JOURNAL OF VIROLOGY, Dec. 1995, p. 7410–7415
0022-538X/95/$04.00+0

Vol. 69, No. 12

Receptor-Induced Conformational Changes in the Subgroup A Avian Leukosis and Sarcoma Virus Envelope Glycoprotein

JOANNA M. GILBERT,[1]† LORRAINE D. HERNANDEZ,[1,2] JOHN W. BALLIET,[3] PAUL BATES,[3] AND JUDITH M. WHITE[2]*

Department of Biochemistry, University of California, San Francisco, California[1]; Department of Cell Biology, University of Virginia, Charlottesville, Virginia[2]; and Department of Microbiology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania[3]

Received 26 May 1995/Accepted 23 August 1995

We recently reported that Tva, the host cell receptor for subgroup A avian leukosis and sarcoma viruses, binds specifically to the subgroup A envelope glycoprotein (Env-A) (J. M. Gilbert, P. Bates, H. E. Varmus, and J. M. White, J. Virol. 68:5623–5628, 1994). Here we have tested the hypothesis that binding of Tva causes conformational changes in Env-A that correlate with its conversion from a fusion-inactive to a fusion-active state. Conformational changes were examined by both a proteolysis and an immunoprecipitation assay. A temperature-dependent conformational change, demonstrated by the generation of a specific thermolysin digestion product of the surface (SU) subunit, occurred when a soluble form of Tva (sTva) was incubated with Env-A. sTva did not induce this conformational change in Env-C or in a noninfectious precursor form of Env-A, Env-A CL. However sTva did induce the conformational change in Env-A CL that had been pretreated in vitro to produce the SU and transmembrane (TM) subunits. Moreover, interaction of Tva with Env-A at 25°C, but not at 4°C, appeared to reveal a previously buried segment of the putative fusion peptide of Env-A. Our results suggest that binding of Tva to Env-A results in specific conformational changes in the Env-A glycoprotein that are relevant to the activation of its fusion function.

To infect a cell, a virus must first bind to the cell surface (17, 28). For enveloped viruses, this task is accomplished by transmembrane glycoproteins that project from the viral membrane (21). The next stage in the life cycle of an enveloped virus is fusion with a cellular membrane. For the well-characterized influenza virus, fusion occurs with the endosomal membrane in response to low pH. Biochemical and biophysical studies with the influenza virus hemagglutinin (HA) have shown that when the HA protein is exposed to low pH, the protein undergoes dramatic conformational changes (3, 32). These alterations transform HA into an active fusion protein. Studies indicate that the conformation changes occur in two steps (33). In the first step, the previously buried fusion peptides are exposed; in the second step, the globular head domains separate substantially from one another (2, 10, 13, 33). The low-pH form of HA interacts with membranes since it is energetically unfavorable for the exposed hydrophobic fusion peptides to exist in an aqueous environment. Interaction of the fusion peptides with the target membrane initiates membrane fusion (11, 25, 26).

Unlike influenza virus, most retroviruses, including the avian leukosis and sarcoma viruses (ALSV), enter cells in a pH-independent fashion (9, 18, 19, 27). Very little is known about the molecular mechanisms of proteins that mediate fusion at neutral pH. It is thought that interactions between neutral-pH fusion glycoproteins and their receptors, perhaps with the aid of additional fusion factor(s), trigger conformational changes that convert them to their fusogenic forms (28, 30, 31). By analogy with HA, such conformational changes would include release of the hydrophobic fusion peptides from the interior of the fusion protein oligomer. Support for the hypothesis of receptor-induced conformational changes in neutral-pH fusion proteins has come from studies on the human immunodeficiency virus (HIV), another pH-independent retrovirus. It has been shown that binding of CD4 to the HIV envelope glycoprotein (Env) results in changes in the reactivity of Env with specific monoclonal antibodies (20, 24) as well as changes proteolytic susceptibility (23). However, the significance of these CD4-induced changes for the fusion activity of the HIV Env are not yet clear (15, 24, 28, 31, 35).

Previously it has been demonstrated that Rous sarcoma virus (RSV), a member of the ALSV family, infects host cells in a pH-independent fashion (9). It was shown that although the virus could bind to host cells at 4°C, fusion would occur only at temperatures greater than 22°C. These studies concluded that there must be postbinding, temperature-dependent changes in order for RSV to fuse with host cells.

The cDNA encoding a receptor for subgroup A ALSV, denoted Tva (for tumor virus a), was recently cloned (1a, 36) and shown to be the product of the genetic locus (*tva*) conferring susceptibility to infection by subgroup A ALSV (1). The predicted amino acid sequence of Tva indicates that it encodes a small transmembrane protein in which the extracellular domain contains a region that has similarity to the ligand binding domain of the low density lipoprotein receptor (1a). Cells transfected with *tva* become susceptible to infection by subgroup A ALSV. We have recently shown that Tva binds specifically to the subgroup A envelope glycoprotein, Env-A (7). Importantly, this specific binding does not disrupt the Env-A oligomer (6a, 7). In addition, a soluble form of Tva binds to subgroup A viruses and blocks the ability of subgroup A viruses to infect susceptible host cells (4). This subgroup specificity indicates that Tva is the primary virus receptor for subgroup A ALSVs.

In this study, we investigated whether the interaction be-

* Corresponding author. Mailing address: Department of Cell Biology, University of Virginia, Health Sciences Center, Box 439, Charlottesville, VA 22908. Phone: (804) 924-2593. Electronic mail address: jw7g@virginia.edu.

† Present address: Departments of Microbiology and Immunology and Gastroenterology, Stanford University School of Medicine, Stanford, Calif.

tween Env-A and its receptor, Tva, results in conformational changes in the Env-A glycoprotein that are related to the activation of its fusion function. We analyzed conformational changes by examining differences in proteolytic susceptibility and anti-fusion peptide antibody reactivity. Our results support a model in which interaction of Tva with Env-A elicits time- and temperature-dependent conformational changes that are relevant to the fusion activity of Env-A.

MATERIALS AND METHODS

Recombinant DNA. The plasmids containing cDNAs encoding either Env-A or the C subgroup envelope glycoprotein (Env-C) of RSV were subcloned into the vector pCB6 as described previously (7). The dibasic cleavage site of Env-A was altered by introducing two amino acid changes as described by Perez and Hunter for Env-C (amino acids 338 and 340 of the mature surface [SU] subunit; RRKR to RSKE [22]) by site-directed mutagenesis (14). This construct is referred to as Env-A CL. The subgroup A receptor, Tva, and the secreted form of Tva, sTva, are as described in reference 7.

Tissue culture. Stable NIH 3T3 cell lines were established by transfection of constructs by the $CaPO_4$-DNA coprecipitation method (34) and selection of Geneticin (Gibco, Grand Island, N.Y.)-resistant colonies as described previously (7). To induce higher levels of protein expression (7), cells were treated with sodium butyrate for 16 h: 25 mM for Env-A and Env-C, 10 mM for Tva, and 5 mM for Env-A CL.

Antibodies and reagents. The rabbit polyclonal antisera against the carboxyl-terminal cytoplasmic tails of Env-A and Env-C are as described previously (7). Polyclonal antibodies against a synthetic peptide corresponding to the putative fusion peptide of the ALSV envelope glycoprotein (amino acids 22 to 37 of the TM domain) were raised in rabbits. Three separate peptides (peptide 2, amino acids 22 to 30; peptide 3, amino acids 27 to 35; and peptide 4, amino acids 29 to 37) comprising overlapping segments of the fusion peptide sequence were individually prepared and coupled to cyanogen bromide-activated Sepharose CL-4B (Sigma Chemical Company, St. Louis, Mo.) as instructed by the manufacturer. Anti-fusion peptide antibodies were affinity purified against each of these peptides and are referred to as fusion peptide antibodies 2, 3, and 4 (see Fig. 5A).

Cell surface labeling, immunoprecipitation, and coimmunoprecipitation. After overnight induction with sodium butyrate, cells expressing Env-A, Env-A CL, Env-C, or Tva were labeled with the membrane-impermeant biotinylation reagent NHS-LC-biotin (Pierce Chemical Company, Rockford, Ill.), and proteins were immunoprecipitated (or coimmunoprecipitated) as described previously (7). Immunoprecipitates were washed and then processed for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (7) at the indicated acrylamide concentration or were used for thermolysin digestion assays (see below). Following electrophoresis, proteins were transferred to nitrocellulose (Schleicher & Schuell, Keene, N.H.) and probed with streptavidin coupled to horseradish peroxidase (Pierce) to detect biotinylated proteins. The horseradish peroxidase signal was detected by enhanced chemiluminescence (Amersham, Arlington Heights, Ill.) (8). All autoradiographs were scanned with a model UC1260 UMAX scanner. The figures were prepared by using the Photoshop and Canvas programs.

Sucrose gradient centrifugation. For sucrose density gradient centrifugation, a cell lysate containing biotinylated Env-A CL was loaded onto a 10 to 30% linear sucrose gradient in *N*-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; Sigma)-buffered saline containing 40 mM octylglucoside and centrifuged in an SW41 rotor for 17 h at 275,000 × *g*. The gradient was fractionated and processed for the detection of Env-A CL protein as described previously (8).

Thermolysin digestion assay. For thermolysin digestion assays, biotinylated cell lysates containing Env glycoproteins were immunoprecipitated with the relevant anti-cytoplasmic tail antibodies, washed, and then incubated with 12.5 μl of sTva (0.25 μg of total protein) or an equal protein concentration of supplemented calf serum (determined by modified Lowry assay [16]) in a final volume of 50 μl of lysis buffer (HEPES-buffered saline containing 1% Nonidet P-40 [Sigma]). Samples were incubated first at 4°C for 30 min and then at 4, 22, or 37°C for the indicated times. Samples were then washed three times in cold lysis buffer, resuspended in cold lysis buffer containing thermolysin (15 μg/ml unless indicated otherwise; Sigma) and 100 μM $CaCl_2$, and digested for 20 min at 4°C. The reactions were stopped by addition of excess lysis buffer containing 1 mM EDTA. The samples were then processed for SDS-PAGE as described previously (7).

Precleavage of Env-A CL. Cells expressing Env-A CL were biotinylated with NHS-LC-biotin, lysed, and immunoprecipitated with the anti-Env-A cytoplasmic tail antibody as described above. The immunobead complexes were washed four times with cold lysis buffer and then incubated at 4°C with 50 μg of thermolysin per ml for 60 min. The reaction was quenched by washing the bead complexes with cold lysis buffer containing 2 mM EDTA.

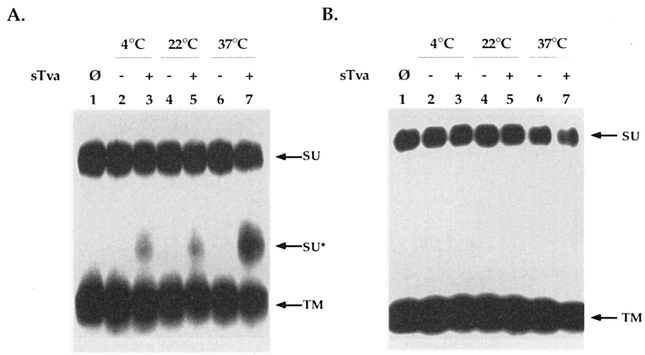

FIG. 1. Thermolysin digestion of Env-A and Env-C in the absence and presence of sTva. Cells expressing Env-A (A) or Env-C (B) were cell surface biotinylated with NHS-LC-biotin, lysed, immunoprecipitated with the appropriate anti-Env cytoplasmic tail antibodies, and then washed. Samples were incubated with either bovine serum protein (−; lanes 2, 4, and 6) or sTva (+; lanes 3, 5, and 7) at 4°C for 30 min and then at the indicated temperature for 30 min. At this time, the samples were digested with thermolysin on ice as described in Materials and Methods for the thermolysin digestion assay. Samples were subjected to SDS-PAGE (11% acrylamide) and transferred to nitrocellulose, and biotinylated proteins were detected by enhanced chemiluminescence as described in Materials and Methods. The SU and TM subunits and SU* migrate as indicated. Control, undigested Env-A (A) and Env-C (B) are shown in lanes 1.

RESULTS AND DISCUSSION

sTva induces a specific thermolysin digestion product in Env-A but not in Env-C. To address whether the binding of Tva induces any conformation changes in Env-A, we performed a series of proteolysis studies. Differences in proteolytic susceptibility have been used to demonstrate conformational changes in a variety of proteins (5, 6). For example, the low-pH form of HA differs from native HA in susceptibility to digestion with trypsin, thermolysin, and proteinase K (5, 29). For experiments with Env, biotinylated Env-A and Env-C were immunoprecipitated from cell lysates with the appropriate anti-cytoplasmic tail antibodies. The washed precipitates were then incubated with either sTva or an equal amount of bovine serum protein, as a negative control, for 30 min at 4°C to allow binding of Env-A to sTva. Previous experiments showed that with this amount of sTva, complete binding to Env-A occurs within 10 min at 4°C (data not shown). Following binding, samples were incubated for an additional 30 min at the indicated temperature, placed on ice, and then digested with the protease thermolysin. A specific proteolytic fragment was produced in samples of Env-A (Fig. 1A, lanes 3, 5, and 7) but not of Env-C (Fig. 1B, lanes 3, 5, and 7). No proteolysis occurred in the absence of sTva (Fig. 1A and B, lanes 2, 4, and 6), and no proteolysis occurred when Env-A and sTva were incubated in the absence of thermolysin (data not shown). The cleavage product clearly results from the digestion of the SU (~80-kDa) subunit of Env-A, since the observed mobility of the band (~50 kDa) is greater than the that of TM (~37-kDa) subunit. Accordingly, we refer to this cleavage product as SU*. The expected corresponding fragment, ~32 kDa, is not seen. Upon examination of the material released from the immunobead complexes during the thermolysin digestion assay, a 32-kDa band, SU**, is detected (not shown). The production of SU* was substantially greater in Env-A samples that had been incubated with sTva at 37°C than in samples incubated at 22 or 4°C (Fig. 1A, lanes 3, 5, and 7). When incubations were performed with two- to fivefold-lower concentrations of sTva, correspondingly less SU* was produced (data not shown). Digestions performed with either higher or lower concentrations of thermolysin (0.15 to 45 μg/ml) did not result in the produc-

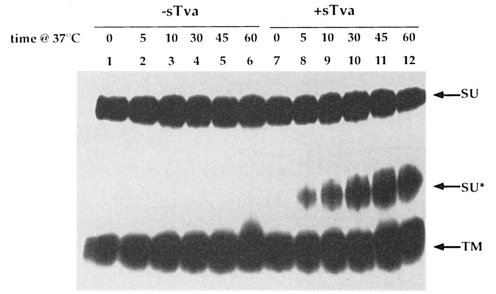

FIG. 2. Time course of SU* production. Biotinylated Env-A was immunoprecipitated and washed as described in Materials and Methods. Samples were then incubated with either bovine serum protein (lanes 1 to 6) or sTva (lanes 7 to 12) at 4°C for 30 min and then at 37°C for the indicated times. Samples were digested with thermolysin on ice as described in Materials and Methods, subjected to SDS-PAGE (11% acrylamide), and transferred to nitrocellulose, and biotinylated proteins were detected as described in the legend to Fig. 1. The SU and TM subunits and SU* migrate as indicated.

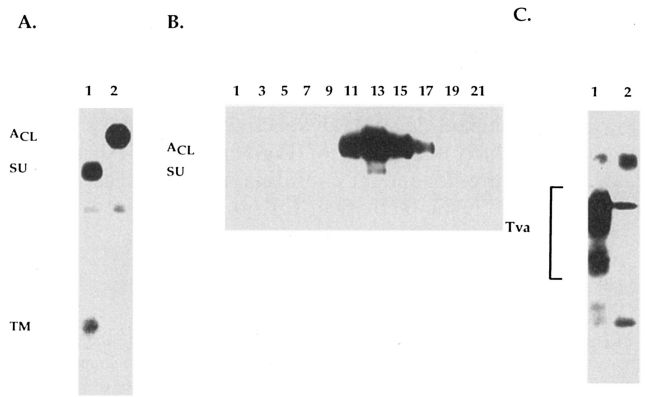

FIG. 3. (A) Cell surface expression. Cell surface-biotinylated Env-A (lane 1) or Env-A CL (lane 2) was immunoprecipitated and washed as described for Fig. 2. Samples were subjected to SDS-PAGE (9% acrylamide) and transferred to nitrocellulose, and biotinylated proteins were detected as described in the legend to Fig. 1. The uncleaved molecule, Env-A CL (A_{CL}), and the SU and TM subunits migrate as indicated. (B) Sucrose density centrifugation. Biotinylated Env-A CL was subjected to sucrose density centrifugation on a 10 to 30% linear sucrose gradient containing 40 mM octylglucoside. Fractions were collected, immunoprecipitated, subjected to SDS-PAGE (9% acrylamide), and transferred to nitrocellulose. Biotinylated proteins were detected as described in the legend to Fig. 1. Env-A CL migrates as indicated, with the peak in fraction 13. This is very similar to the migration of the wild-type Env-A transmembrane glycoprotein (8). Lane 1 contains the lightest fraction; lane 23 contains the heaviest fraction. (C) Interaction with Tva. Cells expressing either Tva or parental NIH 3T3 cells were cell surface biotinylated and lysed. Unlabeled lysates of cells expressing Env-A CL were mixed with lysates of either biotinylated Tva-expressing cells (lane 1) or NIH 3T3 cells (lane 2) and then immunoprecipitated with anti-Env-A cytoplasmic tail antibodies. Samples were subjected to SDS-PAGE (9% acrylamide) and transferred to nitrocellulose, and biotinylated proteins were detected as described in the legend to Fig. 1. Tva migrates as two major heterogeneous bands (7), as indicated. The Env A cytoplasmic tail antibody does not precipitate Tva in the absence of Env A protein (not shown).

tion of either more SU* or any additional sTva-specific bands. At the highest concentration of thermolysin tested (45 μg/ml), nonspecific, sTva-independent digestion of the SU subunit was observed (data not shown).

Time dependence of SU* formation. To examine the time dependence of SU* formation, biotinylated Env-A was immunoprecipitated with anti-Env-A cytoplasmic tail antibodies, washed, and then incubated with either sTva (Fig. 2, lanes 7 to 12) or bovine serum protein (Fig. 2, lanes 1 to 6) at 4°C for 30 min. Samples were then incubated at 37°C for times ranging from 0 to 60 min. The samples were then placed on ice and digested with thermolysin for 20 min. When Env-A was incubated with sTva at 37°C, SU* was produced in a time-dependent fashion. SU* was visible within 5 min of incubation at 37°C and reached an apparent maximum within 45 min (Fig. 2, lanes 8 and 11, respectively). SU* was not detected when Env-A was incubated with bovine serum protein rather than sTva (Fig. 2, lanes 1 to 6). The levels of SU* produced when Env-A was incubated with sTva at either 4 or 22°C for 30 min before digestion with thermolysin (Fig. 1A, lanes 3 and 5) seemed to be lower than the levels seen when Env-A was incubated with sTva for 5 min at 37°C (Fig. 2, lane 8). Thus, the sTva-induced conformational change in Env-A appears to occur more rapidly at 37°C than at temperatures less than or equal to 22°C. This result correlates well with our previous finding that fusion of RSV (a member of the ALSV family) with chicken embryo fibroblasts occurs only at temperatures greater than 22°C (9). Thus, our results indicate that the extent of the observed conformational change in Env-A (production of SU*) is significantly greater under conditions that are permissive for viral fusion.

Expression and characterization of Env-A CL. To substantiate that the digestion product, SU*, was the result of a fusion-related conformational change and not simply due to a binding interaction between Env-A and sTva at elevated temperatures, we created an uncleaved form of Env-A similar to that described by Perez and Hunter for Env-C (22). This form of Env-A, Env-A CL, has two of the four amino acids within its dibasic cleavage site changed such that the processing in vivo from the precursor form, Pr95, to the mature disulfide-bonded subunits, SU and TM, is markedly diminished (Fig. 3A). Similar to Env-A, Env-A CL is expressed at the cell surface as a trimer (Fig. 3B) when examined by sucrose gradient density centrifugation (8). Like Env-A, Env-A CL is also capable of binding Tva, as demonstrated by a coimmunoprecipitation assay (Fig. 3C). However, when the Env-A CL mutant was incorporated into virions, the particles produced were 4 orders of magnitude less infectious than virions with Env-A. Therefore, the Env-A CL mutant is transported to the cell surface as an oligomer (8) and can bind to the ALSV receptor, but Env-A CL cannot undergo subsequent steps that are necessary for genome penetration.

sTva does not induce a conformational change in the Env-A precursor, Env-A CL. Since Env-A CL is able to bind Tva but cannot infect cells, we predicted that Env-A CL is defective in postbinding, fusion-related conformational changes. To test this hypothesis, biotinylated Env-A CL was immunoprecipitated with anti-Env-A cytoplasmic tail antibodies and then washed. Following incubation with either sTva or control bovine serum protein for 30 min at 4°C, to allow binding, the samples were incubated at 4, 22, or 37°C for 30 min. Samples were then digested with thermolysin for 20 min on ice. No SU* was detected in any of the samples (Fig. 4A), although a band that ran at approximately 85 kDa was seen in all samples (see below). These results suggest that the production of SU* in Env-A is not simply the result of binding of sTva at 37°C, since both Env-A and Env-A CL bind sTva. Therefore, the appearance of SU* is indicative of a postbinding conformational change that does not occur in the uncleaved, noninfectious precursor, Env-A CL.

Production of SU* from thermolysin preactivated Env-A CL. During the course of these experiments, we noted that incubation of Env-A CL with 15 μg of thermolysin per ml for 30 min at 4°C (in the absence or presence of sTva; Fig. 4A) resulted in partial cleavage of Env-A CL to a lower-molecular-

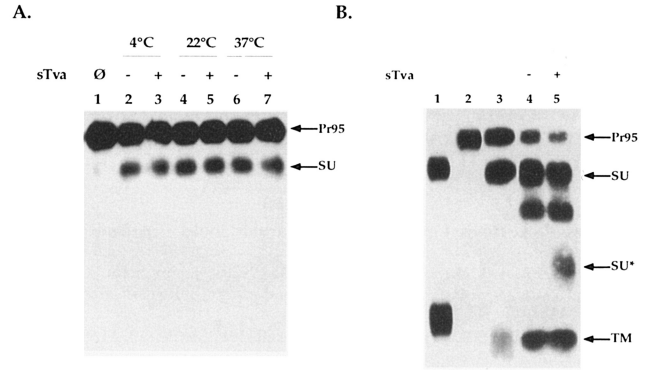

FIG. 4. Thermolysin digestion of Env-A CL in the absence and presence of sTva. (A) Untreated Env-A CL. Cells expressing Env-A CL were cell surface biotinylated, lysed, immunoprecipitated, and washed. Samples were incubated with either bovine serum protein (−; lanes 2 to 6) or sTva (+; lanes 3 to 7) at 4°C for 30 min and then at 4, 22, or 37°C for 30 min and subsequently digested with thermolysin on ice as described in Materials and Methods. Samples were subjected to SDS-PAGE (11% acrylamide) and transferred to nitrocellulose, and biotinylated proteins were detected as described in the legend to Fig. 1. The uncleaved precursor and SU subunit migrate as indicated. Control, undigested Env-A CL is shown in lane 1. (B) Thermolysin-pretreated Env-A CL. Biotinylated Env-A CL was immunoprecipitated, washed, and then precleaved by treatment with 50 μg of thermolysin per ml for 60 min on ice. After addition of EDTA, samples were washed extensively and incubated with either bovine serum protein (−; lane 4) or sTva (+; lane 5) at 4°C for 30 min and then at 37°C for 30 min. Samples were then digested with thermolysin on ice for 20 min as described in Materials and Methods. Samples were subjected to SDS-PAGE (11% acrylamide) and transferred to nitrocellulose, and biotinylated proteins were detected as described in the legend to Fig. 1. The uncleaved precursor, Pr95, the SU and TM subunits, and SU* migrate as indicated. A nonspecific breakdown product of SU, which migrates at approximately 70 kDa, is seen in Env-A CL samples preactivated with thermolysin. The intensity of the 70-kDa band varies from one preparation to another. Control Env-A, undigested Env-A CL, and thermolysin-preactivated Env-A CL are shown in lanes 1, 2, and 3, respectively.

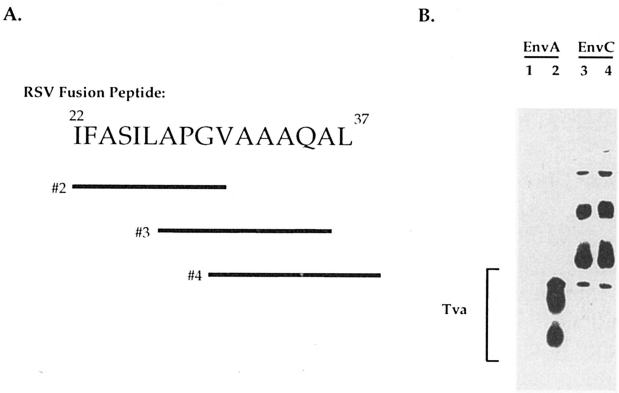

FIG. 5. Reactivity of Env with anti-fusion peptide antibodies. (A) Sequence of the putative fusion peptide of Env and constituent peptides. Amino acid sequences of the putative fusion peptide of RSV Env (amino acids 22 to 37 of the TM subunit) and the peptides used for antibody purification. (B) Tva and Env-A coimmunoprecipitate with anti-fusion peptide 4 antibody at 25°C but not at 4°C. Cells expressing Tva were cell surface biotinylated and mixed with unlabeled lysates of cells expressing Env-A (lanes 1 and 2) or Env-C (lanes 3 and 4). Samples were incubated at either 4°C (lanes 1 and 3) or 25°C (lanes 2 and 4) for 60 min and then immunoprecipitated with anti-fusion peptide antibody 4 as described in Materials and Methods. Samples were then subjected to SDS-PAGE (9% acrylamide) and transferred to nitrocellulose, and biotinylated proteins were detected as described in the legend to Fig. 1. Tva migrates as two major heterogeneous bands, as indicated. The higher-molecular-weight bands in lanes 3 and 4 are nonspecific. If biotinylated lysates from NIH 3T3 cells are mixed with unlabeled Env-C lysates and immunoprecipitated with either peptide 4 antibodies or anti-Env-C cytoplasmic tail antibodies, these same bands are precipitated (data not shown).

weight form that comigrated with SU. This was not unexpected for two reasons: (i) the SU/TM cleavage site must be exposed in the precursor form of the molecule, and (ii) at either side of the native cleavage site, there are residues that fit the specificity of thermolysin. Furthermore, a band corresponding approximately to the size of the TM subunit is observed on longer exposures. Env-A CL could also be cleaved to SU and TM with trypsin (22), but the amount of protein recovered was lower than in digestions performed with thermolysin. In subsequent experiments, we found that treatment of Env-A CL with higher concentrations of thermolysin (50 μg/ml at 4°C, in the absence of sTva) resulted in greater cleavage of Env-A CL. Three bands were seen in all samples of thermolysin-activated Env-A CL: the uncleaved Env-A CL, one that comigrated with SU, and one that migrated slightly faster than TM (Fig. 4B, lanes 1 and 3). If thermolysin cleaves Env-A CL at a site more carboxyl proximal to the native cleavage site, the predicted molecular weight of TM would be decreased. The concomitant increase in molecular weight to SU would not be detected under our SDS-PAGE conditions. We next examined whether, like Env-A, thermolysin-activated Env-A CL could undergo a conformational change upon interaction with sTva. Biotinylated Env-A CL was immunoprecipitated, washed, and then treated at 4°C with 50 μg of thermolysin per ml to precleave Env-A CL to its mature form. The thermolysin digestion was quenched by the addition of EDTA, and following extensive washing, the samples were incubated with or without sTva at 37°C and then processed as described above. When thermolysin-activated Env-A CL was incubated in the presence of sTva at 37°C, SU* was detected (Fig. 4B, lane 5). No SU* was produced when activated Env-A CL was incubated without sTva (Fig. 4B, lane 4). Minimal SU* was produced in samples incubated with sTva at 4 or 22°C (data not shown). An additional band (~70 kDa) was seen in samples treated with or without sTva. The occurrence and intensity of this non-sTva-specific digestion product varied in different experiments. Our results indicate that the Env-A CL molecule can be activated in vitro and that it can subsequently undergo an sTva-induced conformational change similar to that of its wild-type counterpart. These results imply that Env must first be processed to its mature form in order to undergo a Tva-induced conformational change in the SU subunit.

Studies with antibodies against the putative fusion peptide. RSV binds to the host cell surface at 4°C but fuses only when the temperature is raised above 22°C (9). We hypothesize that while some Tva-induced structural rearrangements in Env may occur at 4°C, the transformation of Env to its fusogenic form, with exposed fusion peptides, can be completed only at temperatures above 22°C. In the case of influenza virus HA, the apolar fusion peptides are sequestered in the interior of the trimer until conformational changes are initiated by exposure to low pH (3, 10, 13, 33). By analogy, we examined whether the putative fusion peptide of Env-A is exposed following interaction with Tva at temperatures greater than 22°C. For this purpose we generated anti-peptide antibodies against the putative fusion peptide of RSV, amino acids 22 to 37 of the TM subunit (12) (Fig. 5A). This sequence is absolutely conserved between Env-A and Env-C. The anti-fusion peptide antibodies were then affinity purified, separately, against three distinct but overlapping peptides that span the length of the fusion peptide region (Fig. 5A). The resulting antibodies were tested for the ability to immunoprecipitate Env-A or Env-C in the absence and presence of 0.1% SDS. If, as postulated, segments of the fusion peptides are buried in the native structure, then antibodies to these epitopes should immunoprecipitate Env-A or

Env-C only following SDS denaturation. Antibodies purified against peptide 2 reacted with native Env since they immunoprecipitated Env-A and Env-C in the absence of SDS. Antibodies purified against peptide 3 did not immunoprecipitate Env-A or Env-C under any conditions examined. In contrast, antibodies purified against peptide 4 precipitated Env-A and Env-C but only in the presence of SDS (data not shown). These results indicated that the anti-peptide 4 antibodies react with an epitope that is buried in the native Env oligomer.

Coimmunoprecipitation of Tva with anti-fusion peptide 4 antibody. To examine whether the cryptic peptide 4 epitope is exposed upon binding of Tva to Env-A, we performed a coimmunoprecipitation assay. Cell lysates containing biotinylated Tva were mixed with lysates from an equal number of unbiotinylated Env-A- or Env-C-expressing cells and then incubated for 1 h at either 4 or 25°C. The samples were then immunoprecipitated with affinity-purified anti-peptide 4 antibodies and analyzed for the presence of coimmunoprecipitated, biotinylated Tva. Tva was detected in precipitates that had been incubated with Env-A at 25°C but not at 4°C (Fig. 5B, lanes 1 and 2). Antibodies to peptide 4 did not coimmunoprecipitate Tva in the samples incubated with Env-C at either 4 or 25°C for 1 h (Fig. 5B, lanes 3 and 4). Tva, itself, is not immunoprecipitated by antibodies to peptide 4 (data not shown). We have previously demonstrated that Env-A forms a discrete and stable subgroup-specific complex with Tva at 4°C (7). The finding that the peptide 4 epitope is not exposed when the Env-A–Tva complex is formed at 4°C (Fig. 5B, lane 1) but is exposed when the complex is incubated at 25°C (Fig. 5B, lane 2) indicates that the accessibility of the peptide 4 epitope, a segment of the candidate RSV Env fusion peptide, correlates with the acquisition of fusion competence at temperatures greater than 22°C (9).

Summary. The best-characterized fusion glycoprotein, the influenza virus HA, functions at low pH (reviewed in reference 32). Fusion-inducing conformational changes in HA include the release of the hydrophobic fusion peptides from the trimer interface (3, 10, 13, 33). Many other viruses, including most retroviruses, fuse at neutral pH (9, 18, 19, 27). We and others have hypothesized that an interaction between a retroviral Env glycoprotein and its host cell receptor triggers structural changes necessary to induce fusion (28, 30, 31), but such a mechanism has not yet been demonstrated unequivocally in any case. We have previously documented a specific biochemical interaction between Env-A and its receptor, Tva (7). The Env-A in this complex remains oligomeric and intact (7); there is no shedding of the SU subunit as is seen upon binding of soluble CD4 to the Env glycoproteins of certain strains of HIV (24). In this study, we provide the first evidence that the interaction between Tva and Env-A at fusion-permissive temperatures induces specific conformational changes in both subunits of Env-A: a conformational change in the SU subunit was detected by the generation of a specific proteolytic digestion product, SU*; a conformational change in the TM subunit was detected as the exposure of a segment of the putative fusion peptide. Collectively, our results indicate that binding of the subgroup A receptor, Tva, to Env-A results in conformational changes in Env-A that are relevant to its fusion function. Since sTva alone is sufficient to cause these observed changes in Env-A, additional host factors may not be required to elicit fusion-inducing conformational changes in the ALSV Env glycoprotein.

ACKNOWLEDGMENTS

We thank Tania Chernov-Rogan for generating the anti-fusion peptide antibodies, Luke Hoffman for suggesting thermolysin for the protease assay, and Ramon Tabtiang for help with the figures.

This work was supported by a grant from the National Institutes of Health (RO1 AI22470) to J.M.W. and a Howard Hughes predoctoral fellowship to L.D.H.

REFERENCES

1. **Bates, P., L. Rong, J. A. Young, H. E. Varmus, and L. Crittenden.** Unpublished data.
1a. **Bates, P., J. A. Young, and H. E. Varmus.** 1993. A receptor for subgroup A Rous sarcoma virus is related to the low density lipoprotein receptor. Cell **74:**1043–1051.
2. **Bodian, D. L., R. B. Yamasaki, R. L. Buswell, J. F. Stearns, J. M. White, and I. D. Kuntz.** 1993. Inhibition of the fusion-inducing conformational change of influenza hemagglutinin by benzoquinones and hydroquinones. Biochemistry **32:**2967–2978.
3. **Bullough, P. A., F. M. Hughson, J. J. Skehel, and D. C. Wiley.** 1994. Structure of influenza hemagglutinin at the pH of fusion. Nature (London) **371:**37–43.
4. **Connally, L., K. Zingler, and J. A. Young.** 1994. A soluble form of a receptor for subgroup A avian leukosis and sarcoma viruses (ALSV-A) blocks infections and binds directly to ALSV-A. J. Virol. **68:**2760–2764.
5. **Doms, R. W.** 1993. Protein conformational changes in virus-cell fusion. Methods Enzymol. **221:**61–82.
6. **Fernandes, J., D. Tang, G. Leone, and P. W. K. Lee.** 1994. Binding of reovirus to receptor leads to conformational changes in viral capsid proteins that are reversible upon virus detachment. J. Biol. Chem. **269:**17043–17047.
6a. **Gilbert, J. M.** Unpublished data.
7. **Gilbert, J. M., P. Bates, H. E. Varmus, and J. M. White.** 1994. The receptor for the subgroup A avian leukosis sarcoma viruses binds to subgroup A but not to subgroup C envelope glycoprotein. J. Virol. **68:**5623–5628.
8. **Gilbert, J. M., L. D. Hernandez, T. Chernov-Rogan, and J. M. White.** 1993. Generation of a water-soluble oligomeric ectodomain of the Rous sarcoma virus envelope glycoprotein. J. Virol. **67:**6889–6892.
9. **Gilbert, J. M., D. Mason, and J. M. White.** 1990. Fusion of Rous sarcoma virus with host cells does not require exposure to low pH. J. Virol. **64:**5106–5113.
10. **Godley, L., J. Pfeifer, D. Steinhauer, B. Ely, G. Shaw, R. Kaufmann, E. Suchanek, C. Pabo, J. J. Skehel, and D. C. Wiley.** 1992. Introduction of intersubunit disulfide bonds in the membrane-distal region of the influenza hemagglutinin abolishes membrane fusion activity. Cell **68:**635–645.
11. **Harter, C., P. James, T. Bächi, G. Semenza, and J. Brunner.** 1989. Hydrophobic binding of the ectodomain of influenza hemagglutinin to membranes occurs through the "fusion peptide." J. Biol. Chem. **264:**6459–6464.
12. **Hunter, E., E. Hill, M. Hardwick, A. Bhown, D. E. Schwartz, and R. Tizard.** 1983. Complete sequence of the Rous sarcoma virus *env* gene: identification of structural and functional regions of its product. J. Virol. **46:**920–936.
13. **Kemble, G. W., D. L. Bodian, J. Rose, I. A. Wilson, and J. M. White.** 1992. Intermonomer disulfide bonds impair the fusion activity of influenza virus hemagglutinin. J. Virol. **66:**4940–4950.
14. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods Enzymol. **154:**367–382.
15. **Maddon, P. J., A. G. Dalgleish, J. S. McDougal, P. R. Clapham, R. A. Weiss, and R. Axel.** 1986. The T4 gene encodes the AIDS virus receptor and is expressed in the immune system and the brain. Cell **47:**333–348.
16. **Markwell, M. A. K., S. M. Haas, L. L. Bieber, and N. E. Tolbert.** 1978. A modified procedure to simplify protein determination in membrane and lipoprotein samples. Anal. Biochem. **87:**206–210.
17. **Marsh, M., and A. Helenius.** 1989. Virus entry into animal cells. Adv. Virus Res. **36:**107–151.
18. **McClure, M. O., M. Marsh, and R. A. Weiss.** 1988. Human immunodeficiency virus infection of CD4-bearing cells occurs by a pH-independent mechanism. EMBO J. **7:**513–518.
19. **McClure, M. O., M. A. Sommerfelt, M. Marsh, and R. A. Weiss.** 1990. The pH independence of mammalian retrovirus infection. J. Gen. Virol. **71:**767–773.
20. **Moore, J. P., Q. J. Sattentau, R. Wyatt, and J. Sodroski.** 1994. Probing the structure of the human immunodeficiency virus surface glycoprotein gp120 with a panel of monoclonal antibodies. J. Virol. **68:**469–484.
21. **Nomoto, J. M.** 1992. Cellular receptors for virus infection. Semin. Virol. **3:**77–133.
22. **Perez, L. G., and E. Hunter.** 1987. Mutations within the proteolytic cleavage site of the Rous sarcoma virus glycoprotein that block processing to gp85 and gp37. J. Virol. **61:**1609–1614.
23. **Sattentau, Q. J., and J. P. Moore.** 1991. Conformational changes induced in the human immunodeficiency virus envelope glycoprotein by soluble CD4 binding. J. Exp. Med. **174:**407–415.
24. **Sattentau, Q. P., J. P. Moore, F. Vignaux, F. Traincard, and P. Poignard.** 1993. Conformational changes induced in the envelope glycoproteins of the

human and simian immunodeficiency viruses by soluble receptor binding. J. Virol. **67:**7383–7393.
25. **Stegmann, T., J. M. Delfino, F. M. Richards, and A. Helenius.** 1991. The HA2 subunit of influenza hemagglutinin inserts into the target membrane prior to fusion. J. Biol. Chem. **266:**18404–18410.
26. **Stegmann, T., J. M. White, and A. Helenius.** 1990. Intermediates in influenza-induced membrane fusion. EMBO J. **9:**4231–4241.
27. **Stein, B. S., S. D. Gowda, J. D. Lifson, R. C. Penhallow, K. G. Bensch, and E. G. Engleman.** 1987. pH-independent HIV entry into CD4-positive T cells via virus envelope fusion to the plasma membrane. Cell **49:**659–668.
28. **Weiss, R. A.** 1992. Cellular receptors and viral glycoproteins involved in retrovirus entry. 1-108. Plenum Press, New York.
29. **White, J., J. Kartenbeck, and A. Helenius.** 1982. Membrane fusion activity of influenza virus. EMBO J. **1:**217–222.
30. **White, J. M.** 1990. Viral and cellular membrane fusion proteins. Annu. Rev. Physiol. **52:**675–697.
31. **White, J. M.** 1993. Integrins as virus receptors. Curr. Biol. **3:**596–599.
32. **White, J. M.** 1994. Fusion of influenza virus in endosomes: role of the hemagglutinin, p. 281–301. *In* E. Wimmer (ed.), Cellular receptors for animal viruses. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
33. **White, J. M., and I. A. Wilson.** 1987. Anti-peptide antibodies detect steps in a protein conformational change: Low-pH activation of the influenza virus hemagglutinin. J. Cell Biol. **105:**2887–2896.
34. **Wigler, M., S. Silverstein, L.-S. Lee, A. Pellicer, Y.-C. Cheng, and R. Axel.** 1977. Transfer of purified herpes virus thymidine kinase gene to cultured mouse cells. Cell **11:**223–232.
35. **Yahi, N., S. Baghdiguian, H. Moreau, and J. Fantini.** 1992. Galactosyl ceramide (or a closely related molecule) is the receptor for human immunodeficiency virus type 1 on human colon epithelial HT29 cells. J. Virol. **66:**4848–4854.
36. **Young, J. A., P. Bates, and H. E. Varmus.** 1993. Isolation of a chicken gene that confers susceptibility to infection by subgroup A avian leukosis and sarcoma viruses. J. Virol. **67:**1811–1816.