs that interact with distinct cellular receptors (28). Characterizing the mechanism of entry used by several members of this family should provide insight into the common principles of retrovirus entry.

The ALSV family consists of five major viral subgroups designated A (ALSV-A) through E (ALSV-E). Viruses within each subgroup have the same host range, encode immunologically related envelope (Env) glycoproteins, and demonstrate cross-interference of cellular receptors (9, 13, 15, 19, 24–26). The subgroup specificity of these viruses operates at the level of virus entry and is determined by several noncontiguous regions of the viral surface Env protein (4, 7). Genetic studies with chickens have led to the identification of three ALSV susceptibility loci, *tv-a*, *tv-b*, and *tv-c* (6, 19–21, 23). The *tv-a* and *tv-c* loci are believed to encode receptors for viral subgroups A and C, respectively. The receptors for viral subgroups B, D, and E are predicted to be encoded by different alleles of the *tv-b* locus (28). In addition, there are recessive resistant alleles at each of the susceptibility loci, and these alleles might encode products that are defective for virus entry (28).

Chicken and quail genomic clones that render cells susceptible to infection by ALSV-A have been identified (3, 32). These clones encode cell surface proteins related to the low-density lipoprotein receptor family (3). Expression of these proteins in transfected mouse 3T3 cells allows infection by ALSV-A but not viruses of other ALSV subgroups (3, 32). In addition, an antiserum raised against these proteins blocks

subgroup A virus infection of primary chicken embryo fibroblasts (CEFs) but has no effect on infection of these cells by other ALSV subgroups (3). These data suggest that the cloned gene is probably *tv-a* and that its products are most likely cellular receptors specific for ALSV-A. Similar criteria have been used to define other retrovirus receptors, including CD4 for human immunodeficiency virus (HIV) and simian immunodeficiency virus (SIV) (5). In most of these other systems, it has been shown that the receptors bind specifically to their cognate virus or viral envelope glycoproteins (1, 2, 5, 27).

A soluble ALSV-A receptor was constructed to test whether this protein binds directly to subgroup A virus particles in the absence of other cell surface components. It was necessary to use this approach, rather than test virus binding to cells directly, because of the high background level of ALSV-A binding to cells that do not produce the receptor and are resistant to viral entry (31). The soluble protein comprised the extracellular region of the chicken ALSV-A receptor fused to a 9-amino-acid antibody epitope tag derived from influenza virus hemagglutinin (HA), followed by six histidine residues (Fig. 1A). These two added features allow immunoprecipitation of the protein by the 12CA5 monoclonal antibody (10) and protein purification on a metal-affinity column (16), respectively. Because the chicken genomic clone encoding the receptor (32) has not yet been fully sequenced, the signal peptide and first six amino acids of the mature soluble protein were derived from the quail receptor sequence (Fig. 1A). Transfected monkey COS-7 cells (12) expressing a membrane-bound version of this chimeric quail-chicken protein were susceptible to ALSV-A infection (33).

In order to generate enough soluble protein to study virus-receptor interactions, a pool of stable QT6 cell (18) transfectants expressing this protein was generated. For control purposes, a separate pool of QT6 cells was transfected with a plasmid that encodes only the signal sequence and the first seven amino acids of the soluble receptor protein. Western blot (immunoblot) analysis of culture supernatants from soluble receptor-expressing cells revealed an approximately 30-

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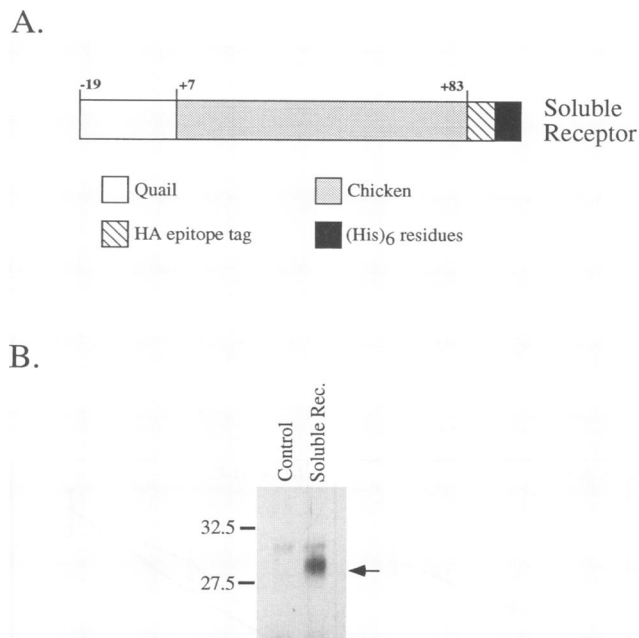


FIG. 1. Soluble form of an ALSV-A receptor. (A) A soluble receptor gene was constructed that encodes the extracellular region of an ALSV-A receptor fused in frame with a 9-amino-acid antibody epitope tag (YPYDVPDYA) from the influenza virus HA protein (10), followed by six histidine residues and a stop codon. This gene was placed under the control of the cytomegalovirus early region promoter in the expression plasmid pCB6 (kindly provided by M. Stinski), generating plasmid pLC126. A control plasmid, pKZ170dIII, was also created by deleting most of the receptor sequences from plasmid pLC126 (described in the text). (B) Approximately 2×10^6 quail QT6 cells were transfected by calcium phosphate precipitation (30) with 15 μ g of plasmid pLC126 or pKZ170dIII. Forty-eight hours after transfection, pools of stable transfectants were selected in medium containing 400 μ g of G418 per ml. Culture supernatants incubated with confluent monolayers of transfected cells for a 60-h period were precleared at 1,200 rpm in a Sorvall RT-6000B tabletop centrifuge and stored at -70°C . Soluble glycoproteins were isolated from 1-ml aliquots of these supernatants following a 3-h incubation at 4°C with 40 μ l of wheat germ agglutinin Sepharose 6MB beads (Sigma), 100 μM MnCl_2 , and 100 μM CaCl_2 . The Sepharose beads were washed three times with 1 ml of Nonidet P-40 lysis buffer (14) containing 100 μM MnCl_2 and 100 μM CaCl_2 . The purified glycoproteins were electrophoresed under reducing conditions on a sodium dodecyl sulfate-12.5% polyacrylamide gel, transferred to a nitrocellulose membrane, and probed at room temperature with antibodies diluted 1:2,500 in Tris-buffered saline (14) containing 0.2% Tween 20 and 1% bovine serum albumin. The antibodies used were a rabbit polyclonal antiserum raised against a receptor-specific peptide sequence (CHPDC-DDGRDEWG; 3) and a donkey anti-rabbit horseradish peroxidase-conjugated antiserum (Amersham). Proteins were detected with the enhanced chemiluminescence system (Amersham) according to the manufacturer's instructions. The filters were exposed to Kodak XAR-5 film for 5 min. The soluble receptor protein (Soluble Rec.) is indicated by an arrow. The numbers to the left of the gel are molecular masses (in kilodaltons).

kDa protein that was not detected in control supernatants (Fig. 1B). We believe that this protein migrates slower than expected from its primary amino acid sequence because of posttranslational modifications; membrane-bound versions of the receptor are highly modified and run as a broad smear on protein gels (3).

To determine whether the soluble protein can interact

directly with subgroup A viruses, culture supernatants containing this protein were assayed for their ability to inhibit ALSV-A infection of QT6 cells and CEFs. Subgroup A-specific viruses that contain a gene conferring resistance to hygromycin B (3) were preincubated with medium containing or lacking the soluble receptor before infection of QT6 cells. Incubation of viruses with culture supernatants containing the soluble receptor reduced the number of hygromycin B-resistant colonies by 96 to 99% compared with the number in control medium (Fig. 2A).

To establish whether the virus-blocking activity of the soluble receptor was specific for subgroup A viruses, medium containing this protein was assayed for its ability to interfere with infection of CEFs by different subgroups of ALSV. QT6 cells could not be used for these experiments because they are resistant to infection by ALSV-B and ALSV-D (28). Equivalent amounts of viruses representing viral subgroups A, B, C, and D were preincubated with soluble receptor-containing medium or control medium prior to infection of CEFs. Following infection, the cells were maintained in medium that contained or lacked the secreted receptor protein and passaged three times to allow virus spread. Culture supernatants were taken at each cell passage and assayed for virus production using a quantitative reverse transcriptase (RT) assay. The medium containing the soluble receptor abolished infection and spread of subgroup A virus but had no effect on infection of CEFs by other subgroups of ALSV (Fig. 2B). Thus, the secreted protein specifically blocks infection of avian cells by ALSV-A.

The ability of the soluble receptor to block infection by ALSV-A was consistent with a model in which this protein binds directly to subgroup A virus particles. To test this idea, viruses of different ALSV subgroups were preincubated in culture supernatants (with or without the HA epitope-tagged soluble receptor), and any resultant virus-receptor complexes were immunoprecipitated with the 12CA5 antibody. The immunoprecipitates were then assayed for RT activity. Approximately 10 to 15% of the total input RT activity associated with the subgroup A virus was immunoprecipitated only in the presence of soluble receptor protein (Fig. 3). In contrast, viruses representing ALSV-B and ALSV-C were not immunoprecipitated by this procedure (Fig. 3). The amount of ALSV-A particles recovered in these experiments was probably an underestimate of the total number of virus-receptor complexes because of inefficient immunoprecipitation of the soluble receptor by the 12CA5 antibody (5a). These data demonstrate that the soluble receptor binds specifically to subgroup A virus particles.

We have shown that culture supernatants containing the soluble receptor block subgroup A virus infection, presumably because this protein binds directly to virus particles. The specific binding interaction between the soluble receptor and ALSV-A adds further strength to the argument that the cloned receptor gene is *tv-a*, the genetically defined receptor locus for ALSV-A. Indeed, preliminary genetic studies have indicated that the cloned gene maps to *tv-a* (3a). Given our findings, it seems likely that the subgroup specificity of ALSV-A infection is determined at least in part by the binding between virus and the ALSV-A receptor. This seems to contradict the results of a previous study which concluded that subgroup A virus-specific infection is determined at a step in virus entry that is subsequent to virus binding (22). In that study, ALSV-A bound equally well to CEFs that were either susceptible or resistant to subgroup A virus infection (22). However, more recent studies have demonstrated that the ALSV-A receptor is expressed at low, almost undetectable levels in CEFs (3), suggesting that the

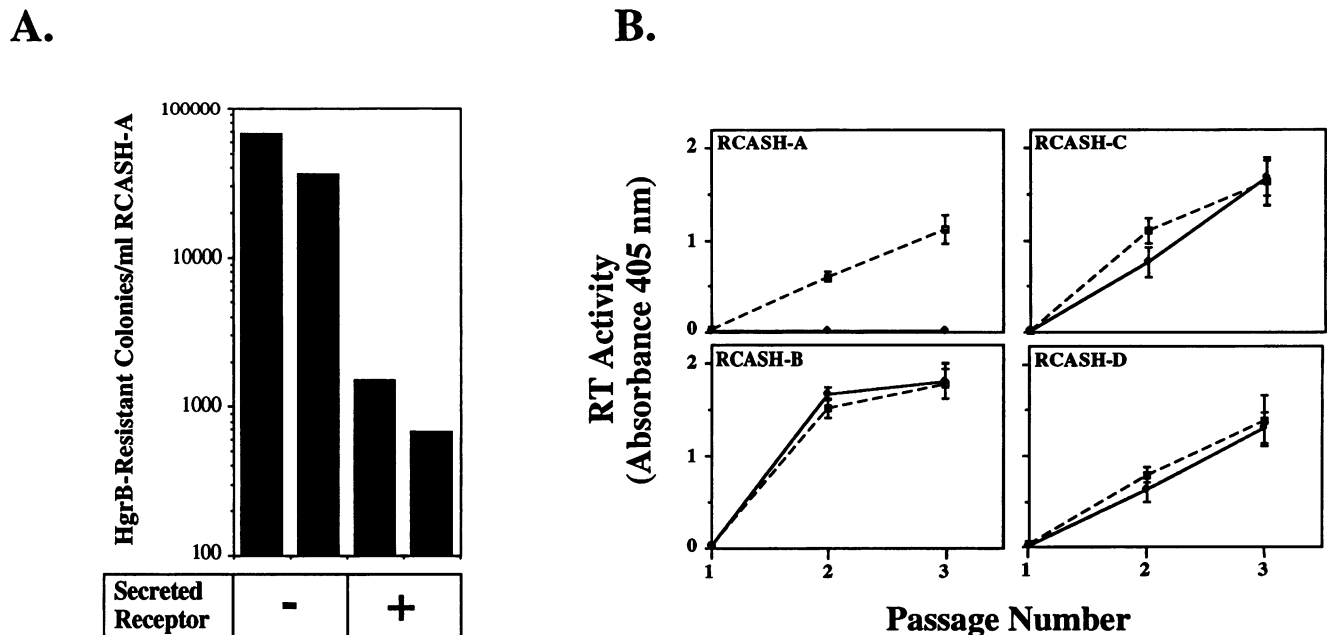


FIG. 2. The soluble receptor blocks ALSV-A infection. (A) Infection of QT6 cells. Aliquots (1.5 ml) of serial 10-fold dilutions of a subgroup A-specific virus that contains a gene conferring resistance to hygromycin B (RCASH-A; 32) were incubated at 4°C for 2 h with 0.5-ml culture supernatants that either contained (+) or lacked (-) the soluble receptor (described in the legend to Fig. 1B). These samples were divided into two 1-ml aliquots that were used to infect approximately 2×10^5 QT6 cells on duplicate plates. Twenty-four hours after infection, cells were incubated in medium containing 300 μ g of hygromycin B (HgrB) per ml and the resultant drug-resistant colonies were counted. The results shown are from two pairs of duplicate plates. (B) Infection of CEFs. RCASH-A (32), RCASH-B (32), RCASH-C (3b), and RCASH-D (3) (representing ALSV-A, ALSV-B, ALSV-C, and ALSV-D, respectively) were used to infect CEFs. Virus (50 to 500 infectious units) in 0.5 ml of medium was incubated at 4°C for 1 h with 1.5 ml of culture supernatants that either contained (—) or lacked (---) the soluble receptor. These samples were divided into two 1-ml aliquots that were used to infect CEFs plated at 20% confluency on 6-well plates. Every 24 h, the medium (with or without soluble receptor) in each well was replaced, and the cells were passaged three times to allow virus spread. Immediately before each cell passage, medium that had been incubated for 24 h with confluent monolayers of cells was precleared of cell debris in an Eppendorf microcentrifuge and assayed for virus by a quantitative RT assay (Boehringer Mannheim). Briefly, virus was pelleted from 0.25-ml aliquots of medium at $120,000 \times g$ for 10 min at 4°C and lysed in 130- μ l portions of RT lysis buffer (Boehringer Mannheim). Three 40- μ l aliquots of each viral lysate were incubated for 2 h with biotin- and digoxigenin (DIG)-labeled dUTP and a poly(A)-oligo(dT)₁₅ template/primer hybrid, according to the manufacturer's instructions. DNA reaction products were bound to streptavidin-coated plates and incubated for 1 h with an anti-DIG-specific peroxidase-coupled antibody. The peroxidase substrate 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) was then added, and peroxidase activity, which is proportional to the amount of DIG-labeled dUTP incorporated into DNA, was measured by A_{405} (minus the background A_{490}) after a 5-min incubation. The data shown represent the means of three measurements from each of two duplicate plates, and the error bars indicate the standard deviations.

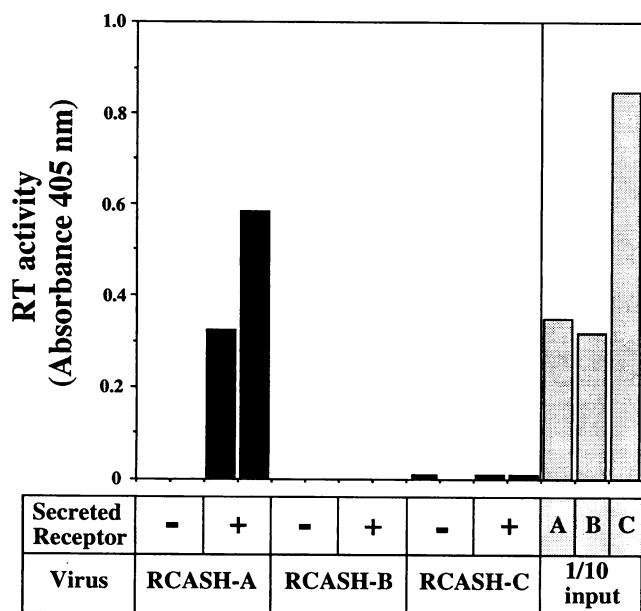


FIG. 3. The soluble receptor binds specifically to ALSV-A. Aliquots (0.5 ml) of RCASH-A, -B, and -C (described in the legend to Fig. 2) were incubated at 4°C for 2 h with 0.25-ml culture supernatants (with [+] or without [-] the soluble receptor) containing 25 μ l of protein A-Sepharose and 7.5 μ g of 12CA5 antibody. The Sepharose beads were pelleted and washed three times with 1-ml portions of phosphate-buffered saline. The precipitated material was then lysed in 40 μ l of RT lysis buffer (Boehringer Mannheim) and analyzed by the quantitative RT assay (see the legend to Fig. 2B). The data shown are from duplicate samples and were measured after a 10-min incubation with 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt. The right panel shows the RT activities associated with 1/10 fractions of the input viruses used in these experiments.

assay used might not have been sensitive enough to detect specific virus binding. Alternatively, if the receptor also mediates postbinding steps of infection, it is possible that proteins encoded by some resistant alleles of the receptor gene might bind virus but have a defect in some subsequent step in viral entry. Experiments are currently in progress to determine whether the cloned receptor also mediates postbinding steps during ALSV-A entry.

Other retroviruses have also been shown to bind to specific host cell surface receptors. The CD4 receptors for HIV and SIV and the receptors for ecotropic murine leukemia virus and bovine leukemia virus initiate virus entry by binding to their cognate viral Env proteins (1, 2, 5, 27). Despite the fact that HIV type 1 and HIV type 2 bind CD4, the entry of these viruses seems to be regulated by distinct HIV type-specific cellular factors that allow the viruses to cross the cell membrane (8). The specificity of these interactions (which operate after virus binding) distinguishes these factors from the ALSV-A receptor described here, which discriminates between different virus subgroups at the level of binding. Presumably, the additional factors required for HIV entry stimulate structural changes in Env protein that are predicted to lead to virus-cell membrane fusion by exposing a fusogenic domain in the viral transmembrane (TM) Env protein (29). We do not yet know whether accessory factors are required in addition to the identified receptor to facilitate ALSV-A entry into cells. However, unlike CD4, the subgroup A virus receptor

can function in cells derived from several different species (3, 32), suggesting that if additional factors are needed, they must be highly conserved.

L. Connolly and K. Zingler contributed equally to these experiments.

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