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The Receptor for the Subgroup A Avian Leukosis-Sarcoma Viruses Binds to Subgroup A but Not to Subgroup C Envelope Glycoprotein

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The putative subgroup A avian leukosis-sarcoma virus (ALSV) receptor (Tva) was recently cloned by gene transfer (P. Bates, J. A. 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). Susceptibility to infection by subgroup A ALSV is conferred on cells upon transfection with cDNAs encoding *tva*. The hypothesis that *tva* encodes a specific receptor for subgroup A ALSV predicts that the Tva protein should bind to subgroup A, but not to subgroup C, envelope glycoprotein. In this study, we examined this prediction by using several biochemical assays. We established stable NIH 3T3 cell lines expressing either Tva, the subgroup A envelope glycoprotein (Env-A), or the subgroup C envelope glycoprotein (Env-C) and used them in conjunction with soluble forms of these molecules to demonstrate specific binding. When cell lysates containing Tva were mixed with lysates of either Env-A or Env-C, an immunoprecipitable complex formed between Tva and Env-A but not between Tva and Env-C. A soluble, oligomeric form of Env-A, not Env-C, binds to cells expressing Tva. Reciprocally, a secreted form of Tva can bind to cells expressing Env-A but not to cells expressing Env-C. A specific and stable complex formed between soluble Env-A and secreted Tva as demonstrated by sucrose density gradient centrifugation. Thus, by three kinds of assays, Tva appears to bind specifically to Env-A, which is consistent with genetic evidence that it serves as the cell surface receptor of subgroup A ALSV and the main determinant of subgroup specificity.

The first step in the life cycle of an enveloped virus is specific binding of the virus to the host cell. This process is mediated by transmembrane envelope glycoproteins in the virus membrane. Only after specific binding occurs can the virus initiate the fusion event that leads to entry of the viral genome into the host cell cytoplasm and virus replication (17, 27). For binding to occur, the host cell surface must contain an appropriate receptor that interacts with an envelope glycoprotein (19). For some enveloped viruses (e.g., influenza virus), no host cell factors other than the primary virus receptor are required for fusion. For other enveloped viruses (e.g., the human immunodeficiency virus [16]), the primary virus receptor is not sufficient, and it has been postulated that additional susceptibility factors or secondary virus receptors are required (26, 28).

For retroviruses, the viral envelope glycoproteins are oligomers of heterodimers consisting of the surface (SU) subunit and the transmembrane (TM) subunit (9). There are five major viral subgroups of avian leukosis and sarcoma viruses (ALSV), subgroups A to E, which are determined solely by amino acid differences in segments of the SU subunit called variable regions (2, 3, 8). Viruses within the same subgroup have identical host ranges, cross interfere, and have immunologically related envelope glycoproteins. Viruses from distinct subgroups have different host ranges and do not cross neutralize (5, 12, 13, 25). Experiments utilizing pseudotyped viruses have shown that viruses of different subgroups can replicate in either susceptible or resistant avian cells, indicating that subgroup specificity is determined at the level of virus entry (23).

Susceptibility of chickens to infection by ALSV is governed by three genetic loci, *tva*, *tvb*, and *tvc* (7, 21). The *tva* and *tvc* susceptibility alleles are thought to encode receptors or susceptibility factors for subgroup A and C viruses, respectively, whereas different alleles of *tvb* may encode receptors (or factors) for subgroups B, D, and E (21). Since susceptibility is dominant over resistance, recessive alleles may produce nonproductive receptors (or factors) or they may be functionally null alleles.

A receptor for subgroup A ALSV was recently cloned by gene transfer (1b, 32) and has been shown to be the product of the *tva* locus (1a). cDNAs representing two alternatively spliced messages that confer susceptibility to infection by subgroup A viruses were obtained by using an exon trap protocol. The predicted amino acid sequences indicate that they encode small proteins (Tva gp12 and Tva gp15) in which the extracellular domain includes a small region partly related to the ligand-binding domain of the low-density lipoprotein receptor. Antiserum raised against one of the gene products (Tva gp15) blocks infection of avian cells in a subgroup-specific manner, indicating that Tva is required for the entry of subgroup A ALSV into host cells. However, it is not clear whether Tva is a receptor that allows specific binding of subgroup A ALSV or a factor that is involved in a post-binding step necessary for virus fusion.

MATERIALS AND METHODS

Recombinant DNA. Plasmids containing genes encoding the subgroup A (pSVenv KS−; Env-A) and subgroup C (pSVenv KX; Env-C) envelope glycoproteins were gifts of J. Young and

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E. Hunter, respectively. The upstream regions of the envelope glycoproteins were replaced to optimize translation of the envelope genes by changing the start site of translation from a poor consensus sequence to one more optimal for translational initiation (15). The *Xho*I-*Cla*I restriction fragment containing the envelope open reading frame without the start site for translation, the signal sequence, and the first five amino acids from the SU subunit were subcloned into vector pSV7denv (gift of N. Landau), replacing the original *Xho*I-*Bam*HI insert in this vector. This manipulation places the subgroup A and C envelope genes adjacent to the native *gag*-spliced leader sequence found in spliced mRNAs in Rous sarcoma virus-infected cells (4), restoring the start site for translation, the signal sequence, and the first five amino acids of the SU subunit. This construction does not alter the amino acid sequence of the mature SU subunit. The resulting Env-A- and Env-C-containing plasmids were then linearized with *Eco*NI and blunted by digestion with mung bean nuclease, and *Bam*HI linkers (Stratagene, La Jolla, Calif.) were ligated onto the ends. The samples then were digested with *Bam*HI, and the envelope-encoding restriction fragments were subcloned into the *Bam*HI site of expression vector pCB6 (gift of M. Stinski). This places the genes that encode Env-A and Env-C downstream of the cytomegalovirus early promoter.

Construction of pCB6 plasmids for expression of chimeric envelope glycoproteins containing a signal for glycosyl phosphatidylinositol (GPI) addition in place of the transmembrane domain and cytoplasmic tail has been previously described (10). These plasmids are referred to as Env-API and Env-CPI. The cDNA encoding Tva gp15 was cloned into pCB6 as previously described (10). The secreted form of Tva (sTva) was cloned into pVTbac and expressed in insect cell lines and will be described elsewhere (1).

Tissue culture. To establish stable cell lines, DNA (50 μg/10-cm-diameter dish) was transfected into NIH 3T3 cells by the $CaPO_4$-DNA coprecipitation method (30). Twenty-four hours after transfection, the cells were placed in selective medium (Dulbecco's modified Eagle's medium [University of California, San Francisco, Tissue Culture Facility], 10% supplemented calf serum [SCS; Hyclone, Logan, Utah]; 500 μg of Geneticin [Gibco, Grand Island, N.Y.] per ml) for 14 days. Geneticin-resistant clones were picked and screened for expression of either envelope or Tva gp15 protein after overnight induction with sodium butyrate (Sigma, St. Louis, Mo.). Optimum sodium butyrate concentrations for protein expression were found to be 25 mM for Env-A-, Env-C-, and Env-CPI-expressing cells, 10 mM for Tva-expressing cells, and 5 mM for Env-API-expressing cells. As expected, the cell line expressing Tva is infectible with subgroup A, but not with subgroup C, Rous sarcoma virus. The parental 3T3 cell line is not infectible with either virus (1).

Antibodies and reagents. The rabbit polyclonal anti-envelope serum used was a gift from M. Stoltzfus. The anti-Tva serum used was raised as described previously (1b). Polyclonal antisera were raised in rabbits against the carboxyl-terminal cytoplasmic tails of both glycoproteins Env-A and Env-C (amino acids 195 to 205 and 184 to 198, respectively, of the TM subunit) by Caltag Laboratories (South San Francisco, Calif.). The anti-tail antibodies used were affinity purified by chromatography over their respective peptides that had been coupled to SulfoLink resin (Pierce, Rockford, Ill.). The anti-A tail antibody recognizes only Env-A, and the anti-C tail antibody recognizes only Env-C (data not shown). Phosphatidylinositol-specific phospholipase C (PI-PLC) was purified from an over-expressing bacterial strain (pIC [14]; gift of P. Bjorkman).

Cell surface labelling, immunoprecipitation, and coimmunoprecipitation. After overnight induction with sodium butyrate, cells were surface labeled as follows. Cells were washed with cold biotinylation buffer (*N*-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) [HEPES]-buffered saline–0.5 mM $MgCl_2$ with the pH adjusted to 7.9) and then labeled with 1 mg of membrane-impermeant biotinylation reagent NHS-LC-biotin (Pierce) per ml for 45 min at 4°C. The excess biotin was quenched with 0.5 mg of bovine serum albumin per ml–100 mM glycine for 5 min. The samples then were washed three times with cold biotinylation buffer with 20 mM glycine. Cells were lysed in HEPES-buffered saline with 1% Nonidet P-40 (lysis buffer) containing a protease inhibitor cocktail for 15 min at 4°C, and then debris was cleared by centrifugation at 12,000 × *g* for 15 min at 4°C. PI-PLC release of GPI-anchored proteins was performed as previously described (10).

For immunoprecipitations, antibodies were precoupled to protein A-agarose (Schleicher & Schuell, Inc., Keene, N.H.) for 90 min at 4°C, washed twice in lysis buffer, and then added to cleared lysates. Samples were precipitated for 90 min at 4°C and then washed three times in lysis buffer and once in RIPA buffer. For coimmunoprecipitations, the cleared lysates of either Env-A or Env-C were mixed with cleared lysates of biotinylated Tva, incubated for 1 h at 4°C, and then precipitated as described above. Samples then were washed, boiled, reduced, and separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE; 9% acrylamide). Proteins were transferred to nitrocellulose (Schleicher & Schuell) and probed with streptavidin coupled to horseradish peroxidase (HRP; Pierce) to detect biotinylated proteins. The HRP signal was detected by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.).

For detection of unlabelled proteins by Western blotting (immunoblotting), samples were lysed as described above, boiled, reduced, and separated by SDS–9% PAGE. After the proteins were transferred to nitrocellulose and probed with the appropriate primary antibody, they were detected with a secondary donkey anti-rabbit immunoglobulin G coupled to HRP (Amersham). The HRP signal was detected by enhanced chemiluminescence.

Binding. GPI-anchored glycoproteins were biotinylated and removed from the cell surface by treatment with PI-PLC as described above and enriched by chromatography over lentil lectin agarose (Vector Labs, Burlingame, Calif.). Samples were concentrated with a Centriprep 30 concentrator (Amicon, Beverly, Mass.) and then added to cells that had been washed previously with cold RPMI 1640 medium (RPMI; Sigma) containing 10% SCS. After 1 h at 4°C, cells were washed three times with cold RPMI–10% SCS and three times with cold RPMI alone and then lysed and processed to detect biotinylated proteins as described above.

Medium was collected from cells infected with either a recombinant baculovirus that expresses sTva or a wild-type (control) baculovirus strain. Samples were diluted into cold RPMI–10% SCS and then incubated with Env-expressing cells or parental 3T3 cells that had been washed previously with cold RPMI–10% SCS. After 1 h at 4°C, cells were washed three times with cold RPMI–10% SCS and three times with cold RPMI alone and then lysed, boiled, reduced, and analyzed on SDS–11% PAGE. The proteins were then transferred to nitrocellulose and Western blotted with anti-Tva serum. The primary antibody was detected as described above.

Sucrose density gradient centrifugation. Cells expressing GPI-anchored envelope glycoproteins were treated with PI-PLC, the released material was concentrated, and half of the samples were incubated with sTva. The rest were not treated. After 1 h at 4°C, the samples were loaded onto 10 to 30%

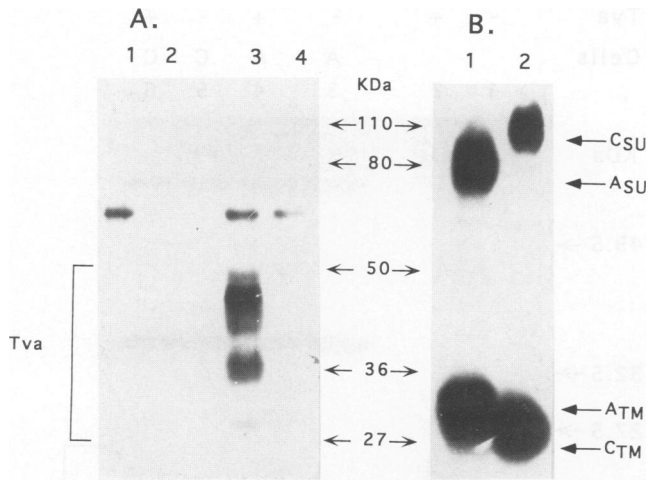

FIG. 1. Coimmunoprecipitation of Tva with the Env-A, but not with the Env-C, glycoprotein. (A) Lysates of Tva-expressing cells were mixed with Env-A (lanes 1 and 3) or Env-C (lanes 2 and 4) cell lysate. Samples were immunoprecipitated with antisera against the carboxyl-terminal tail of either Env-A or Env-C (lanes 3 and 4, respectively) or with the respective preimmune serum (lanes 1 and 2). Tva migrates as three major heterogeneous bands (1b) because of extensive posttranslational modifications (1). (B) A parallel set of cells expressing either Env-A (lanes 1) or Env-C (lane 2) were biotinylated and precipitated with antibodies against either the Env-A (lane 1) or the Env-C (lane 2) carboxyl-terminal tail. Samples were analyzed by SDS–9% PAGE, electroblotted, probed with streptavidin-HRP, and detected by enhanced chemiluminescence. The SU and TM glycoprotein subunits migrated as shown.

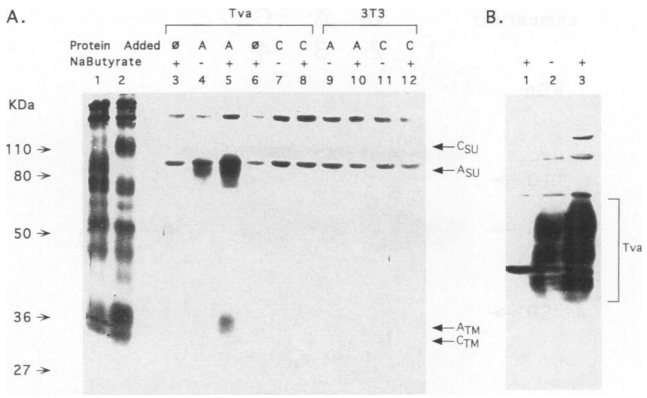

FIG. 2. Soluble Env-API, but not Env-CPI, glycoprotein binds specifically to cells expressing Tva. (A) Cells expressing either GPI-anchored Env-A or Env-C were biotinylated and treated with PI-PLC, and then the released proteins were allowed to bind to cells expressing Tva (lanes 3 to 8) or to parental 3T3 cells (lanes 9 to 12). One-twentieth of the Env-API or Env-CPI protein applied to the Tva-expressing cells is shown in lanes 1 and 2, respectively. No biotinylated glycoproteins were added to the samples of Tva-expressing cells shown in lanes 3 and 6; the two bands detected by streptavidin-HRP represent nonspecific cellular proteins, one of which comigrated with the SU subunit of Env-A. Treatment with sodium butyrate is indicated by the plus and minus signs. The proteins added, either none or biotinylated Env-API or Env-CPI, are labelled Ø, A, and C, respectively. Samples were analyzed as described in the legend to Fig. 1. The Env-API and Env-CPI SU and TM subunits migrated as indicated. (B) Level of Tva expression. Parental 3T3 cells (lanes 1) and uninduced (lane 2) or sodium butyrate-induced (lane 3) cells expressing Tva were analyzed by SDS–9% PAGE, transferred to nitrocellulose, and Western blotted with anti-Tva serum.

linear sucrose gradients in HEPES-buffered saline containing 40 mM octylglucoside (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The samples were centrifuged in an SW41 Ti rotor at 200,000 × *g* for 13 h at 4°C. Fractions were collected, precipitated with a combination of lentil lectin agarose and wheat germ agglutinin agarose (Vector Labs), boiled, reduced, and separated by SDS–12.5% PAGE. Proteins then were transferred to nitrocellulose and Western blotted with the appropriate antibodies as described above.

RESULTS AND DISCUSSION

Coimmunoprecipitations. We first asked whether Env-A and Env-C interact with Tva in crude lysates. Cells expressing Tva were surface biotinylated and lysed. The lysates were mixed with lysates from an equal number of unbiotinylated Env-A- or Env-C-expressing cells and incubated for 1 h at 4°C. The samples were immunoprecipitated with affinity-purified anti-tail antibodies specific for either Env-A or Env-C. The immunoprecipitates were then analyzed for the presence of biotinylated material. Tva was detected in precipitates that had been incubated with Env-A (Fig. 1A, lane 3) but not Env-C (Fig. 1A, lane 4). Furthermore, of all of the biotinylated cell surface proteins, Tva appeared to be the only protein that was coimmunoprecipitated by the anti-A tail antibody. Immunoprecipitations of lysates from biotinylated cells expressing only Env-A or Env-C with the anti-A tail and anti-C tail antibodies indicated similar levels of Env-A and Env-C in the respective lysates (Fig. 1B). This experiment demonstrated a specific interaction between Tva and Env-A. Moreover, these data showed that this interaction withstands the stringent conditions of immunoprecipitation.

Binding of GPI-anchored proteins to Tva-expressing cells. We next examined the interaction of Env-A and Tva under conditions more reminiscent of viral entry by asking whether soluble oligomeric envelope glycoprotein could bind to cells expressing *tva*. We previously reported the construction, expression, and oligomeric assembly of chimeric envelope glycoproteins of both subgroups A and C in which the TM domains and cytoplasmic tails were replaced with GPI tails. These GPI-anchored glycoproteins have biochemical properties similar to those of their wild-type counterparts (10). To analyze binding of the envelope glycoprotein ectodomains to cells expressing Tva, GPI-anchored Env-A and Env-C were biotinylated and then treated with PI-PLC to remove GPI-anchored glycoproteins from the cell surface. The PI-PLC-released molecules, Env-API and Env-CPI, were incubated with cells expressing Tva. Env-API (Fig. 2A, lanes 4 and 5), but not Env-CPI (Fig. 2A, lanes 7 and 8), bound to cells expressing Tva. Neither of the PI-PLC-released envelope glycoproteins bound to parental 3T3 cells (Fig. 2A, lanes 9 and 10 and 11 and 12, respectively). Binding of Env-API was increased (Fig. 2A, lane 4 versus lane 5) when *tva* expression was induced with sodium butyrate (Fig. 2B, lane 2 versus lane 3). In addition, of a mixture of all of the biotinylated cell surface proteins released by treatment with PI-PLC (Fig. 2A, lanes 1 and 2), only the Env-API glycoprotein bound to Tva-expressing cells (Fig. 2A, lanes 4 and 5). These results indicated a specific interaction between the soluble, oligomeric ectodomain of the Env-A glycoprotein and Tva.

To verify that binding of Env-API to cells expressing Tva was specific, binding of biotinylated Env-API to cells expressing Tva was assayed in the presence of an approximately 25-fold

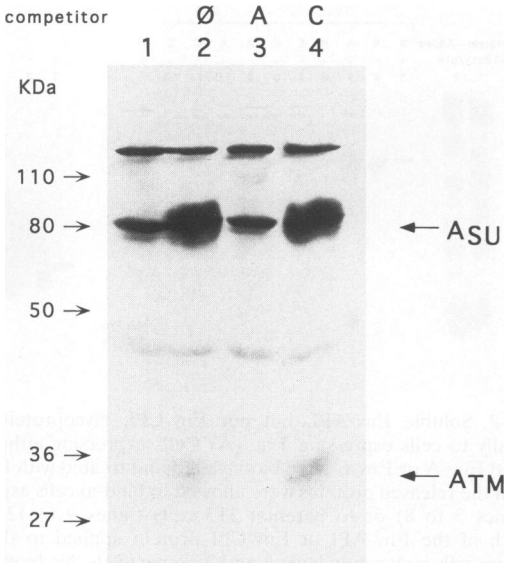

FIG. 3. Binding of biotinylated Env-API to cells expressing Tva is competed for by excess unlabelled Env-API but not Env-CPI. Biotinylated Env-API was mixed with buffer alone (lane 2), with unlabelled Env-API (lane 3), or with unlabelled Env-CPI (lane 4) at 4°C, and then the mixture was immediately bound to Tva-expressing cells, incubated, and processed as described in the legend to Fig. 1. In lane 1, no biotinylated Env-API was added to cells expressing Tva. Ø, no competitor added.

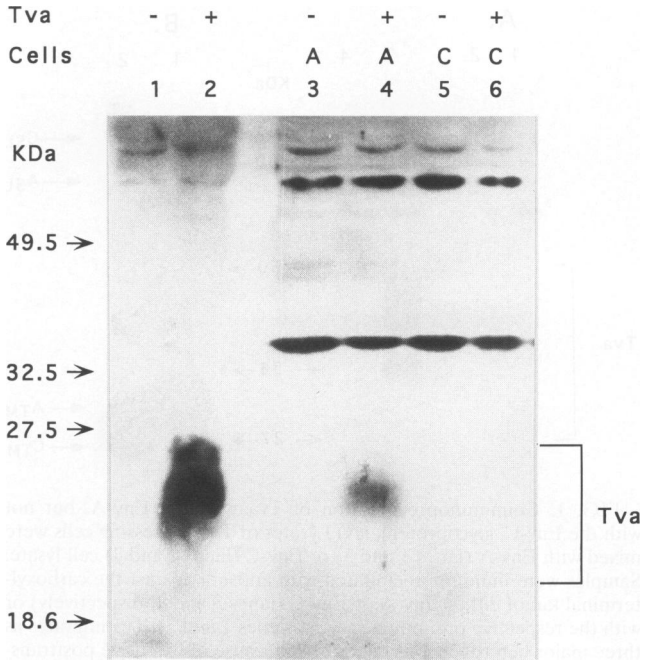

FIG. 4. Baculovirus-expressed sTva binds to Env-A-expressing cells but not to Env-C-expressing cells. One-tenth of the baculovirus control material or sTva added to cells is shown in lanes 1 and 2, respectively. sTva (lanes 2, 4, and 6) or control baculovirus protein (lanes 1, 3, and 5) was diluted and incubated with cells expressing either Env-A (lanes 3 and 4) or Env-C (lanes 5 and 6) for 1 h at 4°C. The cells were then washed, lysed, and processed for Western blotting with anti-Tva serum as described in Materials and Methods.

molar excess of unbiotinylated competing envelope glycoprotein. Unbiotinylated Env-API efficiently competed with biotinylated Env-API for binding to cells expressing Tva (Fig. 3, lane 3). In contrast, unbiotinylated Env-CPI did not compete with biotinylated Env-API for binding to cells expressing Tva (Fig. 3, lane 4). This result further demonstrated the specificity of binding of Env-A to the Tva protein.

Binding of sTva to envelope glycoprotein-expressing cells. Having shown that a soluble form of Env-A could bind to cells expressing Tva, we next conducted the reciprocal experiment and examined the binding of a water-soluble form of the Tva protein to cells expressing the Env-A or Env-C glycoprotein. To do this, we employed supernatants from recombinant baculovirus-infected cells that express sTva, which blocks infection by subgroup A viruses (9a). Supernatants containing sTva were incubated with envelope-expressing cells for 1 h at 4°C. sTva bound to cells expressing Env-A (Fig. 4, lane 4) but not to cells expressing Env-C (Fig. 4, lane 6). This result demonstrated specific binding between soluble recombinant Tva and the membrane-anchored Env-A glycoprotein.

Sucrose density gradient centrifugation of sTva and GPI-anchored envelope glycoproteins. Since Env-A binds specifically to sTva, we next examined whether the soluble envelope glycoproteins could interact with soluble Tva. Cells expressing GPI-anchored Env-A and Env-C were treated with PI-PLC, and the soluble glycoproteins were incubated with sTva for 1 h at 4°C. Samples then were subjected to sucrose density gradient centrifugation. sTva and Env-API formed a complex, as indicated by their comigration (Fig. 5B). The complex migrated in a heavier fraction than sTva alone, which ran at the top of the gradient (Fig. 5E), and it also migrated in a heavier fraction than Env-API alone (Fig. 5A). Env-CPI did not form a complex with sTva (Fig. 5D). This experiment indicated that the soluble ectodomain of Env-A, but not that of Env-C, forms a complex with sTva. Furthermore, this interaction was relatively stable, withstanding the centrifugation conditions employed.

Summary. When transfected into mammalian cells, *tva* confers susceptibility to infection by subgroup A ALSV but not to infection by ALSV belonging to other subgroups (1b, 32). Hence, Tva, the product of the *tva* locus, has been termed the subgroup A receptor. However, it has remained unclear whether Tva serves as a primary virus receptor, conferring subtype-specific virus binding on susceptible host cells, or whether it functions as a secondary virus receptor at a post-binding step in the viral entry pathway. Previous studies examining subgroup-specific binding have shown either no (6, 9a, 22) or only modest (20) increases in virus binding to susceptible versus resistant chicken embryo fibroblasts. The inability to demonstrate specific virus binding to susceptible chicken embryo fibroblasts was most likely due to both the high levels of nonspecific binding and the low level of *tva* expression in chicken embryo fibroblasts (1b, 32).

In this study, we used subgroup A and C ALSV envelope glycoproteins and Tva protein expressed in mouse fibroblasts, in combination with soluble forms of these proteins, to demonstrate specific binding between Env-A and Tva. The interaction is highly specific: Env-C does not bind to Tva, nor does it compete with Env-A for binding to Tva. The interaction also appears to be quite stable; binding is maintained under the stringent conditions of immunoprecipitation, as well as during sucrose density gradient centrifugation. Our study thus provides the first biochemical evidence that Tva is the subgroup A receptor. Our results also suggest that Tva serves as the

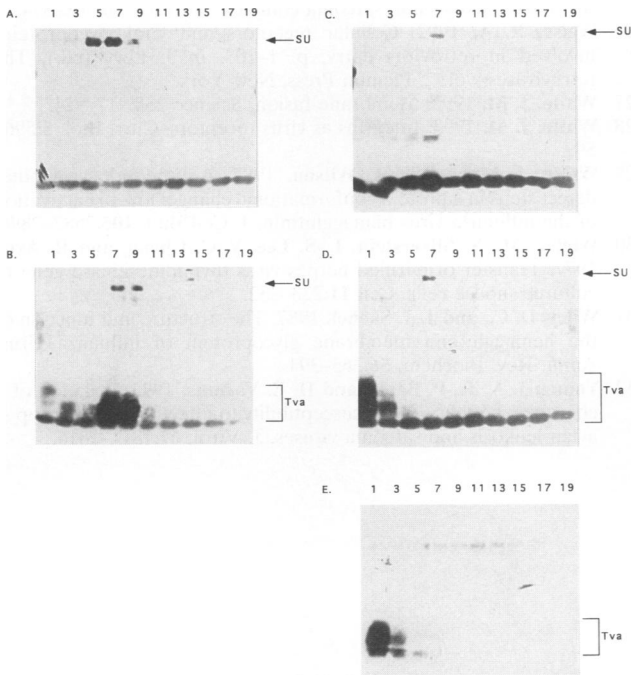

FIG. 5. sTva forms a complex with Env-API but not with Env-CPI. Soluble Env-API and Env-CPI glycoproteins were released from GPI-anchored Env-A- and Env-C-expressing cells with PI-PLC. Half of the samples were mixed with recombinant sTva and incubated for 1 h at 4°C, and the other half were untreated. Samples were then run on sucrose density gradients for 13 h at 200,000 × *g*, fractionated, and analyzed by SDS–12.5% PAGE. Untreated Env-API and Env-CPI are shown in panels A and C, respectively. Samples of Env-API or Env-CPI from the incubation with sTva are shown in panels B and D, respectively. The migration of sTva without addition of either envelope glycoprotein is shown in panel E. The low-molecular-weight band seen in all lanes in panels A to D is due to the lentil lectin used for envelope protein precipitation; this band is detected nonspecifically by the anti-Env antibody. Fractions are as numbered, with lane 1 containing the lightest fraction and lane 21 containing the heaviest fraction.

primary receptor for subgroup A viruses, although it may function in the initiation of membrane fusion as well.

A powerful finding of this study (Fig. 5B) is that a recombinant, secreted form of Tva, expressed in a baculovirus system, interacts stably with a water-soluble oligomeric ectodomain of Env-A. This observation provides a starting point for biochemical, biophysical, and structural analysis of the receptor-binding interaction. In addition, we believe that it will provide a model system for elucidation of how a retroviral envelope glycoprotein mediates membrane fusion. The best-characterized viral fusion glycoprotein, the influenza virus hemagglutinin, functions at low pH (24, 27, 31) via fusion-inducing conformational changes, including release of the apolar fusion peptide from the trimer interface (29). Many other viruses, including most retroviruses, fuse at neutral pH (11, 18). For these viruses, it is not known what triggers fusion. Rous sarcoma virus binds to the host cell surface at 4°C but fuses only when the temperature is raised above 22°C (11). Our working hypothesis is that the interaction between the envelope glycoprotein and its receptor at temperatures greater than 22°C induces conformational changes in the envelope glycoprotein, including release of its fusion peptide, which initiates membrane fusion. The interaction that we have described here, between the ectodomains of Env-A and its receptor, Tva, provides a system that can be used to test this hypothesis.

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