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Receptor-triggered membrane association of a model retroviral glycoprotein

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ABSTRACT **Current models of retroviral entry hypothesize that interactions between the viral envelope protein and the host receptor(s) induce conformational changes in the envelope protein that activate the envelope protein and initiate fusion. We employed a liposome-binding assay to demonstrate directly and characterize the activation of a model retroviral envelope protein (EnvA) from Rous sarcoma virus (RSV). In the presence of purified viral receptor, the trimeric ectodomain of EnvA was converted from a water-soluble form to a membrane-associated form, consistent with conversion of the envelope protein to its fusogenic state. This activation was nonlinear with respect to receptor concentration, suggesting cooperativity within the trimeric envelope protein. The activated EnvA was stably associated with the target membrane through hydrophobic interactions, behaving like an intrinsic membrane protein. The ability of EnvA to associate with membrane was coincident with a loss of receptor-binding activity, suggesting that during viral entry activated EnvA dissociates from the receptor to facilitate membrane fusion. These results provide direct evidence that receptor binding triggers conversion of the EnvA protein to a membrane-binding form, illustrating that RSV is a useful model for the study of retroviral entry and activation of pH-independent fusion proteins.**

Viral envelope glycoproteins play a critical role in viral entry and infection of target cells by attaching the virus to the host cell receptor and mediating fusion of the viral and host cell membranes. Conserved structural features between a diverse group of viral envelope proteins suggest that they employ similar molecular mechanisms to mediate membrane fusion (1). Previous studies, particularly of influenza virus, indicate that viral envelope proteins can exist in at least two conformational states, a native state and an active state. Conversion of the envelope protein from a metastable (nonfusogenic) state to an active (membrane-binding) state (2–4) involves a ''trigger,'' and viral envelope proteins can be categorized based on the nature of this trigger. A number of viral glycoproteins, including influenza virus hemagglutinin (HA), are activated by the low pH environment of the endosome after receptor-mediated endocytosis of the virus (1). Upon activation, dramatic conformational changes in HA release the buried hydrophobic fusion peptide, which inserts into the host cell membrane, beginning the process of membrane fusion (3, 5). In contrast, activation of many other viral envelope proteins, including retroviruses such as Rous sarcoma virus (RSV) (6) and HIV (7), is independent of pH. The molecular mechanisms of neutral-pH fusion are poorly characterized, in part because the trigger for activation remains unclear. It is postulated that interactions between the viral envelope protein and the cellular receptor(s) initiate conversion of the envelope protein to the fusogenic state (8, 9); however, there is no direct evidence of receptor-induced activation of a viral protein to a membrane-binding conformation.

The envelope protein of RSV, EnvA, is a homotrimer with each monomer composed of covalently bound SU and TM subunits. The SU subunit contributes to receptor binding (10), whereas TM is believed to play a role in the fusion activity of the protein and contains a putative internal fusion peptide near its amino terminus (1). EnvA binds specifically to Tva (11), a small (138 aa), type-one integral membrane protein that functions as the viral receptor on susceptible avian cells (12). Expression of *tva* confers susceptibility to RSV entry in all cell types examined including avian, mammalian, amphibian, and fish lines (P.B., unpublished data), and virions pseudotyped with Tva can infect cells expressing EnvA (13). Collectively, these results strongly suggest that Tva is sufficient to initiate the entry process by activating the RSV fusion machinery. Our aim was to provide direct evidence that receptor binding is the trigger that leads to conversion of EnvA to an active, membrane-binding state.

Based on the paradigm of influenza HA, activation of EnvA would result in exposure of the previously buried hydrophobic residues of the fusion peptide in the TM subunit and insertion of this peptide into a target membrane, thereby forming a stable EnvA–membrane complex. To test this hypothesis, we used a cell-free liposome-binding assay to demonstrate that the purified RSV receptor, sTva, caused a soluble, oligomeric form of EnvA to stably associate with a target membrane. These receptor-induced changes in the biophysical properties of EnvA were consistent with a change in the conformation of the protein to a fusogenic state. Furthermore, similar to fusion of RSV virions with susceptible avian cells (6), receptor-triggered conversion was temperature-dependent. EnvA appeared to associate with the target membrane through hydrophobic interactions, with a higher-order structure of the protein contributing to its ability to stably associate with membrane. Conversion of EnvA to a membrane-associated form was nonlinear with respect to the amount of receptor added to the reaction, implying a cooperative transition to the activated state. Activation of EnvA was also associated with an inability to recover envelope-receptor complexes, suggesting that during the transition to a membrane-binding conformation, EnvA releases its cellular receptor. Our results are summarized in a proposed model of the early events in receptor-activated viral entry.

MATERIALS AND METHODS

Lipids. Phosphatidylcholine (PC) from eggs and cholesterol (Chol) were purchased from Sigma. Lipids were stored under argon at −80°C as 100 mg/ml stock solutions in chloroform.

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Abbreviations: RSV, Rous sarcoma virus; HA, hemagglutinin.

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Proteins and Antibodies. EnvA PI was produced as described with modifications (14). NIH 3T3 cells stably expressing a glycosylphosphatidylinositol (PI) linked form of EnvA (EnvA PI) were generously supplied by J. White of the University of Virginia (Charlottesville). Cells were grown in DMEM supplemented with 10% bovine calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and 300 μg/ml G418 (GIBCO/BRL). Subconfluent T150 flasks were induced with sodium butyrate (5 mM) for 16 hr. Cells were washed twice with 1× PBS and one time with Ca^{2+}/Mg^{2+}-free PBS. PI-linked protein was released in Ca^{2+}/Mg^{2+}-free PBS plus protease inhibitors (aprotinin, leupeptin, pepstatin, and PMSF from Sigma) with 50 milliunits/ml phosphatidylinositol-phospholipase C (Boehringer Mannheim) at 37°C for 30 min. Supernatants were clarified by centrifugation, and samples were concentrated approximately 5-fold by using a 30-kDa cut-off Macrosep centrifugal concentrator (Filtron Technology, Northborough, MA). To generate radiolabeled EnvA PI, cells were metabolically labeled with [^{35}S]methionine before harvesting. Samples were stored a 4°C. sTva was produced in insect cells (*Spodoptera frugiperda*) and purified from cellular supernatants by nickel affinity chromatography column (J.C., J. Balliet, and P.B., unpublished data). Protein was stored at 4°C and diluted to the appropriate concentrations in Ca^{2+}/Mg^{2+}-free PBS. Soluble herpes simplex virus gD, anti-gD monoclonal antibody (ID3), and baculovirus-expressed HVEM were gifts from G. Cohen and R. Eisenberg (University of Pennsylvania). Rabbit polyclonal antiserum reactive with SU was generously provided by T. Matthews of Duke University. Goat anti-rabbit HRP-conjugated antibodies were purchased from Boehringer Mannheim. Iodinated protein A (0.1 μCi/μl) was purchased from NEN/DuPont. Trans-label was purchased from ICN.

Preparation of Liposomes. Liposomes were produced by a modification of the protocol previously described (15). Briefly, PC (13 μmol) and Chol (6.5 μmol) in chloroform were mixed at a 2:1 molar ratio and dried under argon in a round-bottom glass flask. Glass beads and absolute ethyl alcohol (preheated to 52°C) were added while vortexing. Lipids were dried to a thin film by heating to 52°C under a vacuum. Liposomes were generated by the addition of 0.5 ml Ca^{2+}/Mg^{2+}-free PBS (preheated to 52°C) while vigorously mixing. Liposomes were sonicated for 60 sec in a water bath sonicator (Heat Systems/Ultrasonics). For localization of liposomes in sucrose gradients, trace amounts of [^{3}H]Chol (NEN/DuPont) were added to the initial lipid films.

Liposome-Binding Assays. The liposome binding assay is a modification of the protocol previously described (5). EnvA PI was incubated with indicated amounts of sTva in a final volume of 40 μl of Ca^{2+}/Mg^{2+}-free PBS on ice for 15 min. Forty microliters of liposomes (preequilibrated to the indicated temperature) was added to the samples and incubated for an additional 15 min. Samples were placed on ice, and 73% sucrose (wt/vol in PBS) was added to the protein–liposome mixture to bring it to 50% sucrose. Samples were overlaid with 150 μl of 40% and 300 μl of 25% sucrose in a 700-μl Ultra-clear centrifuge tube (Beckman). After centrifugation at 269,000 × *g* for 3 hr at 4°C, seven 100-μl fractions were drawn from the air–fluid interface. Gradient fractions 1 through 3 and 5 through 7 were pooled as the top and bottom fractions, respectively. Proteins were precipitated from fractions with the addition of an equal volume of Triton lysis buffer (150 mM NaCl/1% Triton X-100/50 mM Tris, pH 8.0/5 mM EDTA), casein (50 ng/μl), and trichloroacetic acid to 10% (wt/vol). Samples were incubated on ice for 30 min, pelleted, and washed twice with ice-cold acetone. Dried pellets were resuspended in Triton lysis buffer then subjected to SDS/PAGE and Western blotting.

Coprecipitation Assay. EnvA PI and sTva (100 ng) were incubated at 4°C for 15 min. Liposomes in PBS or PBS alone was added and samples were incubated at 37°C for 15 min. Ni-NTA agarose (Qiagen) in PBS was added and samples were rocked at 4°C for 30 min and gently pelleted, and pellets were washed three times with Triton lysis buffer. Imidazole was added to a final concentration of 100 mM, and samples were boiled before analysis by SDS/PAGE and Western blotting.

Detection of Proteins. Western blots were probed with rabbit antisera against SU or Tva. Rabbit antibodies were detected by incubation with goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP). HRP was identified by enhanced chemiluminescence as instructed by the manufacturer (Pierce), and blots were exposed to x-ray film. Alternatively, primary antibody reactivity was detected with [^{125}I]Protein A and quantitated by using a PhosphorImager and IMAGEQUANT software (Molecular Dynamics).

RESULTS

Activation of a pH-dependent viral fusion protein results in its hydrophobic association with the target membrane via the exposed fusion peptide domain. To determine whether receptor binding was sufficient to activate a pH-independent viral fusion protein, we evaluated whether the oligomeric, aqueous ectodomain of RSV envelope (EnvA PI) (14) could be induced

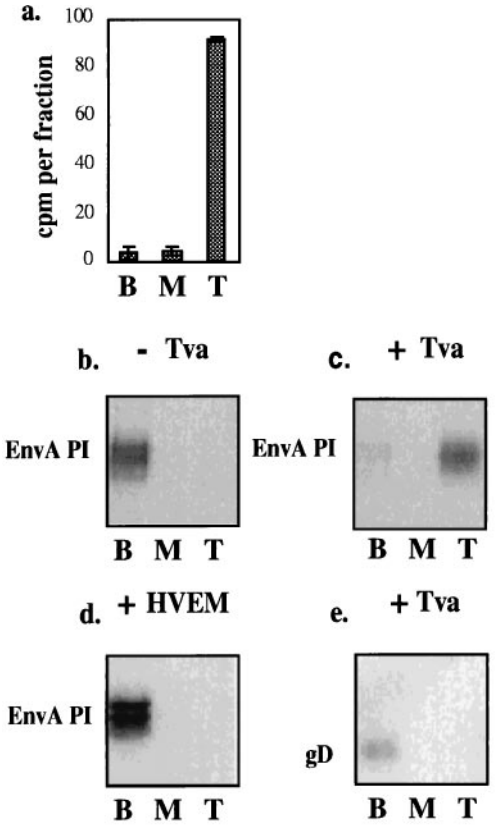

FIG. 1. Envelope colocalized with liposomes in a Tva-dependent manner. (*a*) Liposomes containing ^{3}H-labeled cholesterol were localized within the top fraction of the step-gradient following ultracentrifugation as described in *Methods*. (*b–e*) Liposome-binding assays were carried out as described in *Methods*. The proteins were analyzed by SDS/PAGE and Western blotting. Blots were probed with rabbit anti-serum against SU (*b–d*) or antibodies against gD (*e*). (*b*) EnvA PI alone failed to associate with liposomes. (*c*) Incubation with excess sTva (500 ng) efficiently shifted EnvA PI to the liposome-containing top fraction. (*d*) A control baculovirus-expressed protein (HVEM) was unable to alter the position of EnvA PI in the gradients. (*e*) Tva did not affect membrane association of a control soluble viral glycoprotein, herpes gD.

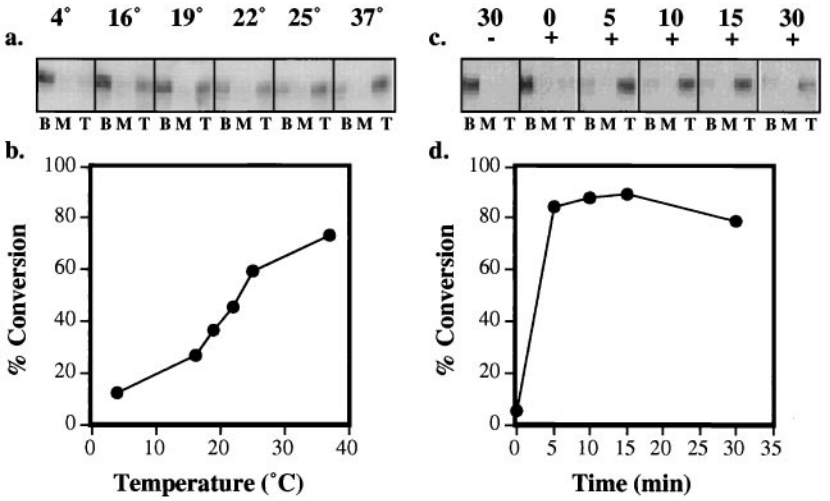

FIG. 2. Tva-triggered conversion of EnvA PI occurred rapidly in a temperature-dependent manner. (*a*) EnvA PI was prebound to sTva (500 ng) by incubating at 4°C. Liposomes preequilibrated to the indicated temperature were added and the samples were shifted to this temperature for an additional 15 min. The extent of binding of EnvA PI to the liposomes was determined by flotation, SDS/PAGE, and Western blot. Blots of gradient fractions were probed with antiserum against SU (*a* and *c*). (*b*) Percentage of EnvA PI converted to a membrane-binding form was plotted as a function of the temperature. (*c*) EnvA PI was incubated in the absence (−) or presence (+) of sTva (500 ng) and liposomes at 37°C for the length of time indicated, shifted to 4°C, and immediately processed on sucrose step-gradients. (*d*) Percentage of EnvA PI converted to a membrane-binding form was plotted as a function of time at 37°C. % Bound = {[(EnvA PI in Top)/(EnvA PI in Top + Middle + Bottom)] ×100}.

to stably interact with a target membrane in the presence of a soluble receptor for RSV, sTva. Liposomes composed of phosphatidylcholine and cholesterol were employed as target membranes and incubated with various combinations of viral glycoproteins and receptors. The buoyant density of these liposomes caused greater than 90% of the liposomes to float to the top of the gradient during ultracentrifugation (Fig. 1*a*) along with any tightly associated proteins. Neither EnvA PI (Fig. 1*b*) nor sTva alone (data not shown) stably associated with liposomes as evidenced by their remaining at the bottom of the gradient. However, incubation of sTva with EnvA PI resulted in efficient colocalization of EnvA PI with the liposomes in the top fraction of the gradient (Fig. 1*c*), suggesting direct conversion of RSV envelope to a membrane-associated state by receptor binding. A purified soluble form of a receptor for herpes simplex virus (HVEM) was unable to convert EnvA PI to a liposome-associated form (Fig. 1*d*). Additionally, a control soluble viral envelope from herpes simplex virus (gD) did not become associated with liposomes in the presence of sTva (Fig. 1*e*). Therefore, the conversion of EnvA PI to a membrane-bound form required sTva, indicating that a specific interaction with the receptor was responsible for the conversion of EnvA PI to an activated, membrane-associated state.

EnvA PI binds sTva stably at 4°C (K. Gendron, J. Huang, and P.B., unpublished data), yet membrane fusion of RSV virions is temperature-dependent with no apparent fusion activity at 4°C (6). This discrepancy points to the existence of a temperature-dependent postbinding event(s) in the entry of RSV into cells. Therefore, the temperature profile and rate of activation of EnvA PI membrane association by sTva were examined to evaluate whether this *in vitro* assay was reflective of the biology of RSV membrane fusion. The thermal profile of receptor-triggered activation was determined by binding EnvA PI and sTva at 4°C and then incubating the complex in the presence of liposomes at increasing temperatures (Fig. 2 *a* and *b*). At or below 16°C, conversion of EnvA PI to a membrane-bound form was inefficient. However, between 16°C and 25°C, the percentage of EnvA PI converted to a liposome-associated form increased significantly (Fig. 2*b*). At 37°C, sTva-triggered EnvA PI binding to liposomes was efficient and plateaued within 5 min, (Fig. 2 *c* and *d*). Prolonged incubation of EnvA PI and liposomes at 37°C in the absence of sTva for 30 min (Fig. 2*c*) or up to 18 hr (data not shown) did not result in conversion of the viral envelope protein to an activated, liposome-associated form. Phosphatidylcholine liposomes are fluid throughout the temperature range evaluated (16); therefore, the temperature dependence of liposome binding was reflective of receptor-triggered conformational changes in envelope protein and not a function of changes in the behavior of the target membrane. Because receptor-induced liposome binding demonstrated a temperature dependence similar to the thermal profile of membrane fusion mediated by intact RSV virions, this cell-free system is likely reflective of early events of viral-host cell membrane fusion.

To characterize envelope during these early events of viral entry, the stability of the EnvA PI–membrane association was evaluated by using agents commonly employed to disrupt protein–protein interactions and strip peripheral membrane proteins (17). Liposome-associated EnvA PI could not be eluted with either high salt (1 M potassium chloride) or with a chaotropic agent (0.1 M sodium carbonate, pH 11.5) (Fig. 3*a*), indicating that EnvA PI was strongly associated with the liposomal membrane and that ionic interactions were not critical for membrane binding. The resistance of liposome-bound EnvA PI to carbonate extraction was characteristic of an intrinsic membrane association. EnvA PI remained associated with liposomes under moderately denaturing conditions (Fig. 3*c*, lanes 8 and 9), but was eluted with the addition of reducing agents (Fig. 3*b*, lanes 5 and 6) or with increases in urea concentration to 6 M (Fig. 3*c*, lane 12), suggesting that the higher-order structure of the protein may contribute to stable association with membranes.

To gain further insight into the requirements for receptor-triggered activation of EnvA to a membrane-bound form, we titrated the amount of sTva added to the liposome-binding assay. With a constant amount of EnvA PI and increasing amounts of sTva, the response to receptor was saturatable, with 75–85% of the total envelope converted to the membrane-associated state (Fig. 4*a*). At low levels of sTva (below 100 ng of sTva), the activation curve increased sharply and was nonlinear (Fig. 4*a Inset*), suggesting that the trimeric EnvA PI binds multiple sTva molecules and that cooperativity within the trimeric EnvA PI contributes to its conversion to a membrane-associated state.

The fate of receptor after activation of EnvA PI was determined by localizing sTva in the liposome-binding gradients using Western blot analysis. Initial experiments utilizing an excess amount of sTva indicated that, although more than 80% of the EnvA PI protein was found associated with liposomes, all of the sTva remained at the bottom of the gradient (data not shown). Our inability to detect any sTva within the EnvA PI-containing fraction of the gradient suggested that the interaction between EnvA PI and sTva was not stable or that the amount of sTva within the top fraction was below the level of detection. The receptor–envelope complex has a half-life of greater than 6 hr when incubated without liposomes (data not shown). Thus, we would predict that if receptor were limiting and the stability of the receptor–envelope interaction were maintained after EnvA PI activation, then sTva would colocalize with EnvA PI in the liposome-containing fraction. To ensure that sTva was not in excess, liposome-binding assays were performed with the minimal amount of sTva (100 ng) required for full conversion of EnvA PI (as determined by titration in Fig. 4*a*). Coprecipitation of EnvA PI with 100 ng of sTva confirmed that sTva was saturated for EnvA PI binding, but was not in excess (data not shown). Using 100 ng of sTva to induce EnvA PI activation, we were unable to detect sTva associated with activated EnvA PI at the top of the sucrose gradient under conditions where as little as 6.5 ng of receptor were detectable. In addition, previous studies demonstrated the majority, if not all, of the sTva protein is competent to stably associate with EnvA PI (11); therefore, the trivial interpretation that only a fraction of the input sTva is capable of binding envelope could be eliminated. These results strongly suggested that receptor-induced conversion of EnvA PI to a membrane-bound form was associated with decreased stability of the receptor–envelope complex.

To evaluate further the effects of envelope activation on receptor binding, EnvA PI was incubated with sTva in the presence or absence of liposomes. sTva was precipitated from the reactions along with bound EnvA PI, and the precipitated proteins were analyzed by Western blot. EnvA PI efficiently coprecipitated with sTva when the proteins were incubated at 4°C with or without liposomes (data not shown) and at 37°C without liposomes. In contrast, incubation of EnvA PI with sTva and liposomes at 37°C consistently resulted in diminished association of EnvA PI with receptor (Fig. 4*c*). Collectively, these results indicated that receptor-triggered activation of EnvA resulted in decreased receptor binding, suggesting that upon conformation change, the viral envelope protein loses its ability to stably associate with the host receptor.

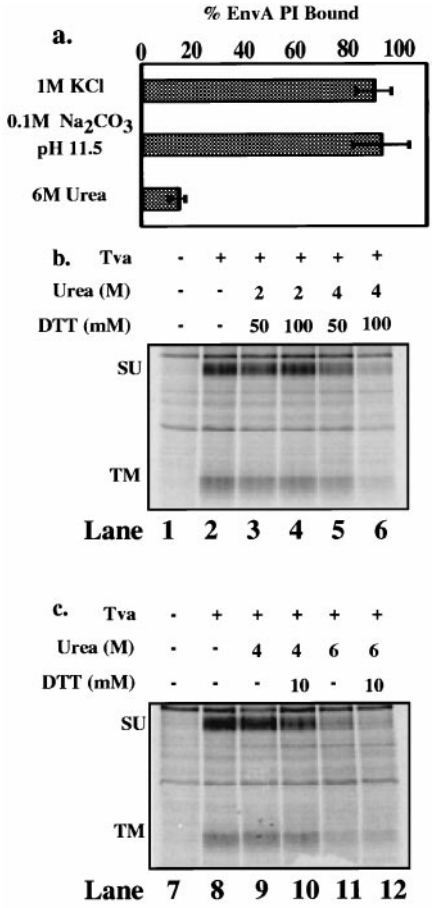

FIG. 3. EnvA PI stably associated with the target membrane. EnvA PI was incubated with sTva as in Fig. 2*a*. KCl (1 M) or urea (6 M) were added to samples followed by incubation at 37°C for 15 min. Alternatively, sodium carbonate (0.1 M), pH 11.5, was added followed by incubation at 4°C for 30 min. Samples were processed on sucrose step-gradients. (*a*) The relative amount of EnvA PI bound to the liposomes following stripping conditions. (*b* and *c*) Metabolically labeled [^{35}S]EnvA PI was incubated with (+) or without (−) sTva (500 ng) and liposomes for 15 min at 37°C. Samples were incubated with or without reducing and denaturing agents for 15 min at 4°C, followed by processing on sucrose step-gradients. Autoradiographs of top fraction from each gradient.

DISCUSSION

The entry of an enveloped virus into a host cell requires fusion of the viral and host cell membranes, a process mediated by specific viral fusion proteins. For viruses that fuse with their target cell at neutral pH, it is postulated that binding to the viral receptor activates the fusion process. We have used RSV and its cellular receptor, Tva, to directly test this hypothesis. Expression of *tva* is sufficient to mediate entry of RSV in all cell types examined (P.B., unpublished data). Furthermore, Tva incorporated into virions will efficiently direct infection of cells expressing RSV envelope. sTva has been shown to induce changes in the protease sensitivity of the SU subunit of RSV envelope (18), suggesting that this receptor may activate the viral envelope glycoprotein. Collectively, these observations strongly suggest that Tva is the sole receptor required for RSV entry. Here we directly demonstrate that receptor binding converts envelope to its active, membrane-binding state. Thus, this viral receptor not only binds the viral envelope, bringing the virus and host cell in close proximity, it also converts envelope to a membrane-binding state at the interface between the viral and cell membranes (Fig. 5). Because Tva functions as both an anchor and a trigger, it ensures that the fusion machinery is active only in the appropriate temporal and spatial context.

Activation of EnvA PI by sTva resulted in the conversion of the ectodomain from a water-soluble conformation to a hydrophobic conformation consistent with the activation of the RSV envelope glycoprotein. Similar changes in hydrophobicity and membrane-association occur when the soluble form of influenza virus hemagglutinin is exposed to low pH and the protein adopts its fusogenic conformation (5). The stable complex formed between EnvA PI and the liposomal membrane was likely mediated by hydrophobic interactions between the RSV envelope fusion peptide and the lipid core, as demonstrated by its resistance to stripping with high salt and a chaotropic agent. Only under highly denaturing conditions or denaturing and reducing conditions were both the SU and TM subunits of EnvA PI removed from the target membrane, suggesting that the conformation of the protein was critical for stable association. A soluble form of the influenza viral fusion protein, BHA, is stripped from liposomal membranes under high pH conditions and is retained under denaturing conditions (5). The differences in behavior between these fusion

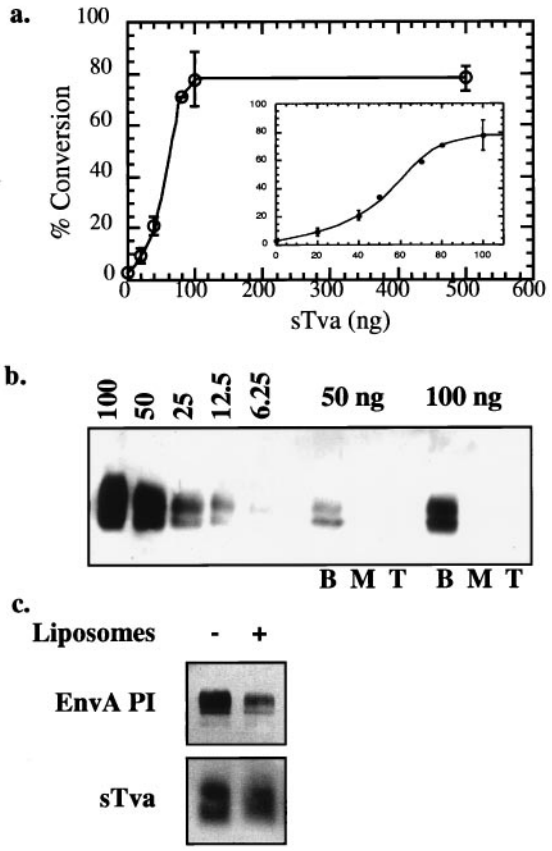

FIG. 4. EnvA PI–liposome association is nonlinear with respect to sTva concentration. Liposome-binding assays were performed with constant amounts of EnvA PI and increasing amounts of purified sTva, and then processed as described in Fig. 2*a*. (*a*) The percentage of EnvA PI converted to a membrane-binding form as a function of sTva concentration. (*Inset*) Titration curve between 0 and 100 ng of sTva. The mean and standard deviation of three experiments (open circle) were plotted by using KALIOGRAPH software (Abelbeck Software). Data points at 50 and 70 ng represent single experiments. (*b*) Liposome-binding assays were performed with subsaturating (50 ng) and saturating (100 ng) amounts of sTva. Fractions were analyzed by SDS/PAGE and Western blotting using anti-Tva antibodies and were compared with sTva mass standards. (*c*) EnvA PI was incubated with 100 ng of sTva for 15 min at 4°C. Liposomes in PBS (+) or PBS alone (−) were added, and samples were shifted to 37°C for 15 min. Precipitation of sTva with bound EnvA PI was analyzed by SDS/PAGE and Western blotting with antibodies specific for Tva and EnvA.

proteins may reflect differences in the nature of the protein–membrane interactions of the internal fusion peptide and amino-terminal fusion peptide of RSV and influenza virus, respectively.

The response of EnvA PI to sTva was nonlinear, suggesting that activation of envelope by receptor may favor engagement of multiple monomers within the envelope trimer. Examples of cooperative interactions between monomers in oligomeric envelope proteins have been demonstrated with both pH-independent and -dependent viruses. For HIV, binding of the primary cellular receptor, CD4, results in conformational changes in the viral envelope including dissociation and release of the SU subunit (gp120) (19). However, efficient fusion by HIV envelope requires coreceptors (CXCR4, CCR5, and others), suggesting that CD4-induced shedding of gp120 may reflect partial or premature activation of the envelope protein (8, 20). Moore *et al.* (21) demonstrated that CD4 must bind multiple viral glycoprotein (gp120) subunits in the oligomeric HIV envelope to induce gp120 shedding. Additionally, analysis of hybrid trimers of HA demonstrated that acid-induced

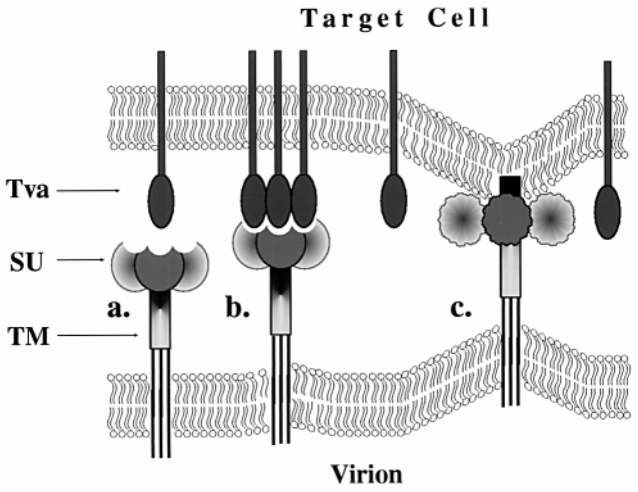

FIG. 5. Model of RSV EnvA-mediated fusion. (*a*) Trimeric EnvA composed of covalently bound SU and TM subunits initiates infection by binding to the host cell receptor protein, Tva. (*b*) Binding of multiple receptor molecules by the SU subunits triggers cooperative conformational changes in both SU and TM subunits. (*c*) Active conformation of the retroviral envelope protein after triggering with the hydrophobic fusion peptide of TM inserted into the host membrane. Tva is released by the SU subunit, allowing lateral diffusion of the TM molecule for subsequent formation of the fusion pore.

conformational changes occur in a concerted and cooperative manner (22). Our results suggest that cooperative interactions also play a role in receptor-induced activation of the RSV EnvA protein and may allow for the unified and rapid receptor-induced activation of the trimeric envelope. Experiments using mixed oligomers of EnvA composed of wild-type and mutant monomers unable to bind receptor will be generated to definitively address the requirements for cooperative interactions during RSV entry.

Receptor-induced activation of EnvA PI was coincident with changes in the envelope–receptor interaction. In the absence of a target membrane, RSV envelope binds sTva rapidly and forms a stable complex with a half-life of greater than 6 hr (K. Gendron, J. Huang, and P.B., unpublished data). In contrast, the presence of a target membrane appears to promote dissociation of the activated viral envelope–receptor complex. During HA-mediated membrane fusion, membrane-attached HA molecules undergo lateral reorganization to form a fusion pore (23) composed of multiple HA oligomers (24, 25). By analogy, it is likely that fusion mediated by RSV EnvA will involve multiple envelope trimers that, after insertion of the fusion peptide into the target membrane, must diffuse within the lipid membrane to form the fusion pore. The RSV SU and TM subunits are covalently associated by a stable disulfide bond; therefore, release of the receptor by the SU subunit would allow for lateral diffusion of the membrane-associated TM subunit. For a number of other retroviral glycoproteins, including HIV-1, the association of SU and TM is much more labile. Perhaps these viruses shed the receptor-bound SU subunit to free the activated envelope protein from the receptor.

The difference in behavior of EnvA PI and sTva when incubated in the presence of membrane also suggests that lipids may act as a cofactor in the conversion reaction, altering the rate or outcome of the reaction. Lipids play a role as cofactors in the activation of other viral fusion proteins. For example, Semliki forest virus requires specific lipids to undergo pH-triggered conformational changes and to enter cells (17). For RSV, the requirement of a lipid cofactor may act to ensure that full activation of the viral envelope protein occurs when the glycoprotein is in close proximity to the cellular membrane.

Other viral fusion proteins may use protein cofactors that lie flush with the membrane to achieve this effect, as has been suggested for HIV-1 and its coreceptors, CCR5 and CXCR4 (8). Similarly, a number of viruses use primary receptors that lie close to the membrane surface such as the multimembrane-spanning receptors for ecotropic and amphotropic MLVs and gibbon ape leukemia virus.

Organizational and structural features are conserved among retroviral glycoproteins and between the viral glycoproteins of distinct viral families, such as Orthomyxoviridae and Filoviridae (1, 4, 26, 27). Such conservation suggests a common molecular mechanism is used to mediate fusion. The interaction between RSV and its receptor provides a powerful model of early events in retroviral entry and may further elucidate the molecular mechanisms of receptor-dependent fusion. By increasing our understanding of these critical, early events in the infectious cycle, we may gain new insight into how to block viral entry.

Note Added in Proof. Hernandez *et al*. (28) have recently published work demonstrating a similar activation of RSV EnvA by Tva.

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