Activation of Vesicular Stomatitis Virus Fusion with Cells by Pretreatment at Low pH*

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Fusion of vesicular stomatitis virus (VSV) with Vero cells was measured after exposure of the virus to low pH under a variety of experimental conditions. The method of relief of fluorescence self-quenching of the probe octadecylrhodamine was used to monitor fusion. Incubation of the virus at pH 5.5 prior to binding to cells led to significant enhancement of fusion at the plasma membrane, whereas fusion via the endocytic pathway was inhibited. Fusion of pH 5.5-pretreated VSV showed a similar pH threshold for fusion as non-pretreated virus, and it was blocked by antibody to VSV G protein. Activation of VSV by pretreatment at low pH was only slightly dependent on temperature. In contrast, when VSV was first bound to target cells and subsequently exposed at 4 °C to the low pH, activation of the fusion process did not occur. The pH 5.5-mediated activation of VSV could be reversed by returning the pH to neutral in the absence of target membranes. The low pH pretreatment also led to aggregation of virus; large aggregates could be pelleted by low speed centrifugation and only the effects of the supernatant, which consist of single virions and/or microaggregates, were considered. The data were analyzed in the framework of an allosteric model according to which viral spike glycoproteins undergo a pH-dependent conformational transition to an active (fusion-competent) state. Based on that analysis we conclude that the conformational transition to the active state is rate-limiting for fusion and that the viral spike glycoproteins are fusion-competent only in their protonated form.

Vesicular stomatitis virus (VSV) enters cells by receptor-mediated endocytosis through coated pits (1, 2). In the endocytic vesicle, rapid acidification occurs, a process which triggers fusion of the viral membrane with that of the endosome, and the nucleocapsid is ejected into the cytosol. VSV can also be made to fuse directly with the plasma membrane by lowering the pH of the medium (3). We have studied fusion of VSV with Vero cells (4) using a fusion assay based on fluorescence self-quenching of octadecylrhodamine (R18) incorporated into the intact virus (5). Relief of R18 self-quenching occurs as a result of fusion of virus with target membranes. We examined the kinetics, extent, temperature dependence, effect of osmotic strength of the medium, effect of antibody to VSV G protein, and the reversibility of the fusion reaction (4).

During a study aimed at examining mechanisms of viral fusion and at developing controls for the fusion activity of the VSV spike glycoprotein reconstituted in lipid vesicles, we preincubated virions at different pH values and temperature, or pretreated virus with different chemical agents and enzymes. We then examined the effect of those preincubation conditions on the rate and extent of fusion. In the case of influenza virus, pretreatment at low pH causes inactivation of fusion (6). To our surprise, we obtained completely opposite results with VSV; fusion was enhanced after pretreatment at low pH. In this paper we will describe this observation in detail and discuss it in the framework of a recently developed allosteric model for viral spike glycoproteins (7).

EXPERIMENTAL PROCEDURES

Materials—Octadecylrhodamine B chloride (R18) was obtained from Molecular Probes (Junction City, OR), Triton X-100 from Aldrich, and cell culture media and trypsin EDTA from GIBCO. [3H]Leucine and [3H]glucosamine were obtained from Amersham Corp.

Cell Cultures—Vero cells were grown to confluency in Dulbecco’s modified Eagle’s medium supplemented with 10% calf serum in 75-cm² plastic dishes. Cells were grown to confluency and then suspended in X-100 virions at different pH values and temperature, or pretreated virus with different chemical agents and enzymes. The virus was grown on monolayer cultures of baby hamster kidney (BHK-21) cells and purified by sucrose velocity and density gradients to approximately 1 mg of VSV protein/ml. 1H-Labeled VSV virus was prepared by infecting BHK cells in the presence of [3H]leucine or [3H]glucosamine (5 μCi/ml growth medium). The specific activity ranged from 200 to 2000 cpm/μg VSV protein (see Ref. 4).

Virus-cell Binding Assay—1H-Labeled VSV (10-50 μg of protein) was added to 2 × 10⁵ Vero cells in 1 ml of NaCl-Hepes buffer at pH 7.4 and incubated at 4 °C for different time intervals. Free virus was separated from virus-cell complexes by centrifugation of duplicate 100-μl samples through 300 μl of phthalate oils (dibutylphthalate and bis(2-ethylhexyl)phthalate (1:2, v:v) (Eastman, Rochester, NY) in a 400-μl polyethylene tube at 2000 × g for 1 min (8). This procedure separates bound from free virus without an aqueous wash. Control experiments indicated that all cells were pelleted into the tip of the centrifuge tube, but none of the free virus entered the oil phase under those conditions. The percentage of virus bound to cells was determined from the radioactivity in the tip of the centrifuge tube, which was cut and dropped directly into a scintillation vial. The concentra-
tion of free virus was determined from a 50-µl aliquot of the supernatant.

Envelope Fusion Assay—VSV was labeled with R18 as described previously (4). R18VSV was preincubated with Vero cells at 4 °C for 30 min to form VSV-Vero complexes, and unbound virus was removed by centrifugation in a 12 × 75-mm polystyrene tube at 300 × g. The pellet containing VSV-Vero complexes was resuspended in a NaCl-Hepes buffer and kept on ice. Small aliquots of R18VSV-cell complexes were added to 2 ml of buffer prewarmed at 37 °C. Subsequently the pH was lowered by adding 20–100 µl of a 1 M MES buffer. Percent fusion was calculated according to Eq. 1:

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\% \text{ fusion} = \frac{F - F_0}{F_i - F_0} \times 100
\]

where \( F_i \) and \( F \) are the fluorescence intensities at time 0 and at a given time point. \( F_0 \) is the fluorescence after disruption of virus and cells by detergent divided by 1.5, a correction factor to account for the difference in quantum efficiency of R18 in plasma membrane versus Triton micelles (4). The pH was always measured at the end of the experiment. Fluorescence was measured using an SLM 8000 spectrofluorometer with 1-s time resolution at 560 and 585 nm excitation and emission wavelengths, respectively. A 570 nm cut-off filter was used at the emission to reduce scatter contributions.

Antibodies to the VSV Spike Glycoprotein (G Protein)—The antibodies were prepared and tested as described previously (4). G protein was extracted from purified virus with 30 mM octyl-β-D-glucopyranoside and purified by sucrose density centrifugation. About 1 mg of purified G protein in 2 ml of NaCl bicarbonate buffer with 60 mM octyl-β-D-glucopyranoside was mixed with Freund’s adjuvant and injected into a rabbit, boosted 4 weeks later with Freund’s incomplete adjuvant and bled 8 days later. The activity of the antibody was tested by incubating at different dilutions with pHVSV for 60 min at 4 °C. Subsequently the incubation mixture was centrifuged in a 1.5-ml tube at 6000 × g for 15 min, and radioactivity in the supernatant was counted. Two µl of antiserum was sufficient for complete pelleting of 5 µg of VSV. To test for inhibition of fusion, 24 µl of antiserum was incubated for 30 min at 4 °C with R18VSV-Vero complexes containing 24 µg of VSV. Unbound antibody was removed by centrifugation and the R18VSV-Vero-antibody complexes were assayed for fluorescence dequenching as above.

Pretreatment at Low pH—R18VSV was exposed to pH 5.5 at 0.2–0.6 mg/ml viral protein at a given temperature for 10 min. This treatment gives rise to formation of viral aggregates as observed by the application of intensified quantitative fluorescent video optical microscopy. This technique measures fluorescence intensity and area from individual fluorescent points in a given field with sufficient accuracy that the number of optically unresolved particles producing the fluorescence in the spot can be estimated. Isolated individual VSV particles are detected. Analysis of the VSV cluster size distributions of control and pH-treated virus showed clear differences: The pH-treated VSV population was dominated by large cluster sizes (4–8 VSV), as well as “superclusters” (greater than 8 VSV), while the control population showed predominantly small clusters (1–3 VSV).2 The large VSV aggregates (superclusters) were removed by centrifugation in a 1.5-ml polypropylene tube for 3 min at 600 × g, and the supernatant R18VSV was used for the fusion assay described above. About 50% of pH 5.5-pretreated virus was pelleted under those conditions. Fig. 1 shows the percent pelleting as a function of pretreatment pH. The pH dependence of aggregation was very similar to the pH dependence of VSV fusion, indicating that similar changes in the VSV spike glycoprotein (G protein) trigger both fusion and aggregation.

The spectrofluorometric method for measuring fusion does not distinguish contributions to fusion between monomeric virus and smaller aggregates. Experiments using quantitative video optical microscopy are under way to measure the fusion of individual particles or small aggregates with cells.

**RESULTS**

Rates of Fusion—Fig. 2, a and b, shows the time course for fusion at various pH values of untreated and pH 5.5-pretreated VSV. Fusion of untreated VSV was about 10% in 400 s at pH 5.7 (Fig. 2a). The pattern with pH 5.5-pretreated virus was entirely different (Fig. 2b) in that fusion initially proceeded at a much faster rate. After about 400 s, the fast process leveled off at about 70% fusion. Subsequently, fusion proceeded at a much slower rate to about 100% (data not shown). Fusion of both control and pretreated virus was pH-

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1 J. Lowy, unpublished observations.
dependent with a similar threshold of about pH 6.2

We had previously shown that fluorescence dequenching measured at neutral pH represents entry of VSV via the endocytic pathway followed by fusion at low pH with the membrane of the endosome (4). Comparison of Fig. 2, a and b shows that entry of pH 5.5-pretreated VSV via the endocytic pathway (i.e. at pH 7.4) occurred at a much slower rate than entry of untreated virus. Our working hypothesis for this phenomenon is that the pH-pretreated VSV population is dominated by larger clusters (4-8 VSV), and that those clusters are too large to enter the coated pits. However, the clustering does not prevent VSV from fusing with the plasma membrane. This hypothesis is supported by measurements of intensified fluorescent video microscopy (see “Experimental Procedures”) and will be subject to further investigation. For the purpose of this study we will focus on fusion at the plasma membrane of untreated and pH-pretreated virus.

Inhibition by Antibody to the VSV G Protein—The pH dependence of fusion (Fig. 2b) indicates that the enhanced R18 dequenching observed with pH 5.5-pretreated virus reflects the biological activity of the viral spike glycoprotein in inducing membrane fusion. In order to confirm this notion, we used antibody raised against the VSV G protein. The antibody was added to the R18VSV-Vero complex formed at 4°C. It did not remove bound VSV from Vero cells (data not shown). The R18VSV-Vero-antibody complex was then added to a cuvette prewarmed to 37°C and the pH was lowered after 60 s in the same way as the R18VSV-Vero complexes were treated to induce fusion.

Fig. 3 shows a very low level of dequenching of low pH-pretreated VSV in the presence of the antibody to the G protein, indicating that the enhanced dequenching observed with the pH-pretreated virus was not due to nonspecific dye transfer. Incubation of R18VSV-Vero complexes with preimmune serum did not affect the rate of fluorescence dequenching (data not shown).

Pretreatment in the Presence and Absence of Target Membranes—We hypothesize that lowering the pH during the preincubation period induces a conformational transition of the viral spike glycoproteins to an active state (see “Discussion”). This transition occurred within a few minutes even at temperatures below 37°C. Fig. 4 shows that pH 5.5 pretreatment at room temperature or at 0°C resulted in a similar rate and extent of fusion between VSV and Vero cells.

On the other hand, when VSV was prebound to Vero cells and the complex was subsequently exposed to low pH at 4°C for 15 min, no increase in the fluorescence dequenching was observed. Only after raising the temperature to 37°C was fusion observed at about the same rate as that of untreated virions (see Fig. 4).

The experiment indicates that the hypothesized conformational transition is slowed down considerably when the virus is bound to the target membrane. Moreover, the observation that the rate of fusion is very rapid with activated virus bound to the target indicates that the conformational transition is the rate-limiting step in the fusion of untrated VSV.

Reversibility of the Activation Process—In order to test whether the conformational change initiated by protonation could be reversed by returning the pH to neutral, the pH 5.5-pretreated VSV was incubated at pH 7.4 and 4°C for different periods of time in the absence of target membranes. During incubation of pH 5.5-pretreated VS at neutral pH, viral aggregation took place and the large aggregates had to be removed by pelletting before addition of the Vero cells (see “Experimental Procedures”).

Fig. 5 shows fusion of pH 5.5-pretreated VSV, which had
been incubated at pH 7.4 for different periods of time prior to binding to Vero cells. The results indicate that at 4 °C the activation can be reversed completely in less than 2 h. The reversal occurred at room temperature and 4 °C at similar rates (data not shown).

**Binding to Target Membranes** — We hypothesize that when activated VSV is bound to the target membrane, the bound viral spike glycoproteins are frozen in their active conformation (see “Discussion”). To achieve sufficient VSV binding in the activated state, the rate of binding to target membranes should be faster than that for reversal to the inactive state. In order to test this hypothesis, we measured rates of VSV binding to Vero cells using the oil tube assay (see “Experimental Procedures”). Fig. 6 shows binding of control and pH 5.5-pretreated virus to Vero cells. The rate and extent of binding were larger for pretreated virus, but the overall pattern was quite similar. The observation that the half-time for binding was about 5 min (see Fig. 6) supports our notion that binding occurred more rapidly than reversal to the inactive state at pH 7.4.

**DISCUSSION**

The pattern of fusion of pH-activated virus can be interpreted in the framework of an allosteric model for membrane fusion mediated by viral spike glycoproteins (7). This model does not involve a detailed mechanistic description for bringing lipid membranes together and fusing them. That is a complex process involving apposition, deformation, dehydration, and destabilization (9). The model rather deals with the effects of ligand binding at regulatory sites, of cooperativity, and of protein conformational changes of their function. This is analogous to the analysis of allosteric enzymes, where regulatory properties are considered, without requiring a detailed analysis of the mechanism of catalysis (10).

In the model the viral spike glycoproteins are assumed to be arranged as oligomers, i.e. they consist of a number (n) of subunits (Fig. 7). The oligomer is assumed to undergo a “concerted” conformational change from a T state to an R state. Each subunit in the oligomer contains a regulatory site for a ligand (H⁺). We are considering an “exclusive” model, i.e. the T state does not induce fusion or bind the ligand, although it does bind to the target membrane. The equilibrium constant for the conformational change between T and R states of the oligomer is large, so that in the absence of ligand the oligomer is predominantly in the T state and inactive. Binding the ligand shifts the conformational equilibrium toward the active form, thereby enabling the fusion process to occur. The process is cooperative in that binding to one subunit stabilizes the active conformation of n subunits. The forward and backward rate constants for the conformational change are k₁ and k₋₁, respectively, and the rate of protonation is assumed to be instantaneous. The transition to the R state brings membranes together and induces their fusion with a rate constant kₚ.

Fig. 7 shows the proposed sequence of transitions resulting in activation: Since the equilibrium constant for the T to R transition is assumed to be large, initially the majority of the oligomers will be in the T state. However, ligand (H⁺) binding to the oligomers shifts the population of R states to the R₂ states, thus leading to activation of the virus. Preincubation at low pH in the absence of target causes the transition to the protonated R⁺ state at temperatures lower than 37 °C (see Fig. 4). When the pH is brought back to 7.4 at 4 °C the population of viral spike glycoproteins will instantaneously shift to the R state, and then gradually return to the T state with a rate constant k₋₁. However, binding to target membranes at pH 7.4 and 4 °C is relatively fast (see Fig. 6). Since reversal to the T state is slow (see Fig. 5), binding will result in the ollogomers in the T ↔ R equilibrium states initially occupied by low pH pretreatment in the absence of targets. The observation that preincubation of VSV-Vero complexes at pH 5.5 and 4 °C does not result in subsequent enhancement of the fusion rate (see Fig. 4) indicates that the T → R transition of the oligomer is very slow when it is bound to the target membrane. In this sense the binding site on the target membrane serves as a ligand interacting with the allosteric binding epitope on the viral protein.

The pH 5.5 pretreatment of VSV, followed by neutralization and binding to target membranes, freezes a large portion of the oligomers in the R state. However, the pH 5.5 pretreated VSV did not fuse with the plasma membrane at 37 °C and pH 7.4 (Fig. 2). This observation indicates that the unprotonated R form is not fusion-competent. Fusion was only induced by lowering the pH of pretreated VSV-Vero complex, indicating that only the protonated R⁺ state is fusion competent. In other words, fusion requires both a conformational change and the low pH (i.e. protonation of the R state).

Since a portion of the oligomers had already been brought to the R state by pH pretreatment and protonation is instantaneous, the only kinetic barrier to fusion of pH 5.5-pretreated VSV is the R⁺ → F transition, defined in our model by the rate constant kₚ. On the other hand, the fusion kinetics of untreated VSV are dominated by the rate of T → R transition which has a rate constant k₁ (Fig. 7). From Fig. 2, a and b, we can derive values for fusion rate constants at pH 5.7 of 5.7 × 10⁻⁷/s and 6.4 × 10⁻⁷/s for untreated and pH 5.5-pretreated
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VSV, respectively, yielding a ratio of \( k_f/k_l \) of about 10. This indicates that, in the absence of pretreatment, the rate-limiting step for fusion is the conformational transition of the oligomer.

We had previously shown that the process of triggering fusion is reversible in that fusion activated at pH 5.5 can be arrested by returning the pH to 7.4 (4). Based on the experiments presented in this study, we can interpret that observation in the following manner: Since the conformational change is rate-limiting, pH neutralization arrests fusion by preventing more viral spike glycoproteins from undergoing a conformational change. Moreover, those oligomers which had been committed to the R state will not induce fusion at neutral pH, since, according to the model, fusion requires both a conformational change and low pH (i.e. protonation of the R state).

To examine whether the oligomers can be brought back to the T state, we incubated preactivated virus for various lengths of time at pH 7.4 and 4 °C in the absence of target membranes and subsequently measured the fusion rate after binding to Vero cells. These incubation conditions resulted in a gradual change in fusion rate from that of activated virus to that of untreated virus with complete reversal after about 2 h (Fig. 5). This experiment indicates that the activation process can be reversed, i.e. the oligomers can be brought back to the T state with a half-time of about 1 h, i.e. \( k_1 \), is about \( 2 \times 10^{-4}/s \).

Our observation that VSV fusion is activated by low pH pretreatment is different from that seen with influenza virus, which is inactivated by low pH pretreatment (6). Assuming that inactivation of influenza virus is not due to aggregation of virus, we are led to propose another, desensitized, state in the allostery model in the case of the hemagglutinin protein of influenza virus. Such a desensitized state has also been proposed in an allosteric model for the acetylcholine receptor (11). The desensitized state of hemagglutinin might involve self-aggregation of the proteins, which have exposed their hydrophobic portion to the aqueous environment. VSV does not have any obvious long hydrophobic stretches in its extracytoplasmic non-membrane spanning sequence and therefore might not be desensitized that easily. However, our observation that the extent of fusion of pH 5.5-pretreated virus is quite variable among experiments (60, 20, 40, and 65% in Figs. 2b, 3, 4, and 5, respectively) might be due to a heterogeneous virus population containing partially activated, aggregated, and desensitized viral proteins.

Examination of the hypotheses presented here regarding the regulatory properties of the viral spike glycoproteins in mediating membrane fusion requires further detailed studies of the kinetics of fusion mediated by untreated and pretreated VSV and of the physicochemical states of those proteins in the intact virus, using a variety of biophysical techniques.

REFERENCES