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Fusion of Rous Sarcoma Virus with Host Cells Does Not Require Exposure to Low pH

JOANNA M. GILBERT, DIANE MASON, AND JUDITH M. WHITE*

Department of Biochemistry and Biophysics, Department of Pharmacology and the Cell Biology Program, University of California, San Francisco, San Francisco, California 94143-0450

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We investigated whether Rous sarcoma virus (RSV) infects cells through a pH-independent or a low-pH-dependent pathway. To do this, the effects of lysosomotropic agents and acid pretreatment on RSV infectivity of, and fusion with, chicken embryo fibroblasts (CEFs) were studied. High concentrations of lysosomotropic agents (ammonium chloride and monensin) did not inhibit virus infectivity: equal titers of RSV were produced in the presence and absence of these agents. Similarly, low-pH pretreatment did not inhibit RSV infectivity. In parallel experiments, lysosomotropic agents and acid pretreatment completely abolished the ability of influenza virus to infect CEFs. To monitor the fusion activity of RSV directly, the viral membrane was labeled with the fluorescent lipid probe octadecyl rhodamine at a self-quenching concentration. Upon fusion with a host cell, the probe is diluted in the cell membrane, resulting in fluorescence dequenching (D. Hoekstra, T. de Boer, K. Klappe, and J. Wilschut, Biochemistry 23:5675–5681, 1984). In this assay, fusion of RSV with CEFs was found to occur in both a time-dependent and a strictly temperature-dependent fashion. No fusion occurred unless cells with prebound virus were warmed to temperatures greater than 20°C. Fusion, but not binding, was abolished if virus was pretreated with low concentrations of glutaraldehyde. High concentrations of ammonium chloride had no effect on fusion of RSV with CEFs but greatly diminished the ability of influenza virus and Semliki Forest virus to fuse with CEFs. Similarly, acid pretreatment of RSV had no effect on fusion with CEFs while markedly inhibiting fusion of both influenza and Semliki Forest viruses. Collectively, our results show that RSV fusion with and hence infection of CEFs does not require exposure of the virus to low pH. In this respect, RSV resembles another retrovirus, human immunodeficiency virus.

To initiate an infection, all enveloped animal viruses must fuse with a cellular membrane (38, 46). Viral fusion reactions can be divided into two general classes, low pH dependent and pH independent. Influenza virus, an orthomyxovirus, is the best-characterized virus with low pH-dependent fusion activity. The fusion glycoprotein of influenza virus, the hemagglutinin (HA), undergoes an irreversible conformational change upon exposure to mildly acidic pH, thereby converting it to its fusogenic form (38, 46, 50). This conformational change occurs within acidic organelles after receptor-mediated endocytosis of the virus particle (20). Other viruses such as paramyxoviruses fuse with cells in a pH-independent fashion (46). For viruses that enter cells by a pH-independent pathway, fusion occurs at the plasma membrane but may occur in endosomes as well. Viruses with pH-independent activity also possess specific fusion glycoproteins in their envelopes, such as the F protein of Sendai virus (24). However, the mechanism by which any pH-independent fusion protein functions remains to be determined.

As a family, the retroviruses may differ from other enveloped viruses. Whereas one retrovirus, mouse mammary tumor virus, requires low pH to manifest fusion activity (29), another retrovirus, human immunodeficiency virus (HIV), fuses with cells in a pH-independent fashion (21, 41). Some reports have suggested that murine leukemia viruses require low pH to infect cells (1), but others suggest that these viruses enter cells in a pH-independent fashion (26, 27).

Rous sarcoma virus (RSV) is a transforming avian retrovirus (45). The RSV envelope contains a single glycoprotein, the *env* glycoprotein, which consists of two disulfide-linked glycoproteins, gp85 and gp37. gp85 and gp37 are derived from a common precursor, $Pr95^{env}$ (43), and assemble into higher-order oligomers (7). Like the influenza virus HA and several other viral glycoproteins, the RSV *env* glycoprotein is thought to function both to bind the virus to specific cell surface receptors and to induce fusion with host cells (6, 16, 46). Although the regions of *env* that dictate host range specificity have been mapped (6), little is yet known about how the RSV *env* glycoprotein mediates fusion.

As a first step in elucidating the fusion mechanism of the RSV *env* glycoprotein, we have investigated the pH requirements for RSV fusion with and infection of host cells. We present evidence that exposure to low pH is not required for penetration of the RSV genome into host cells.

MATERIALS AND METHODS

Cells and viruses. Embryonated chicken eggs of genotype C/O (chicken helper factor negative) were purchased from Spafas, Inc., Norwich, Conn. Chicken embryo fibroblasts (CEFs) prepared from 11-day-old embryos were grown in Dulbecco modified Eagle medium (DME H-21; 4.5 g of glucose per liter) plus 10% tryptose phosphate broth (GIBCO, Grand Island, N.Y.), 5% supplemented calf serum, and 1% chicken serum (GIBCO) in a 38°C CO_2 incubator. For viral propagation, polybrene (4 μg/ml; Sigma Chemical Co., St. Louis, Mo.), 1% dimethyl sulfoxide (Fisher Scientific, Inc., Santa Clara, Calif.), penicillin, and amphotericin B were added to the standard medium. Unless specified, the ingredients of all tissue culture media were obtained from the University of California-San Francisco Tissue Culture Facility. Tissue culture dishes were from Corning Plastics (Corning, N.Y.). CEFs were infected with RSV of the Prague C

* Corresponding author.

(PrC) strain or transfected with infectious cDNA clones encoding the Bryan high-titer strain *gag* and *pol* genes and the *env* gene from either a PrC or Schmidt-Ruppin A strain (15). RSV was purified from culture supernatants essentially according to Smith (34). Influenza virus (X:31 strain) and Semliki Forest virus (SFV) were grown and purified as described previously (5, 48).

Syncytium assay. Two types of syncytium assays were performed. Cells with prebound virus (fusion from without [FFWO]) or highly infected cells (fusion from within [FFWI]) were incubated with medium adjusted to various pH values. For FFWO, CEFs on six-well tissue culture plates (2×10^6 to 4×10^6 cells per well) were washed two times with cold binding medium: RPMI (GIBCO) containing 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; Sigma), 5 mM 2-(*N*-morpholino)ethanesulfonic acid (MES; Sigma), and 5 mM succinate (Sigma), pH 7.4. After the cells were chilled on ice for 15 min, virus, either RSV (from 10 to 150 μg of viral protein in 200 μl of cold binding medium) or influenza virus (10 μg of viral protein in 200 μl of cold binding medium) was added and allowed to bind for 1 h on ice. For FFWI, CEFs on six-well tissue culture plates were infected with RSV (viral stocks possessed an endpoint titer of 10^6 to 10^8 infectious units per ml). When the cells reached ~80% confluency (3×10^6 cells per well), they were washed two times with cold binding medium and cooled for 15 min on ice. From this point on, FFWO and FFWI were assayed as follows. The cold binding medium was aspirated, and 2.5 ml of prewarmed binding medium adjusted to pH values ranging from pH 4.8 to 8.0 was added. After incubation at 37°C for 90 s, the pH-adjusted media were removed and the cells were incubated in normal growth medium for 3 to 18 h in a 38°C CO_2 incubator. Cells were then fixed with methanol for 2 h at 4°C, stained with a 10-fold dilution of filtered Giemsa stain (Fisher) in phosphate-buffered saline (PBS), and examined with a light microscope.

Infectivity assays. To measure RSV infectivity, CEFs were plated in 24-well tissue cluture plates and infected with virus-containing samples when they reached 80 to 90% confluency. Serial 10-fold dilutions of virus (in 125 μl of CEF medium) were added to cells; after 1.5 h in a 38°C CO_2 incubator, the cells were overlaid with 1 ml of CEF medium and incubated for an additional 5 h. At this time, low-serum medium (medium with only 1% supplemented calf serum and 0.2% chicken serum) was added. After 8 days, cell supernatants were assayed for reverse transcriptase activity (30; as modified by J. Tuttleman).

To monitor influenza virus infectivity, CEFs (4×10^6 per well in six-well plates) were infected with 1 ml of X:31 allantoic fluid (~10^4 hemagglutinating units [HAU]/ml) diluted 1:1 in CEF medium. After incubation for 1.5 h in a 38°C CO_2 incubator, unbound virus was removed and fresh medium was added for an additional 5 h. At this time, the medium was replaced with DME H-21 containing 10% tryptose phosphate broth and 10 μg of tosylphenylchloroketone-treated trypsin (Sigma) per ml. After an additional 18 h at 38°C in a CO_2 incubator, cell supernatants were removed, cleared of cell debris by centrifugation at 12,000 × *g* for 1 min at room temperature, and assayed for hemagglutinating activity (11).

Incorporation of octadecyl rhodamine B chloride into viral membranes. Octadecyl rhodamine B chloride (R_{18}) was obtained from Molecular Probes (Eugene, Ore.). Stock solutions (6.8 mM in absolute ethanol) were stored at −70°C. The RSV envelope was labeled by adding 37.5 pmol of R_{18} per nmol of viral phospholipid. Influenza virus and SFV envelopes were labeled by adding 57 pmol of R_{18} per nmol of viral phospholipid and purified as described below for RSV. In a typical reaction, 6.6 μl of 6.8 mM R_{18} was injected into a 1-ml solution of RSV (3 mg/ml) in PBS during vortexing and then incubated at room temperature in the dark for 1 h. Unincorporated R_{18} was separated from R_{18}-viruses by chromatography on a column (0.75 by 16 cm) of Sepharose CL-4B (Sigma). After column purification, all R_{18}-virus preparations were centrifuged at 12,000 × *g* for 3 to 5 min at 4°C to remove viral aggregates. R_{18} has been used previously to label RSV for binding studies, and the R_{18}-labeled RSV was shown to be fully infectious (25). The percentage of fluorescence self-quenching was determined by comparing the fluorescence of labeled virus in the absence and presence of 1.0% Nonidet P-40 (NP-40; Sigma). In all preparations of R_{18}-labeled viruses, the degree of fluorescence self-quenching ranged from 75 to 85%.

FdQ assay. The R_{18}-RSV fluorescence dequenching (FdQ) assay was modeled after an assay first used to monitor fusion between influenza virus and BHK cells (40). CEFs on 60-mm tissue culture plates (4×10^6 to 6×10^6 cells per dish) were washed two times at 4°C with cold Hanks-HEPES buffer, pH 7.4 (H/H 7.4; 40), and chilled on ice for 15 min. Then 60 to 80 μg of R_{18}-RSV in 600 μl of binding medium (H/H 7.4 plus 4 μg of Polybrene per ml) was bound to CEFs on ice at 4°C for 1.5 h. Influenza virus and SFV (60 to 80 μg of R_{18}-virus in 600 μl of H/H 7.4 without Polybrene) were bound to CEFs on ice at 4°C for 1.5 h. Samples used for binding determinations were washed two times with cold H/H 7.4 and lysed with 1 ml of H/H 7.4 containing 1.0% NP-40, and the fluorescence was measured. After binding, samples for fusion analysis were washed in the cold two times with cold H/H 7.4 and further incubated in 2 ml of H/H 7.4. Unless otherwise stated, postbinding incubations were conducted at 38°C in a CO_2 incubator. At the indicated times, cells were placed on ice, washed two times with cold H/H 7.4, and then gently scraped into 1.2 ml of H/H 7.4. The amount of fluorescence in the cell suspension was measured. The cells were then lysed in 1.0% NP-40 for 30 min at room temperature in the dark, and the total cell-associated fluorescence was measured. FdQ is the amount of fluorescence measured before addition of NP-40 as a percent of the total cell-associated fluorescence (measured after addition of NP-40).

Low pH pretreatment. For infectivity studies, viral samples containing 10^6 to 10^9 infectious units of RSV or 10^4 HAU of influenza virus per ml were diluted with an equal volume of CEF medium containing 5 mM MES, 5 mM HEPES, and 5 mM succinate at pH 7.4. The pH was adjusted to 4.8 by the addition of predetermined amount of 0.5 N HCl. After incubation at 37°C for the indicated time, samples were reneutralized to pH 7.4 by the addition of a predetermined amount of 1 N NaOH, diluted into an equal volume of CEF medium, and assayed for infectivity as described above. For R_{18} FdQ assays, R_{18}-labeled viruses (0.3 mg/ml) were brought to pH 4.8 with a predetermined amount of 1 N acetic acid. After incubation at 37°C for the indicated time, samples were reneutralized to pH 7.4 by the addition of a predetermined amount of 1 N NaOH, diluted with an equal volume of H/H 7.4, and assayed as described above for FdQ.

Treatment of cells with lysosomotropic agents. NH_4Cl and monensin were purchased from Fisher and Calbiochem-Behring (San Diego, Calif.), respectively. A stock solution of monensin (10 mM in dimethyl sulfoxide; stored at −20°C) was diluted to the indicated concentration in CEF medium, and the pH was readjusted to 7.4 just before use. Solid

NH_4Cl was added to CEF medium or H/H 7.4, and the pH was readjusted to 7.4 just before use. For infectivity assays, CEFs were preincubated in the presence of lysosomotropic agents for 1 h at 38°C in a CO_2 incubator to neutralize acidic intracellular compartments. Cells were then infected with RSV or influenza virus in the continued presence of these reagents for 1.5 h. Unbound virus was removed by washing with medium containing lysosomotropic agents and then incubated for an additional 5 h in the continued presence of lysosomotropic agents. At this time fresh medium, without lysosomotropic agents, was added, and the cultures were incubated and assayed for virus production as described above. For the R_{18} FdQ assay, cells were preincubated for 2 h in H/H 7.4 plus the indicated concentration of NH_4Cl. Virus binding and fusion were carried out as described above but in the continued presence of NH_4Cl. For all experiments using lysosomotropic agents, neutralization of the pH of endocytic organelles was confirmed by treating parallel cultures with acridine orange and observing them in an Olympus inverted-stage fluorescence microscope (2).

Glutaraldehyde treatment of RSV. RSV and R_{18}-RSV (0.8 mg/ml) in PBS were treated with 0.025% glutaraldehyde (Fisher) for increasing lengths of time at 37°C. The samples were chilled on ice and then centrifuged at 4°C in a Beckman airfuge at 30 lb/in^2 (178,000 × *g*) for 10 min through a 20% sucrose–PBS cushion. The viral pellets were washed twice with cold PBS, resuspended in PBS, and then centrifuged at 12,000 × *g* for 3 to 5 min to remove any viral aggregates. Virus was then assayed in CEFs for infectivity, binding, and FdQ.

Fluorescence measurements. Monitoring of R_{18} fluorescence was carried out with a Perkin-Elmer LS 5B luminescence spectrometer (excitation and emission wavelengths of 560 and 590 nm, respectively). The sample chamber was equipped with a magnetic stir motor. The final volume for all samples was 1.2 ml.

Miscellaneous assays. Protein concentrations were determined by the method of Lowry et al. (19). Phospholipid was measured by the method of Folch et al. (8).

RESULTS

RSV does not induce CEFs to form syncytia. Cells that are highly infected with or have high concentrations of enveloped viruses bound at their surface can, in most cases, be induced to fuse with each other. Viruses that enter cells through a low pH-dependent pathway require low pH to induce syncytia (48). Conversely, viruses that enter cells by a pH-independent pathway induce polykaryon formation at neutral pH (46). We examined whether RSV is capable of inducing CEFs to form syncytia over the pH range 4.8 to 8.0. Neither CEFs with high concentrations of RSV prebound (up to 150 μg per 2 × 10^6 cells) nor CEFs infected with RSV to high titer (up to 10^8 infectious units per ml) formed polykaryons at any pH (4.8 to 8.0) tested (data not shown). Addition of amphotericin, which is required for ecotropic murine leukemia virus-induced syncytium formation (26), did not enable RSV to induce CEF polykaryons (data not shown). Parallel CEF cultures with prebound influenza virus (10 μg per 2 × 10^6 cells) formed syncytia when briefly treated at pH 5.0 (data not shown). These results indicated that RSV is incapable of inducing CEFs to form syncytia over the pH range 4.8 to 8.0.

NH_4Cl and monensin do not inhibit RSV infectivity. Infection of host cells by influenza virus and other viruses in the low pH-dependent class requires the acidic pH of endocytic organelles. Lysosomotropic agents (agents that raise the pH of acidic intracellular compartments) such as the weak bases NH_4Cl and chloroquine and the carboxylic ionophore monensin reversibly neutralize the pH of endosomes, thereby blocking the fusion activity and hence infectivity of viruses such as SFV and influenza virus. Thus, although viruses with low pH-dependent fusion activity can bind to and are internalized by cells treated with lysosomotropic agents, they are incapable of fusing with such cells and their infectivity is therefore greatly diminished (20). Conversely, neutralization of acidic intracellular compartments by lysosomotropic agents has no effect on the infectivity of viruses such as HIV which enter cells in a pH-independent fashion (21, 41).

To determine whether RSV requires exposure to the acidic environment of an endosomal compartment in order to infect host cells, we examined the effects of NH_4Cl and monensin on RSV infectivity. CEFs were infected with RSV for a total of 7.5 to 8 h in the presence of these agents and, after return to medium lacking lysosomotropic agents, assayed for progeny virus production as described in Materials and Methods. Concentrations of NH_4Cl as high as 40 mM and concentrations of monensin as high as 10 μM had no effect on the amount of RSV PrC produced as compared with untreated cells (endpoint titer of 1.6 × 10^8 in all cases). This lack of effect of lysosomotropic agents was also observed for RSV containing the *env* glycoprotein from the Schmidt-Ruppin A strain (not shown). In sharp contrast, the presence of 40 mM NH_4Cl or 10 μM monensin completely inhibited the ability of influenza virus to infect CEFs (0 HAU/ml, compared with 10^4 HAU/ml for nontreated cultures). These data suggest that RSV does not require the acidic pH of endosomes to infect host cells.

Low pH pretreatment does not inhibit RSV infectivity. Viruses that enter cells by receptor-mediated endocytosis fuse rapidly and efficiently when they encounter low endosomal pH. If viruses in this category, such as West Nile virus (10), SFV (21), and certain strains of influenza virus (37, 47), are acidified before addition of target membranes, their fusion activity, and hence infectivity, is irreversibly inactivated. Inactivation is presumably due to premature triggering of the fusion-inducing conformational change in the viral fusion protein (37, 49). Conversely, low pH pretreatment of a virus with pH-independent fusion activity (e.g., HIV) has no effect on its ability to infect host cells (21).

We investigated whether preincubation of RSV at acid pH has any effect on its ability to infect CEFs. To do this, RSV was acidified to pH 4.8 at 37°C for 5 min. After reneutralization to pH 7.4, virus infectivity was titered by endpoint dilution reverse transcriptase assays as described in Materials and Methods. No differences in titer were observed between low-pH-pretreated and nontreated RSV (1.6 × 10^8 in each case). In parallel experiments, pretreatment of influenza virus at pH 4.8 totally abolished the ability of influenza virus to infect CEFs (0 HAU/ml, compared with 10^4 HAU/ml for nontreated cultures). These data indicate that unlike its effect on influenza virus, acid pretreatment does not irreversibly inhibit the ability of RSV to infect host cells.

Binding and FdQ of R_{18}-RSV with CEFs. To examine the pH dependence of RSV fusion with host cells, we used an assay devised by Hoekstra and co-workers (12) that employs the fluorescent probe R_{18}. R_{18} can be easily and efficiently incorporated into the outer bilayer of biological membranes at self-quenching concentrations. Upon fusion with a target membrane, R_{18} is diluted and fluorescence increases as a

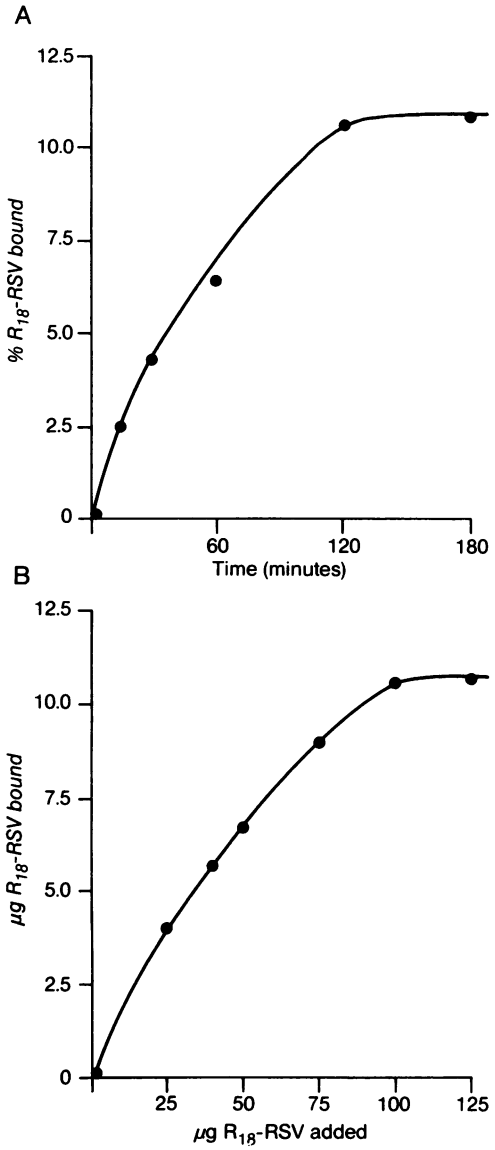

FIG. 1. Binding of R_{18}-RSV to CEFs. RSV was labeled with R_{18} and allowed to bind to CEFs at 4°C. The amount of virus bound during 90 min as a function of the amount of virus added (A) and the amount of virus bound as a function of time (B) were determined as described in Materials and Methods. Experiments were conducted on CEFs in 60-mm tissue culture dishes with 4×10^6 cells per dish. In panel B, 70 μg of virus was added per dish. Triplicate dishes were analyzed for each datum point.

result of relief of self-quenching (12). Variations of this assay have been used to examine the fusion activity of many other enveloped viruses (3, 17, 22, 31, 33, 40).

R_{18}-RSV was prepared as described in Materials and Methods. Labeled virus was bound to CEFs at 4°C. Binding of R_{18}-RSV was saturable in terms of both time (Fig. 1A) and concentration of added virus (Fig. 1B). In a typical experiment, about 10% of the added virus bound. Therefore, based on the molecular weight of RSV (35), about 3,000 virus particles bound per cell. After binding in the cold, unbound virus particles were removed by washing. The cells were warmed to 38°C, and at the indicated times the amount of FdQ was measured as described in Materials and Methods.

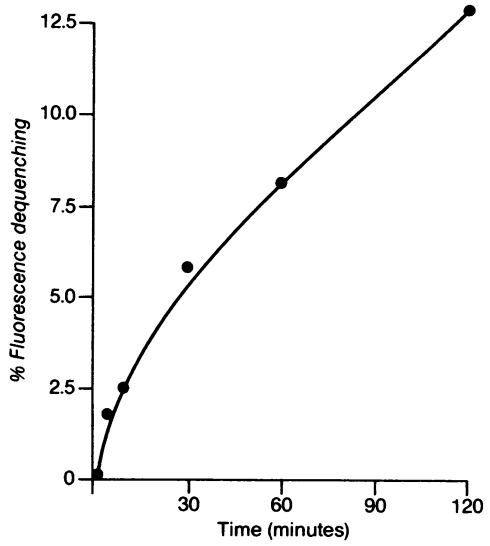

FIG. 2. FdQ of R_{18}-RSV. RSV was labeled with R_{18} and allowed to bind to CEFs at 4°C for 90 min. After washing to remove unbound virus, cells were warmed to 38°C and incubated in a CO_2 incubator. At the indicated times, cells were returned to ice, washed, and analyzed for the amount of FdQ that had occurred as described in Materials and Methods. The cells used were as described in the legend to Fig. 1; 60 μg of R_{18}-RSV was added per dish. Triplicate dishes were analyzed for each datum point.

FdQ was observed upon warming of the cells to 38°C (Fig. 2). The maximum amount of FdQ observed over a 2-h period ranged from 10 to 20% in different experiments.

R_{18}-RSV FdQ is temperature dependent. We next examined the temperature dependence of FdQ. R_{18}-RSV was bound to CEFs for 1.5 h at 4°C. After washing in the cold, the cells were warmed to the indicated temperatures for 1.5 h. We observed a strict temperature dependence for R_{18}-RSV FdQ (Fig. 3). No dequenching was observed unless the cells with prebound R_{18}-RSV were warmed to temperatures greater than 20°C. Such a strict temperature dependence of FdQ has been observed previously for R_{18}-labeled Sendai virus (14). We did not observe any R_{18}-RSV FdQ with CEFs maintained at 4°C for up to 4 h (not shown).

Glutaraldehyde abolishes FdQ without affecting binding. To demonstrate that the FdQ signal that we observed upon incubating R_{18}-RSV with CEFs at >20°C was due to fusion between viral and host cell membranes, we sought to establish conditions under which R_{18}-RSV could bind to cells but there would be no transfer of R_{18} lipid probe into the host cell membrane. One way in which this has been done previously (for Sendai virus) is by treatment with low concentrations of glutaraldehyde (4, 42). We therefore tested the effects of low concentrations of glutaraldehyde on the binding and FdQ of R_{18}-RSV. R_{18}-RSV was treated with 0.025% glutaraldehyde for various amounts of time and then washed extensively as described in Materials and Methods. As little as a 5-min treatment with 0.025% glutaraldehyde dramatically decreased (74%) the FdQ signal compared with the untreated control (Fig. 4). Conversely, treatment of RSV with 0.025% glutaraldehyde for up to 25 min had no effect on the amount of R_{18}-RSV that bound to CEFs. Interestingly, we observed a strict correlation between the loss of FdQ and the loss of infectivity of glutaraldehyde-treated RSV. From these data, we conclude that the FdQ observed upon interaction of R_{18}-RSV with CEFs most likely represents fusion

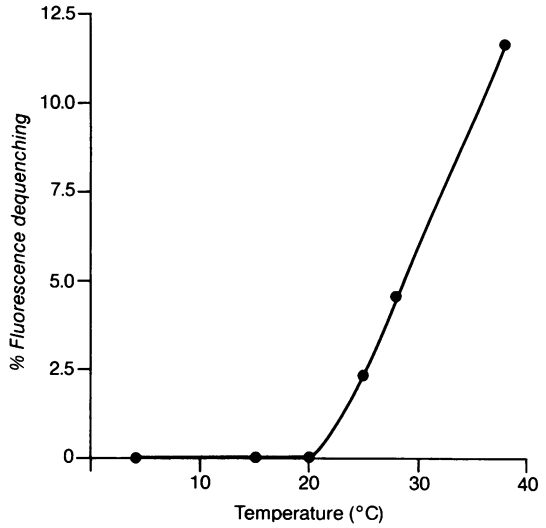

FIG. 3. Temperature dependence of R_{18}-RSV FdQ. R_{18}-RSV was allowed to bind to CEFs at 4°C for 90 min. Unbound virus was removed by washing at 4°C, and the cells were further incubated at the indicated temperatures. After 90 min, the amount of FdQ that had occurred was determined as described in Materials and Methods. Triplicate dishes were analyzed for each datum point.

between the viral and host cell membranes, leading to infectivity. On the basis of the percent of bound virions that fused (Fig. 2), we calculate that ~250 virus particles fused per CEF cell in a 60-min period at 38°C.

NH_4Cl does not inhibit fusion of R_{18}-RSV with CEFs. Once conditions optimal for R_{18}-RSV binding and FdQ were established, we examined whether the presence of a lysosomotropic agent affected the efficiency of fusion of R_{18}-RSV.

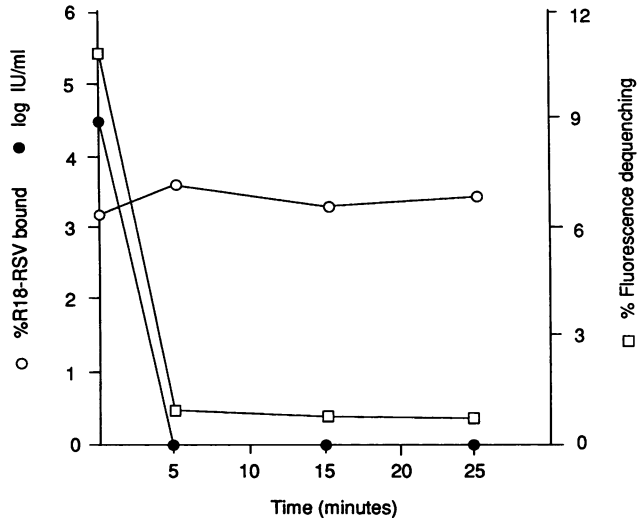

FIG. 4. Glutaraldehyde treatment of RSV: effect on infectivity, binding, and FdQ. RSV and R_{18}-RSV were treated with 0.025% glutaraldehyde at 37°C for increasing amounts of time. Serial 10-fold dilutions of glutaraldehyde-treated RSV were then assayed for infectivity on CEFs (●) as described in Materials and Methods. Samples (60 μg) of glutaraldehyde-treated R_{18}-RSV were added to 60-mm dishes of CEFs. Binding (○) and FdQ (□) were determined as described in Materials and Methods.

TABLE 1. Effect of acid pretreatment and lysosomotropic agents on FdQ

R_{18}-labeled virus	% Inhibition of FdQ	
	pH 4.8, 37°C, for 5 min	50 mM NH_4Cl
RSV	6	3
Influenza virus	100	68
SFV	72	75

CEFs were preincubated with NH_4Cl (50 mM) for 2 h. R_{18}-RSV was then allowed to bind at 4°C for 1.5 h in the presence of 50 mM NH_4Cl. This concentration of ammonium chloride had no effect on the amount of virus bound (not shown). After binding, the cells were warmed to 38°C for 1.5 h in the continued presence of NH_4Cl. The extent of R_{18}-RSV fusion with CEFs was not affected by the presence of NH_4Cl (Table 1). However, in parallel experiments, fusion of R_{18}-influenza virus and R_{18}-SFV with CEFs was markedly reduced by the presence of ammonium chloride (Table 1). These results suggest that RSV does not require the low pH of acidic endocytic organelles in order to fuse with host cells.

Low pH pretreatment does not inhibit fusion of R_{18}-RSV with CEFs. To determine whether the fusion activity of R_{18}-RSV could be inactivated by low pH pretreatment as occurs with low pH-dependent viruses such as influenza virus and SFV, R_{18}-RSV was acidified to pH 4.8 and incubated at 37°C for 5 min. After reneutralization, samples were allowed to bind to and fuse with CEFs as described above. Treatment for 5 min at pH 4.8 at 37°C had no effect on the percent of virus bound (not shown) and very little effect on the amount of FdQ observed (Table 1). Parallel experiments with R_{18}-influenza and R_{18}-SFV demonstrated that these viruses were highly sensitive to low pH pretreatment, as demonstrated by significant decreases in their FdQ (Table 1). These results suggest that acid pretreatment does not irreversibly inactivate the fusion activity of RSV. Moreover, they suggest that the mechanism by which RSV fuses with and hence infects host cells is different than those employed by influenza virus and SFV.

DISCUSSION

RSV is a prototypic retrovirus. Although much is known concerning its replication strategy and transforming capability, little is known about how RSV fuses with and infects host cells. To examine this mechanism, it is first important to know whether RSV enters host cells by a low pH-dependent or a pH-independent route. To address this question, we used a combination of techniques that have been used previously to study this problem for other enveloped animal viruses. We sought to establish conditions necessary for virus-induced syncytium formation (48), we assessed the effects of lysosomotropic agents and low pH pretreatment on viral infectivity (21), and we examined the effects of lysosomotropic agents and low pH pretreatment on fusion between the viral and host cell membranes (40).

We first investigated whether RSV could induce CEFs to form syncytia under a variety of conditions. Neither CEFs with high concentrations of RSV prebound nor CEFs highly infected with RSV yielded polykaryon formation at any pH (4.8 to 8.0) or under any condition examined. CEFs are not inherently resistant to syncytium formation, as evidenced by the ability of influenza virus to induce CEF syncytia at acidic

pH. Although we were unable to observe RSV-induced syncytium formation, a previous study reported that certain strains of RSV, but not others, produced a limited number of small syncytia within the foci of highly transformed cells (28). Presumably this fusion occurred at neutral pH. It should be noted that RSV is not unique among enveloped viruses in its inability (or at least impaired ability) to induce syncytia. Strains of bovine parainfluenza virus (32) and herpesvirus (36) exist that are unable to induce polykaryon formation yet are fully infectious. A genetically engineered mutant of the influenza virus HA is capable of inducing fusion with erythrocytes yet unable to mediate polykaryon formation (9). Therefore, the apparent inability of RSV to induce syncytia does not indicate that RSV is not fusion competent.

Since we could not determine the pH requirements for RSV fusion on the basis of simple assays of syncytium formation, we next assessed the effects of lysosomotropic agents and low pH pretreatment on RSV infectivity. Lysosomotropic agents raise the pH of endosomes. They have been shown to inhibit the infectivity of all enveloped viruses tested that display low pH-dependent fusion activity because they raise the endosomal pH above the pH required to elicit fusion (20). Lysosomotropic agents have no effect on the infectivity of viruses such as HIV that do not require low pH to fuse (21). We therefore tested the effects of two lysosomotropic agents, NH_4Cl and monensin, on RSV infectivity. Neither lysosomotropic agent had any effect on RSV infectivity. Conversely, both agents completely abolished the infectivity of influenza virus in CEFs. These data indicate that whereas influenza virus requires the low endosomal pH for genome penetration and hence infection, RSV does not. In our protocol, the lysosomotropic agents were present only during the first 7.5 h of infection, thereby precluding effects that these agents might have on later events in the viral life cycle. In a previous study, another lysosomotropic agent, amantadine, was found to inhibit RSV infectivity by 1 order of magnitude. In the earlier study, however, amantadine was present during the initial infection and for the subsequent 7 days (in the agar overlay) before foci were counted (44). Therefore, the previously observed inhibitory effect of amantadine on RSV infectivity may have been due to aberrations in posttranslational events resulting in the production of virus particles of lowered infectivity. Such a phenomenon has been documented for HIV (21).

Acid pretreatment inhibits the infectivity of many enveloped viruses with low pH-dependent fusion activity (10, 21, 37, 47) while having no effect on those with pH-independent fusion activity (21). We found that incubation of RSV at pH 4.8 at 37°C for up to 5 min had no effect on the ability of RSV to infect CEFs, whereas identical treatment of influenza virus totally inhibited its ability to infect CEFs.

Collectively, our infectivity studies indicate that, unlike influenza virus but similar to HIV, RSV infects cells through a pH-independent mechanism. To corroborate these findings, we developed an assay that monitors fusion between RSV and CEFs directly. This assay employs a fluorescent lipid probe, R_{18}, incorporated into the RSV membrane at a self-quenching concentration. Upon fusion with a target membrane, the probe is diluted, resulting in fluorescence dequenching. This assay was modeled after ones used previously to monitor fusion of viruses with both low-pH-dependent activity, including influenza virus (39, 40), vesicular stomatitis virus (3), and Sindbis virus (31), as well as viruses with neutral pH fusion activity such as Sendai virus (14, 17), HIV (33), and Epstein-Barr virus (22).

After binding R_{18}-RSV to CEFs at 4°C, we observed strictly temperature dependent fusion as assayed by FdQ. Samples of CEFs incubated with R_{18}-RSV at 4°C for up to 4 h showed no FdQ. For an FdQ signal to be observed, CEFs with bound R_{18}-RSV had to be incubated at temperatures greater than 20°C. Such a strict temperature dependence of fusion has been observed previously for R_{18}-labeled Sendai virus (14). We also observed that the FdQ activity of R_{18}-RSV with CEFs could be abolished by treatment with low concentrations of glutaraldehyde without any loss of virus-binding capacity. Thus, we have established two conditions under which R_{18}-RSV binds to cells but under which we see no FdQ: incubation at temperatures less than 20°C and treatment of virus particles with low concentrations of glutaraldehyde. We interpret these observations to mean that the FdQ signal that we observe with R_{18}-RSV is due to fusion of the viral membrane with the host cell membranes. Our calculations indicate that ~250 RSV particles fuse per CEF cell in 60 min at 38°C. This value is comparable to the number of influenza virus particles observed previously to fuse with BHK-21 cells (~500) or LLC-MK_2D cells (~250) during the same time period (40).

Using the R_{18}-RSV FdQ assay, we found that high concentrations of NH_4Cl had little effect on the extent of fusion between RSV and CEFs. In contrast, NH_4Cl reduced markedly the extent of fusion of R_{18}-influenza virus and R_{18}-SFV with CEFs (by 68 and 75%, respectively). These results indicate that, unlike SFV and influenza virus, RSV does not require the acidic pH of endosomal compartments in order to fuse with host cells.

Using the FdQ assay, we also found that pretreatment of RSV at low pH had no effect on fusion between RSV and CEFs. Low-pH pretreatment of influenza virus and SFV greatly diminished the ability of these viruses to fuse with CEFs. These results indicate that RSV *env* glycoprotein is not inactivated by exposure to acid pH as are the influenza virus HA and the SFV spike glycoproteins. Therefore, on the basis of our studies of both viral infectivity and viral fusion activity, we conclude that RSV does not require exposure to low pH in order to fuse with and thereby infect host cells.

For most enveloped viruses, the pH requirement for fusion appears to be a common property of the virus family. For example, all orthomyxoviruses and all togaviruses studied to date require low pH to fuse. Similarly, all paramyxoviruses studied to date are able to fuse at neutral pH. The retroviruses may be different in this respect (46). The envelope glycoprotein of mouse mammary tumor virus appears to require low pH in order to manifest its fusion function (29). Conversely, it is now clear that HIV does not require exposure to low pH in order to fuse with and infect host cells (21, 41). On the basis of the studies presented in this report, we conclude that RSV resembles another C-type retrovirus, HIV, in fusion pH requirements.

The fusion protein of RSV is its envelope glycoprotein. This glycoprotein is a complex of two polypeptides, gp85 and gp37, that arises by cleavage of a larger precursor (43) and exists on the viral surface as an oligomer (7). The *env* glycoprotein is also responsible for binding virus to receptors on CEF membranes (6). In these respects, the *env* glycoprotein resembles the well-characterized fusion protein of influenza virus, the HA (38, 46, 50). However, with respect to fusion activity, the *env* glycoprotein differs from HA in at least two important respects. First, as shown here, the protein does not require exposure to acidic pH in order to function as a fusogen. Second, the *env* glycoprotein lacks

a characteristic amino-terminal fusion peptide such as is found at the amino terminus of the HA2 subunit of HA (16, 46). In this respect, the RSV *env* glycoprotein also differs from the HIV *env* glycoprotein, which possesses a fusion peptide at the amino terminus of gp41 (18, 23). Rather than being at the amino terminus of gp37, the putative fusion sequence of the RSV *env* glycoprotein is internal to gp37 and is located between residues 22 and 37 (16, 46). Future studies are aimed at elucidating the molecular mechanism of fusion of the RSV *env* glycoprotein. It will be interesting to see how the mechanism of the RSV *env* glycoprotein compares with those of the influenza virus HA and the *env* glycoprotein of HIV.

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