JOURNAL OF VIROLOGY, Nov. 1996, p. 7510–7516 Vol. 70, No. 11
0022-538X/96/$04.00+0

Residue Trp-48 of Tva Is Critical for Viral Entry but Not for High-Affinity Binding to the SU Glycoprotein of Subgroup A Avian Leukosis and Sarcoma Viruses

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Received 29 March 1996/Accepted 21 July 1996

Previously, mutant Tva receptors were classified as either partially or completely defective in mediating subgroup A avian leukosis and sarcoma virus (ALSV-A) entry (C. Bélanger, K. Zingler, and J. A. T. Young, J. Virol. 69:1019–1024, 1995; K. Zingler, C. Bélanger, R. Peters, D. Agard, and J. A. T. Young, J. Virol. 69:4261–4266, 1995). To specifically test the abilities of these mutant Tva proteins to bind ALSV-A surface (SU) protein, binding studies were performed with a subgroup A SU-immunoadhesin. This fusion protein is composed of the subgroup A Schmidt-Ruppin SU protein fused in frame to a rabbit immunoglobulin constant region. This reagent was conjugated to fluorescein isothiocyanate and used for flow cytometric analysis with transfected human 293 cells expressing different forms of Tva. The SU-immunoadhesin bound the wild-type Tva protein with a K_D of approximately 1.5 nM. Amino acid substitutions that reduced viral entry at Asp-46 and at Cys-35 and Cys-50, which are predicted to form an intrachain disulfide bond in Tva, drastically reduced the binding affinity for the SU-immunoadhesin. Thus, the effects on viral entry of some mutations could be explained solely by changes in the binding affinity for ALSV-A SU. However, this was not true for other mutations tested, especially those with amino acid substitutions that replaced Trp-48. Compared with the wild-type receptor, these latter mutations led to approximately 43- to 200-fold reductions in viral infectivity but only to approximately 2.5- to 3.4-fold reductions in the binding affinity for the SU-immunoadhesin. These results support a role for Trp-48 of Tva in mediating steps of viral entry subsequent to binding ALSV-A SU.

Retroviral infection is mediated through interactions between the viral envelope protein (Env) and specific receptors present on the surface of the host cell. Following binding of viral surface (SU) Env proteins to receptors, conformational changes in Env that expose fusion peptide regions of the transmembrane (TM) Env protein are thought to drive fusion of viral and cellular membranes (26, 42, 50, 51). This model of retroviral entry is derived mainly from studies of the influenza A virus hemagglutinin (HA), on the basis of similarities between these viral glycoproteins. However, there must be important differences between the molecular details of how these two proteins mediate viral entry into cells, because HA-mediated entry is a pH-dependent process (10, 12, 51) whereas the entry of retroviruses is generally pH independent (23, 32, 44).

A number of different retroviral receptors have so far been identified. Binding has been demonstrated between the human CD4 receptor and the SU glycoproteins of human immunodeficiency virus (HIV) and simian immunodeficiency virus (29, 33), between the murine ATRC1 receptor and ecotropic murine leukemia virus SU (1, 48), between the Tva receptor and subgroup A avian leukosis and sarcoma virus (ALSV-A) Env (14, 21, 40), between the RaPit2 receptor and amphotropic murine leukemia virus SU (27), and between the bovine leukemia virus receptor and bovine leukemia virus SU (4). The major binding determinants of the CD4 receptor have been defined (2, 3, 7, 9, 13, 28, 35, 36, 38, 41, 43, 49), and the effects on viral entry of mutations at the most important positions can be explained solely by changes in the binding affinity for HIV type 1 SU (8).

Determinants that are important for viral entry have also been defined for the ATRC1 receptor (1, 30, 53); for the Tva receptor (6, 40, 55); for the PiT2, RaPiT2, and HaPiT2 receptors for amphotropic murine leukemia virus (17, 34, 37); for the PiT1 receptor for gibbon ape leukemia viruses, subgroup B feline leukemia viruses, and simian sarcoma-associated viruses (34, 37, 46); and for the HaPiT2 receptor for gibbon ape leukemia viruses (17). The precise functions of these determinants during viral entry, however, remain to be established.

We are attempting to understand the process of retroviral entry by using ALSV-A–Tva interactions as a model system. The Tva receptor has a small 83-amino-acid extracellular domain which contains a motif (residues 11 to 50) that is highly related to the seven ligand binding repeat regions of the low-density lipoprotein receptor (LDLR) (5). The major viral interaction determinants of Tva are located at the C-terminal end of the LDLR-related motif (6, 40) and consist of a three-amino-acid stretch (Asp-46, Glu-47, and Trp-48) and a putative disulfide bond between Cys-35 and Cys-50 (6, 55). Although a disulfide bond between Cys-35 and Cys-50 in Tva remains to be formally demonstrated, nuclear magnetic resonance studies have confirmed that the equivalent cysteines in the first and second ligand binding repeat regions of the LDLR form a disulfide bond pair (15, 16). Efficient receptor function was also observed with mutant receptors in which the acidic character of residues 46 and 47, and the aromatic character of residue 48, were maintained (55).

A soluble Tva protein is capable of inducing conformational changes in ALSV-A Env that appear to be similar to those expected of the fusion-active form of the viral glycoprotein

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(22). This finding suggests that in addition to binding ALSV-A SU, Tva might also stimulate events that lead to activation of the fusogenic potential of subgroup A Env to drive virus-cell membrane fusion. In further support of this proposition, we now show that the effects of mutations of Tva on viral entry cannot be explained solely by changes in binding affinity for ALSV-A SU.

MATERIALS AND METHODS

Cells and transfections. Human 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. These cells were plated on 100- or 150-mm-diameter plates so that they were at approximately 20% confluency 1 day before transfection. The cells were transfected by the calcium phosphate method (52) with plasmid vectors encoding SUA-rIgG (see below), and wild-type or mutant HA-Tva protein. After incubating with the calcium phosphate precipitate for 18 h, the cells were washed with Dulbecco's phosphate-buffered saline (DPBS) and incubated with Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum before analysis 22 to 30 h later.

Construction of the *SUA-rIgG* gene. PCR mutagenesis was used to introduce a *Bam*HI site in the subgroup A Schmidt-Ruppin *env* gene, at a position immediately following Arg-338 (19), and the sequence of the amplified fragment was confirmed by using a PCR-based dideoxy sequencing protocol (Bethesda Research Laboratories). The same protocol was used to introduce a *Bam*HI site in the rabbit immunoglobulin gamma heavy-chain gene (SwissProt data bank accession number P01870), at a position immediately upstream of Ala-96. The two constructs were ligated together at the introduced *Bam*HI sites, generating a fusion gene that encodes a mature 568-amino-acid protein (SUA-rIgG) that consists of ALV-A SU (amino acids 1 to 338) fused to the constant region of rabbit immunoglobulin G (amino acids 96 to 323). The fusion gene was placed under the control of the cytomegalovirus early-region promoter in the pCB6 expression plasmid (kindly provided by M. Stinski).

Construction of the *HA-Tva* gene. A DNA fragment encoding three copies of the nine-amino-acid HA epitope tag (YPYDVPDYA) was introduced at a *Cla*I site engineered between the codons for Ser-7 and Leu-8, in the extracellular region of the synthetic quail *Tva* gene (6, 47, 55). The *Cla*I site was introduced by a PCR mutagenesis protocol, and the sequence of the final product was confirmed as described above. The epitope-tagged protein was designated HA-Tva. The mutations at residues Asp-46, Glu-47, Trp-48, Cys-28/Cys-35, Cys-41/Cys-50, and Cys-28/Cys-41, were transferred into HA-Tva from previously described Tva constructs (6, 55). The wild-type and mutant *HA-Tva* genes were placed under the control of the cytomegalovirus early-region promoter in the pCB6 expression plasmid.

Protein precipitations. The SUA-rIgG protein was precipitated from 500 μl of extracellular supernatants of transfected 293 cells, using 20 μl of a 1:3 mix of protein A-Sepharose and protein A-Sepharose CL-4B (Sigma), 5 μl of wheat germ agglutinin-coupled Sepharose, or 5 μl of concanavalin A-coupled Sepharose. The lysis and wash buffers for the last two precipitations were supplemented with 100 μM $CaCl_2$ and 100 μM $MnCl_2$.

Immunoblotting. Nitrocellulose membranes were incubated with Tris-buffered saline supplemented with 0.1% Triton X-100 and 8% powdered milk to block nonspecific protein binding sites. All subsequent steps were performed in Tris-buffered saline containing 0.1% Triton X-100. To detect immunoadhesins, these membranes were incubated with a 1:3,000 dilution of a horseradish peroxidase (HRP)-conjugated donkey antibody specific for rabbit immunoglobulins (Amersham) and washed four times, and the bound antibodies were detected by enhanced chemiluminescence (Amersham).

Purification of SUA-rIgG. For large-scale production of the SUA-rIgG protein, 15-cm-diameter plates of 293 cells were transfected with 45 μg of plasmid DNA. After being washed in DPBS for 18 h, the cells were incubated with medium containing 10% fetal calf serum that had been previously precleared two times over a protein A column (Pharmacia) to remove bovine immunoglobulins. The medium was harvested from the cells after a 24- to 30-h incubation, when it was replaced with additional protein A-precleared medium. This second batch of medium was harvested 24 h later. The harvested media were centrifuged, filtered through a 0.45-μm-pore-size membrane to remove cellular debris, and then passed over a 1-ml protein A column (Pharmacia) at a rate of 1 to 2 ml/min. The column was then washed with 20 mM $NaPO_4$ (pH 7) for 15 min at a rate of 4 ml/min, and the SUA-rIgG protein was eluted at the same rate in 0.1 M citric acid (pH 3) and immediately neutralized in 1 M Tris (pH 9.5). The purified SUA-rIgG protein was then dialyzed against calcium- and magnesium-free DPBS and stored at 4°C. The concentration of SUA-rIgG was determined by using a Bradford assay and confirmed by Coomassie blue staining, compared with rabbit immunoglobulin G and bovine serum albumin standards electrophoresed on sodium dodecyl sulfate (SDS)–8% polyacrylamide gels.

Conjugation of SUA-rIgG to FITC. The SUA-rIgG protein was conjugated with fluorescein isothiocyanate (FITC) by using a commercially available kit (Boehringer Mannheim) for 4 h at room temperature. Two separate stocks were prepared with an FITC/protein ratio of 2.2:1 or 1.9:1. FITC concentrations were determined as specified by the manufacturer (Boehringer Mannheim). Excess FITC was removed either by using a desalting column or by dialysis, and the fluoresceinated SUA-rIgG reagents were stored at 4°C in DPBS supplemented with 0.1% sodium azide. The conjugation of FITC to SUA-rIgG had no effect on the ability of the fusion protein to bind Tva, as determined by flow cytometric analyses similar to those described below.

Flow cytometry. All of the mutant receptors were analyzed twice for their abilities to bind fluoresceinated SUA-rIgG. Most receptors were tested once with each batch of fluoresceinated protein. The receptors with W48A, W48L, W48I, and the cysteine mutations were tested twice with the stock containing 2.2 FITC molecules per molecule of protein. Approximately 48 h after transfection, the 293 cells were removed from tissue culture dishes, using calcium- and magnesium-free DPBS supplemented with 1 mM EDTA. The cells were washed once with DPBS containing either 1 or 5% bovine calf serum (BCS).

In the experiments presented in Fig. 2, 2×10^5 293 cells were incubated on ice for 30 min with approximately 1 μg of SUA-rIgG (contained in 200 μl of crude extracellular supernatants) and 800 μl of DPBS–1% BCS. These cells were washed once and then incubated for 30 min on ice with 1 ml of DPBS–1% BCS containing 5 μl of FITC-conjugated swine anti-rabbit antibody (DAKO). The cells were washed once with DPBS–1% BCS and resuspended in this same medium supplemented with 40 ng of propidium iodide per ml to stain for dead cells. Five thousand live cells were analyzed on a Becton Dickenson FACScan, using Lysis II software and a fluorescence channel 1 gain of 380.

In the experiments presented in Fig. 3, flow cytometry was performed in sets of five, with transfected cells expressing either wild-type Tva, one of three mutant forms of Tva, or no Tva. Aliquots of 1.2×10^5 transfected 293 cells were incubated on ice for 120 min with increasing amounts of fluoresceinated SUA-rIgG, or with 1.5 μg of monoclonal antibody 12CA5 (Babco) and 5 μl of fluoresceinated goat anti-mouse antibody (DAKO), in a total volume of 500 μl of DPBS–5% BCS. Following these incubations, the cells were washed twice with DPBS–5% BCS and resuspended in 500 μl of the same medium supplemented with 400 ng of propidium iodide per ml. These cells were analyzed on an Ortho Diagnostics Cytofluorograf System 2150 FACscan, using Cyclops software (Cytomation Inc.) and a fluorescence channel 1 gain of 4.5.

RESULTS

Construction, expression, and purification of an ALSV-A SU-immunoadhesin. To facilitate studies of SU-Tva binding, an ALSV-A SU-immunoadhesin was generated. The SU-immunoadhesin (designated SUA-rIgG) contains amino acid residues 1 to 383 of the subgroup A Schmidt-Ruppin SU protein fused in-frame to the constant region (amino acid residues 96 to 323) of a rabbit immunoglobulin gamma chain. Similar types of immunoadhesins were used previously to study a variety of binding interactions, including those between HIV type 1 gp120 and CD4 (11), Fas and Fas ligand (45), interleukin-1β and its receptor (39), ICAM-1 and rhinoviruses (31), and gamma interferon and its receptor (20, 24).

The SU-immunoadhesin was expressed in transiently transfected human 293 cells. Crude extracellular supernatants prepared from these cells were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions, and the immunoadhesin was detected by immunoblotting using an HRP-coupled antibody specific for rabbit immunoglobulins (Fig. 1A). The approximately 100-kDa immunoadhesin was detected only in samples prepared from transfected cells expressing SUA-rIgG, not in samples from control human 293 cells (Fig. 1A). As expected for a glycosylated immunoglobulin fusion protein, the SU-immunoadhesin was efficiently precipitated from aliquots of the extracellular supernatants by protein A-Sepharose, wheat germ agglutinin-Sepharose, and concanavalin A-Sepharose (Fig. 1A). When supernatants containing the immunoadhesin were analyzed by SDS-PAGE under nonreducing conditions, the immunoadhesin migrated as an approximately 200-kDa protein species (Fig. 1B), consistent with the formation of disulfide-linked homodimers similar to those formed by other immunoadhesins (11, 24, 39).

To purify the SU-immunoadhesin for binding studies, extracellular supernatants containing this protein were passed over a protein A column. The bound SU-immunoadhesin was eluted from the column by using acid conditions which did not impair the ability of the protein to bind Tva (see Materials and

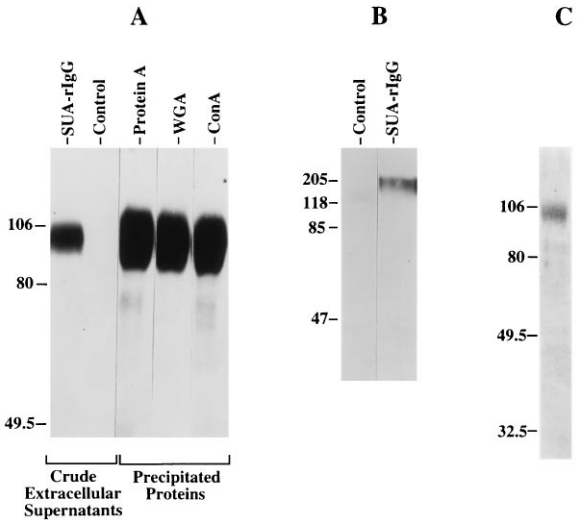

FIG. 1. Expression and purification of the ALSV-A SU-immunoadhesin. (A) Immunoblot analysis of 20-μl fractions of crude extracellular supernatants from transfected 293 cells expressing SUA-rIgG, and from control mock-transfected 293 cells, and of proteins precipitated from SUA-rIgG-containing supernatants by protein A-Sepharose, wheat germ agglutinin-Sepharose (WGA), and concanavalin A-Sepharose (ConA). The samples were reduced with β-mercaptoethanol, subjected to electrophoresis on an 8% polyacrylamide gel containing SDS, and transferred to a nitrocellulose membrane (25). The membrane was incubated with HRP-coupled antibodies specific for rabbit immunoglobulins, and the bound antibodies were detected by enhanced chemiluminescence (Amersham). (B) Immunoblot analysis of 25-μl fractions of crude extracellular supernatants from transfected cells expressing SUA-rIgG or from control 293 cells. These experiments were performed as for panel A except that the samples were not reduced prior to electrophoresis. (C) A 2-μg fraction of SUA-rIgG protein that was purified by using a protein A column (as described in Materials and Methods) was subjected to electrophoresis under reducing conditions on an 8% polyacrylamide gel containing SDS. The polyacrylamide gel was stained with Coomassie Blue and photographed. Sizes are indicated in kilodaltons.

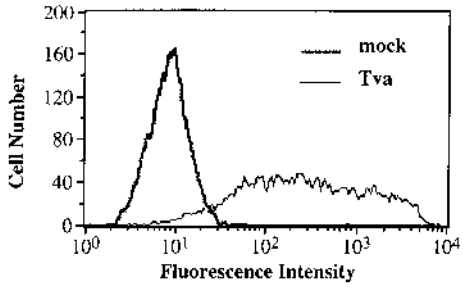

FIG. 2. ALSV-A SU-immunoadhesin specifically bound transfected human 293 cells expressing HA-Tva. Human 293 cells were transfected with 20 μg of plasmid DNA encoding the wild-type HA-Tva protein. The transfected cells and control mock-transfected 293 cells were incubated with crude extracellular supernatants containing SUA-rIgG and with a fluoresceinated antibody specific for rabbit immunoglobulins. The mock-transfected cells (gray line) had a mean fluorescence of only 9.4, while those expressing HA-Tva (black line) had a mean fluorescence of 695.5.

Methods). Analysis of an aliquot of the purified sample by SDS-PAGE under reducing conditions, followed by Coomassie blue staining of the polyacrylamide gel, revealed that the SU-immunoadhesin was highly purified by this procedure (Fig. 1C). The identity of the approximately 100-kDa protein (Fig. 1C) as the immunoadhesin was confirmed by its immunoreactivity with HRP-conjugated antibodies specific for rabbit immunoglobulins and by the fact that this protein was not recovered when the same protocol was used to purify proteins from extracellular supernatants of control human 293 cells (data not shown).

Measurement of the binding affinities between the ALSV-A SU-immunoadhesin and different forms of Tva. Flow cytometric analysis was used to measure the binding affinities of the SU-immunoadhesin and different forms of Tva expressed at the surfaces of transfected 293 cells. To compare levels of receptor expression, wild-type and mutant versions of a Tva protein (designated HA-Tva) were used, with three copies of an epitope tag specific for the 12CA5 monoclonal antibody introduced near the amino terminus of the extracellular region (see Materials and Methods). The relative cell surface levels of each of these receptors were determined by flow cytometry using the 12CA5 antibody and FITC-conjugated antibodies specific for mouse immunoglobulins (see Materials and Methods). This modification to Tva had no effect on the ability of the protein to mediate subgroup A viral entry (data not shown).

The initial experiments were performed by incubating transfected 293 cells that expressed wild-type HA-Tva, and control 293 cells, with crude extracellular supernatants containing the SU-immunoadhesin and with FITC-conjugated antibodies specific for rabbit immunoglobulins. The SUA-rIgG protein bound specifically to cells expressing HA-Tva (Fig. 2). Virtually all of the transfected human 293 cells expressed HA-Tva, demonstrating a transient transfection efficiency of nearly 100% in these experiments (Fig. 2).

Following these preliminary studies, several improvements were made to the flow cytometry protocol. First the SU-immunoadhesin was purified to an estimated 80 to 90% homogeneity (Fig. 1C), and the purified protein was conjugated to FITC. This modification did not affect the ability of SUA-rIgG to bind Tva (data not shown) but allowed direct measurements of binding affinities without any contributions from fluoresceinated secondary antibodies. Second, reduced amounts of plasmid DNA encoding HA-Tva proteins were used for transfection, so that less of the SU-immunoadhesin would be required for saturating levels of binding.

To determine the binding affinity for wild-type HA-Tva, transfected 293 cells expressing this protein were incubated with different amounts (0.3 nM to 0.3 μM) of the fluoresceinated SU-immunoadhesin (Fig. 3A). The SUA-rIgG protein bound to the cells in a concentration-dependent manner, and saturable levels of binding were obtained with concentrations in excess of 30 nM (Fig. 3A). These data were used to measure a K_D of 1.5 nM for the interaction between the fluoresceinated SU-immunoadhesin and wild-type HA-Tva (Fig. 3A). These results were corrected for nonspecific binding by subtracting the values of fluorescence obtained after incubating the SU-immunoadhesin with mock-transfected 293 cells (Fig. 3A). The levels of nonspecific binding were minimal but increased slightly when 0.3 μM to 1 μM SU-immunoadhesin was used (Fig. 3A).

The same type of analysis was used to measure the affinities of binding between the SU-immunoadhesin and different mutant forms of Tva (Fig. 3B to F). Saturable levels of binding were obtained with the W48I, W48L, W48Y, W48F, W48V, and C28A/C41A mutant receptors (Fig. 3D to F) but not with the D46E, D46N, D46A, E47Q, E47D, E47A, W48A, C41A/C50A, and C28A/C35A mutant receptors (Fig. 3B, C, D, and F). These experiments were performed under equilibrium binding conditions: cells expressing each of the mutant receptors demonstrated maximal levels of binding to a performed complex of the SUA-rIgG protein (final concentration of 30 nM), and an FITC-conjugated antibody specific for rabbit immunoglobulins, after a 60-min incubation period at 4°C (data

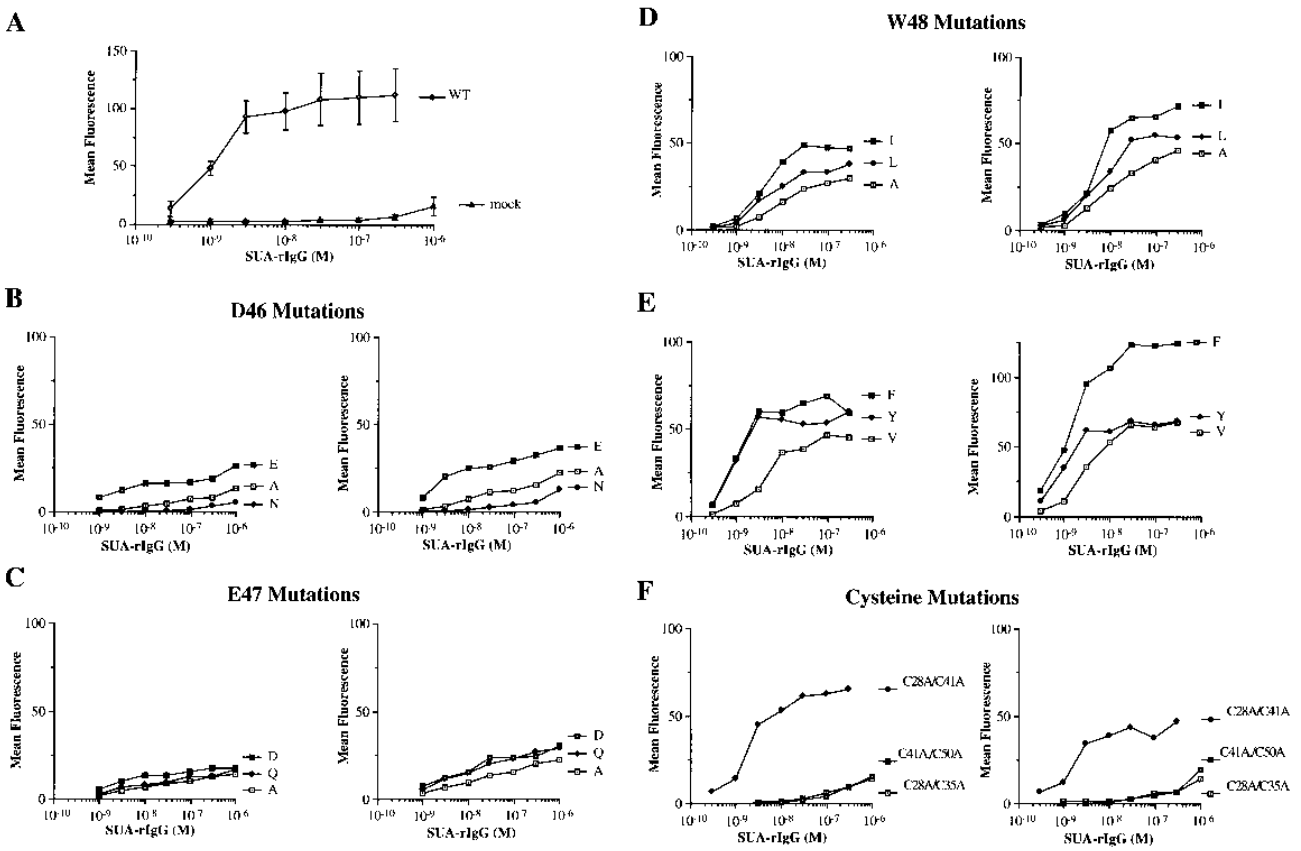

FIG. 3. Measurement of the relative binding affinities of different mutant Tva proteins for the ALSV-A SU-immunoadhesin. Human 293 cells were transfected with 2.5 μg of plasmid DNA encoding wild-type and mutant HA-Tva proteins. These cells were incubated with different amounts of the fluoresceinated SUA-rIgG protein and analyzed by flow cytometry. (A) Comparison of the mean fluorescence values obtained in 10 independent experiments with cells expressing wild-type HA-Tva compared with the values obtained with mock-transfected 293 cells. The levels of nonspecific binding to mock-transfected cells at each concentration of the SU-immunoadhesin used were subtracted from the datum points shown for the cells expressing HA-Tva. (B to F) Two separate experiments in which transiently transfected 293 cells expressing different HA-Tva proteins were incubated with different amounts of the fluoresceinated SUA-rIgG protein and the cells were analyzed by flow cytometry. These measurements were corrected for nonspecific binding of the SU-immunoadhesin as described above. Total levels of receptor expression varied by no more than a factor of 2 within each group of mutations tested, as judged by flow cytometry using the 12CA5 antibody and an FITC-conjugated antibody specific for mouse immunoglobulins and also by immunoblot analysis using the 12CA5 antibody and HRP-conjugated antibodies specific for mouse immunoglobulins (data not shown).

not shown). The K_D measurements were precise for those receptors that achieved saturable levels of binding; for the other receptors, these values represent the highest possible binding affinities for the SU-immunoadhesin and are accurate within a 5-fold to 10-fold range with the concentrations of the SU-immunoadhesin used. Although it is likely that the latter class of mutant receptors have even lower binding affinities, it was not possible to perform these experiments with increased amounts of the SU-immunoadhesin because of the large amount of nonspecific binding when cells were incubated with higher concentrations of this reagent (Fig. 3A).

Previous studies demonstrated the importance of an acidic residue at position 46 of Tva for efficient subgroup A viral entry (55). There is a similar requirement for an acidic residue at this position for high-affinity binding to the SU-immunoadhesin. Replacement of Asp-46 with alanine or asparagine led to at least 44.9- or 138.4-fold reduced binding affinity, respectively (Table 1). However, replacing Asp-46 with another acidic residue, glutamic acid, reduced the binding affinity only by approximately 2.5-fold (Table 1). The putative disulfide bond between Cys-35 and Cys-50, which is predicted to be important for subgroup A viral entry (6), also appears to be important for high-affinity binding. Compared with the wild-type receptor, the binding affinities of the C41A/C50A and C28A/C35A mutant receptors, which are unable to form this putative disulfide bond, were at least 191.8- and 103.6-fold reduced, respectively (Table 1). In contrast, the C28A/C41A mutant receptor, which has the potential to form the putative disulfide bond, bound the SU-immunoadhesin with an affinity that was approximately 1.5-fold lower than that obtained with the wild-type receptor (Table 1).

The Glu-47 residue, which was less important for viral entry (55), also appeared to be less important for binding ALSV-A SU. Replacement of this residue with aspartic acid, glutamine, and alanine led to at least 3-, 8.9-, and 12.1-fold reductions in binding affinity for the SU-immunoadhesin (Table 1). However, as described above, it is possible that these mutant receptors bind the SU-immunoadhesin with affinities even lower than those measured, and therefore this residue might be more important for binding ALSV-A SU than these data suggests.

Substitutions at Trp-48 that drastically reduced viral entry had relatively little effect on binding the SU-immunoadhesin compared with other residues tested. Substituting this residue with valine, isoleucine, leucine, and alanine led to approximately 2.5-, 3.1-, 3.4-, and 6.7-fold reductions in binding affinity for the SU-immunoadhesin, whereas replacing this residue

TABLE 1. Affinities of binding between wild-type and mutant Tva proteins and the SUA-rIgG protein[a]

Receptor	K_D (nM)		
	Expt 1	Expt 2	Avg
WT	NA	NA	1.5
D46E	3.9	3.7	3.8*
D46N	214.0	212.4	213.2*
D46A	78.5	59.8	69.2*
E47D	2.7	6.4	4.6*
E47Q	15.6	11.8	13.7*
E47A	18.0	19.2	18.6*
W48Y	1.3	1.0	1.1
W48F	1.5	1.4	1.4
W48V	4.7	2.9	3.8
W48I	4.3	5.4	4.8
W48L	5.4	5.1	5.3
W48A	9.6	11.0	10.3*
C28A/C41A	2.6	2.0	2.3
C28A/C35A	141.8	177.3	159.6*
C41A/C50A	229.1	361.7	295.4*

[a] The K_D values were calculated from the data in Fig. 3 for wild-type (WT) and mutant receptors, using the previously described equation $K_D = \{([\text{SU-IgG}] \cdot F_L\text{max})/F_L - [\text{Su-IgG}]\}$ where $F_L$max is the maximum measured mean fluorescence for each receptor analyzed and F_L represents the mean fluorescence at a given concentration of fluoresceinated SUA-rIgG (36, 54). The use of this equation to calculate relative binding affinities is based on two assumptions: first, that the amount of fluoresceinated SUA-rIgG bound to the receptor is small compared with the level of free reagent, and second, that the levels of fluorescence accurately reflect the relative level of fluoresceinated SUA-rIgG binding to the receptor, up to a measurable maximum ($F_L$max). For those receptors that achieved saturable binding, this analysis allowed an accurate measurement of their binding affinities for the immunoadhesin. For those receptors that did not reach saturable binding, the maximum fluorescence value obtained was used as $F_L$max, allowing a reasonably accurate (within 5- to 10-fold) measurement of their binding affinities (indicated by asterisks). To calculate the K_D values, the values of F_L used for each receptor were those within the linear ranges of SUA-rIgG binding. The average binding affinity measured for the wild-type receptor was calculated from the data in Fig. 3A. NA, not applicable.

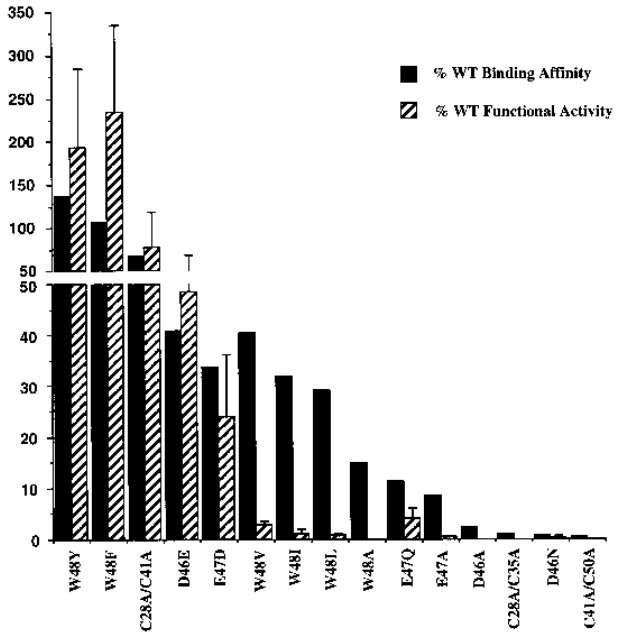

FIG. 4. Comparison of the functional activities of mutant Tva proteins and their relative binding affinities for the ALSV-A SU-immunoadhesin. The binding affinities of each mutant receptor were expressed as a percentage of the wild-type receptor (WT) levels, using the data from Table 1. For this analysis, the maximum possible binding affinities obtained for the C41A/C50A, C28A/C35A, W48A, Asp-46, and Glu-47 mutant receptors were used. The functional activities of the different mutant receptors were also expressed as a percentage of the wild-type receptor levels and were taken from previously described data (6, 55).

with tyrosine and phenylalanine actually increased the binding affinity by 1.4- and 1.1-fold, respectively (Table 1). Consistent with their higher binding affinities, the W48Y and W48F receptors also functioned at slightly better than wild-type levels (55).

To determine whether the effects of mutating Tva on viral entry can be explained solely by changes in the binding affinity for ALSV-A SU, the relative functional activities of each Tva protein and their relative binding affinities for the SU-immunoadhesin were directly compared. The relative functional activities of the W48Y, W48F, and C28A/C41A mutant receptors were closely correlated with their relative binding affinities for the SU-immunoadhesin (Fig. 4). For example, compared with the wild-type receptor, the C28A/C41A mutant receptor bound the SU-immunoadhesin with an approximately 1.5-fold-lower affinity and permitted viral entry at approximately 1.7-fold-lower levels (Fig. 4). The same relationship seems to hold true for the relative functional activities and relative maximal binding affinities of the D46E, E47D, E47Q, and D46N mutant receptors (Fig. 4).

In contrast, the binding affinities of the W48V, W48I, and W48L mutant receptors correlated poorly with their functional activities (Fig. 4). These mutant receptors displayed approximately 2.5- to 3.4-fold reductions in binding affinity for the SU-immunoadhesin but permitted 43- to 200-fold-lower levels of viral entry than the wild-type receptor (Fig. 4). The relative functional activities of the E47A and W48A mutant receptors also did not appear to be correlated with their binding affinities (Fig. 4). Furthermore, the functional activity of the D46A mutant receptor cannot simply be explained by a corresponding reduction in the binding affinity for ALSV-A SU. This completely defective receptor appeared to bind the SU-immunoadhesin with an affinity higher than that observed with the D46N mutant receptor, which functioned at partial levels (Fig. 4). However, more precise binding measurements are needed for the E47A, W48A, and D46A mutant receptors before a more accurate comparison can be made with their relative functional activities.

DISCUSSION

In these experiments, the binding affinities of mutant Tva proteins for an ALSV-A SU-immunoadhesin were measured. These studies have demonstrated that for high-affinity binding to ALSV-A SU, residue Asp-46 and the putative disulfide bond between Cys-35 and Cys-50 are important determinants, Glu-47 appears to be less important, and Trp-48 is the least important residue. Not surprisingly, the highest-affinity interactions were obtained with mutant Tva proteins that preserved the acidic character of residues at position 46 and 47, the aromatic character of the residue at position 48, and the putative disulfide bond between Cys-35 and Cys-50 (Fig. 3 and 4). These are the same amino acid side chain requirements as those described previously for most efficient receptor function (6, 55).

If Tva serves only as a binding receptor for ALSV-A, the functional activities of all of the mutant receptors tested should have been closely correlated with their relative binding affinities for the subgroup A SU-immunoadhesin. For example, effects that mutations of CD4 have on viral entry can be explained by corresponding changes in the binding affinity for

HIV type 1 SU (8). However, this was not the case for a number of the mutant Tva receptors tested, especially those with amino acid substitutions at Trp-48. There are several possible explanations for the lack of a direct correlation between the functional activities of mutant Tva proteins and their relative binding affinities for the ALSV-A SU-immunoadhesin. First, these binding measurements, obtained with the homodimeric SU-immunoadhesin may not precisely reflect the binding properties of virus-associated SU: ALSV-A SU proteins are normally produced as a trimeric complex in association with the viral TM proteins on the surfaces of cells and viral particles (18). Testing this possibility will require the use of either large amounts of a soluble ALSV-A Env trimer to perform similar binding studies with cells expressing different forms of Tva or, alternatively, the use of large amounts of soluble wild-type and mutant Tva proteins for binding studies with cell surface-associated Env or virus-associated Env. Second, the binding measurements performed at 4°C might not faithfully reflect the affinities between the Env and Tva proteins at the higher temperatures used for viral infection assays (37°C). We were unable to use the 293 transient transfection system to study these binding interactions at this higher temperature because these cells did not tolerate incubation at 37°C in BCS supplemented phosphate-buffered saline (data not shown). Third, perhaps more subtle differences in binding (e.g., different on rates and off rates) that were not revealed by the binding affinity measurements might be crucial for the viral entry process.

The fourth and most interesting possibility is that Tva not only binds ALSV-A SU but also participates in postbinding events that lead to viral entry. This idea is consistent with the recent observation that a soluble form of Tva is capable of inducing temperature-dependent structural alterations in a soluble ALSV-A Env trimer that are similar to the changes expected for the fusogenic form of the viral protein (22). If this model is correct, the entry defects associated with the W48V, W48I, W48L, and W48A mutant receptors could be mainly due to deficiencies at a step subsequent to binding SU, because each of these mutations significantly reduced or abolished viral infectivity without a correspondingly large decrease in the binding affinity for the SU-immunoadhesin. In summary, if Tva participates in postbinding steps of viral entry, residue Trp-48 is predicted to be at least one important determinant for stimulating these events.

ACKNOWLEDGMENTS

This work was supported by NIH grant R29 CA62000 and in part by funds provided by the Gladstone Institute of Virology and Immunology.

We thank members of the Young laboratory and David Knipe and Cathy Lee for critical reading of the manuscript, and we thank Wylie Marshall and Mark Feinberg for generously providing reagents and for stimulating discussions. Also, we thank Amy Imrich at the Harvard School of Public Health for providing excellent assistance with the flow cytometry.

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