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RSV entry inhibitors block F-protein mediated fusion with model membranes

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Abstract

RSV fusion is mediated by F-protein, a major viral surface glycoprotein. CL-309623, a specific inhibitor of RSV, interacts tightly with F-protein, which results in a hydrophobic environment at the binding site. The binding is selective for F-protein and does not occur with G-protein, a surface glycoprotein that facilitates the binding of RSV to target cells, or with lipid membranes at concentrations in the sub-millimolar range. Using an assay based on the relief of self-quenching of octadecyl rhodamine (R18) incorporated in the RSV envelope, we show that the virus fuses efficiently with large unilamellar vesicles containing cholesterol, in the absence of specific receptor analogs. Fusion of *cp-52*, a mutant virus lacking the G and SH surface glycoproteins, with vesicles is inhibited by CL-309623 and RFI-641 due to specific interactions of the inhibitor(s) with the fusion protein. Both virus–vesicle and virus–cell fusion are inhibited with equal potency. The formation of the binary complex of CL-309623 with F-protein in its native state, resulting in the inhibition of fusion and entry of virus, is a prerequisite for the observed anti-RSV activity in cell cultures. © 2002 Published by Elsevier Science B.V.

Keywords: RSV; *cp-52*; Inhibition; Large unilamelar vesicles; F-protein; G-protein; Lipid composition; Dequenching assay

1. Introduction

Respiratory syncytial virus (RSV) is a member of the paramyxovirus group of enveloped viruses and is a major and widespread cause of lower respiratory tract illness during infancy and childhood (Collins et al., 1996). Serological evidence indicates that approximately 95% of children have been exposed to RSV by 2 years of age with approximately 91 000 infants hospitalized annually with RSV infection in the US (Hall, 1999; LaVia et al., 1993). Since the immune response to RSV infection is not protective, RSV infections reoccur throughout adulthood. RSV in the institutionalized elderly can be more serious and characterized by severe pneumonia and mortality rates up to 20 and 78%, respectively (Dowell et al., 1996; Falsey and Walsh, 2000). Significant mor-

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tality has been observed in immunocompromised patients, particularly those undergoing bone marrow transplantation. Regular outbreaks of RSV are well characterized and predictable, occurring between October and May each year with peak occurrences in January and February. Currently, the only small molecule inhibitor of RSV is ribavirin (Aylward and Burdge, 1991). However, utilization of ribavirin is limited due to efficacy and toxicity concerns (Ohmit et al., 1996). Due to the lack of effective and safe anti-RSV agents there has been a significant increase in research to identify new drugs.

Enveloped viruses enter the cell by fusing their membranes with those of the plasma or endosomel membrane. Retroviruses and paramyxoviruses bind to cell surface receptors, followed by fusion with the target cell membrane. In the case of influenza, the virus particle is internalized into the endosomes where fusion occurs with the membrane at low pH (Matlin et al., 1981; Marsh, 1984; White et al., 1983). With paramyxoviruses, activation of fusion occurs via interactions with cell surface receptors at neutral pH. With the paramyxovirus, receptor binding and fusion are due to two distinct cell surface glycoproteins, attachment and fusion proteins, respectively. Glycoprotein receptors containing sialic acid residues and asialoganglioside receptors are required for fusion of sendai virus with HEp-2 cells (Markwell et al., 1985). Sialic acid containing glycoproteins or glycolipids are also receptors for influenza virus (Millar et al., 1999). Recent work with sendai virus has shown that fusion is maximal at 55 °C and that an increase in temperature is a trigger for fusion (Wharton et al., 2000). Recently, it was shown that RSV attachment and infectivity mediated both by interaction with F and G proteins (Feldman et al., 2000). In RSV the viral envelope includes two major surface glycoproteins: G and F. G is a highly glycosylated protein and is responsible for binding of the virion to cells. F, the fusion protein, induces membrane merging, and release of viral capsid into the cytoplasm of the targeted cell through the fusion pore. Membrane merger is a lipid-mixing event in virus–cell fusion process. A mutant cold-passaged RSV (*cp-52*) consisting of the fusion protein as the only viral envelope protein has been shown to be infectious in cell culture (Karron et al., 1997).

Several years ago a screening program at Wyeth-Ayerst Research identified a potent and specific inhibitor of RSV (see Fig. 1), the disulfonated stilbene CL-309623, from a diverse compound library. The lead compound in the RSV program, RFI-641, a biphenyl analog was shown to have potent anti-RSV activity in virus and plaque growth assays (Aulabaugh et al., 2000; Ding et al., 1998). We had concurrently demonstrated that the inhibitor binds specifically to purified preparations of fusion protein thereby supporting the hypothesis that the fusion protein is the target for these inhibitors. RFI-641 has also been tested in animal models of RSV infection. When administered prophylactically, RFI-641 reduced viral titers in mouse, cotton rat and African green monkey animal models (Huntley et al., 2002). More recently we have shown that these dendrimer-like molecules inhibit an early event of lipid mixing during virus–cell fusion (Razinkov et al., 2001). RFI-641 binds and inhibits both the wild type and the cold-passaged mutant forms thereby supporting the observation that the fusion protein is the primary target. To further evaluate the mechanism of action of the inhibitors with respect to the functional aspects of the fusion protein, we investigated the effect of inhibitors on

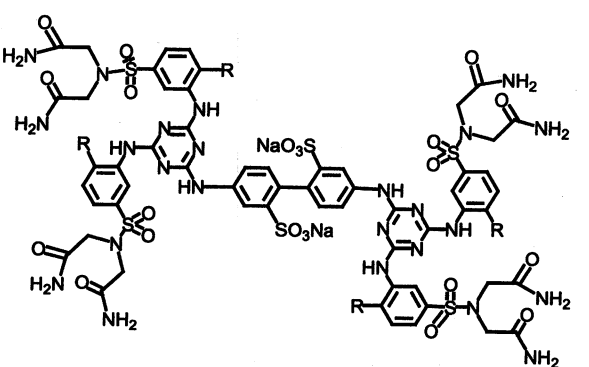

Fig. 1. Chemical structures of RSV inhibitors.

the fusion of virus with vesicles. Fusion of virus with cells was monitored using a Rhodamine R18 fluorescence dequenching assay (Hoekstra et al., 1984). The R18 assay is based upon the dequenching of fluorescence of Rhodamine R18 probe, incorporated in the viral membrane, upon fusion with vesicles (or cells). In this assay, viral membrane is labeled with a fluorescent lipid probe at a self-quenching concentration. After fusion with unlabeled membrane, mixing and dilution of the probe results in the dequenching of fluorescence. The R18 fluorescence dequenching assay has been widely used for study of the interaction of enveloped viruses with target cell membranes (Hoekstra et al., 1984; Razinkov et al., 2001).

In order to differentiate the inhibitory effects on F proteins from G-proteins, we have employed the cold-passaged RSV subgroup B designated RSV B1/*cp-52*, which contains a deletion that prevents the synthesis of both the G and SH proteins. It has been shown that *cp-52* replicates in tissue culture (Karron et al., 1997) and grows very poorly in animal models (Crowe et al., 1996). These findings suggest that the F-protein alone can mediate infectivity of RSV. We have, therefore, used this virus strain to characterize fusion between the viral envelope and lipid bilayer of large unilamellar vesicles (LUV) and determined the inhibitory effects of RFI-641 and CL-309623. The binding characteristics of CL-309623 with purified preparations of F- and G-proteins were also investigated to confirm the target specificity of the inhibitor. The results described here show that the CL-309623 and RFI-641 inhibit RSV via interactions with F-protein in its native state and this interaction primarily accounts for the observed inhibitory effects in vitro.

2. Materials and methods

2.1. Viruses

Human RSV A2 strain was obtained from Wyeth-Lederle Vaccines, Pearl River, NY. *cp-52*, cold-passaged RSV mutant was kindly provided by Valerie Randolph from Wyeth-Lederle Vaccines.

2.2. Virus purification

Purification of RSV was done as previously described with some modifications. Virus stock solutions were prepared by infecting Vero cells in roller bottles at low multiplicity of infection (moi) of 0.01 plaque forming units (pfu) per cell. Infected cells were incubated at 37 °C until extensive syncytium formation, for 3–4 days, then freezing medium (MEM, 10% FBS, 1 × SPG, 25 mm Hepes) was added and the bottles were quickly frozen in dry ice–methanol. Infected cells were thawed quickly in 37 °C water bath, sonicated for 2 min in a Bronson 1210 sonicator and Dounce homogenized. Cell debris was pelleted by centrifugation at $5000 \times g$ at 4 °C for 20 min. Polyethylene glycol (PEG) 6000 was added to supernatent to a final concentration of 6.5% (w/v) concentration and stirred at 4 °C for 1.5 h. The virus was collected by centrifugation at $5000 \times g$ at 4 °C for 1 h and resuspended in NTE buffer (100 mm Tris–HCl, pH 7.5, 100 mm NaCl, 10 mm EDTA) by Dounce homogenization. The resuspended virus was then added to a tube containing two layers of sucrose in NTE buffer. Eight ml of 40% sucrose was layered on the top of the tube and 1 ml of 60% sucrose at the bottom. The virus was centrifuged against the two sucrose bands at 25 000 rpm in a SW41 Ti rotor at 4 °C for 2 h. The virus band sedimenting at the interface of 40 and 60% sucrose layer was harvested, resuspended in 20% sucrose in NTE buffer and centrifuged on a discontinuous gradient of 30–60% sucrose at 30 000 rpm in a SW41 Ti rotor at 4 °C for 2 h. The purified virus band was collected and 50 μl aliquots were stored at − 70 °C.

2.3. Plaque assay

Monolayer cultures of VERO cells in six well plates were infected at room temperature with serially diluted RSV preparations in DMEM (2% serum). After adsorption for 1.5 h, the inoculum was removed and replaced with medium containing methylcellulose. After 5 days, the cells were fixed with methanol:acetone (1:1) and viral plaques were visualized by immunoperoxidase staining. Primary antibody was mouse anti-RSV

(1:1000 in 1 × PBS), and the secondary antibody was peroxidase-conjugated anti-mouse IgG (Sigma; 1:1000 dilution in 1 × PBS). 3,3′-Diaminobenzidine (DAB)/Peroxide solution was used for development of plaques. Plaques numbers were quantified by visual counting.

2.4. Yield reduction assay

The antiviral activity of CL-309623 and RFI-641 against RSV strains A2 and *cp-52* was evaluated in a yield reduction assay, a direct measurement of the amount of virus produced during a single round of viral infection. Vero cells were infected at an MOI = 0.3 of RSV in media (DMEM containing 2% fetal calf serum (FCS)) and freshly prepared compound at concentrations of 0, 0.1, 0.25, 0.5, 1.0, 2.0, 5.0, or 10.0 μM. After a 90-min adsorption period at room temperature, the inoculum was removed and replaced with fresh media containing CL-309623 or RFI-641 at the appropriate concentrations. After a 30-h incubation at 37 °C, progeny virus were harvested by the addition of 0.5 ml of RSV freezing media (1.36 × Minimal Essential Media, 0.02 M HEPES, 10% FCS, 7.6 mM KH_2PO_4, 14.4 mM K_2HPO_4, 10 mM glutamate, 0.44 M sucrose) to each well and the plates were stored overnight at − 70 °C. The cells were thawed, scrapped with a rubber policeman, transferred to microfuge tubes, and sonicated for 1 min (Branson model 1200 water bath sonicator). Cellular debris was pelleted for 10-min at 80 × *g* and 4 °C. The virus containing supernatant was titered by serial dilution on Vero cells as above.

2.5. R18 labeling of RSV

The purified virus was labeled with the lipid probe octadecyl rhodamine B chloride, R18 (Molecular Probes Inc., Eugene, OR), according to the procedure described previously (Hoekstra et al., 1984) with slight modifications. Virus (300 μg of total protein) in 300 μl of NTE buffer was incubated with 5 μg of R18 (5 μl of ethanol solution) for 45 min in the dark at room temperature. The labeled virus was separated from unincorporated R18 probe by gel filtration on a G-25 Sephadex microspin column (Amika Corporation, Columbia, MD, USA). The column was spun at 1500 × *g* for 4 min. The virus recovered corresponded to a protein concentration no less than 80% of input protein concentration using a BCA kit (Pierce, Rockford, IL).

2.6. Preparation of vesicles

Large unilamellar vesicles (LUV) were prepared from egg phosphatidyl choline (egg PC) and cholesterol (Chol) as follows. In a typical experiment a 1 mg/ml of vesicle solution was prepared as follows: 86 μl of a 20 mg/ml stock solution of egg PC in chloroform was combined with 28 μl of a stock solution of 10 mg/ml of cholesterol and the mixture was dried for 1 h using a vacuum rotor. Then, 2 ml of NTE buffer was added to lipid under nitrogen atmosphere and the suspension mixed vigorously for 1 min to form multilamellar vesicles (MLV). This procedure produces a 3:1 molar ratio of egg PC to cholesterol at a total lipid concentration of 1.5 mm. When other lipids were used in the preparation of the vesicles they were combined with the egg PC/Chol mixture as indicated above. MLV vesicles were extruded 15 times through 200 nm polycarbonate filter on a syringe extruder (Avanti Polar Lipids) to obtain LUVs with narrow size distribution according to the manufacturer's procedure. LUV preparations were prepared and stored under an atmosphere of nitrogen and used the same day.

2.7. Fluorescence dequenching assay

Two μg (in terms of total protein) of R18 labeled RSV was added to 0.5 ml of NTE buffer in 0.5 × 0.5 cm quartz cuvette. After a 5-min pre-incubation at 37 °C, vesicles were added to virus solution at a final lipid concentration of 0.05 mm. Fluorescence intensity was continuously recorded after vesicle addition on a FluoroLog (Jobin Ivon Inc, Edison, NJ) spectrofluorimeter with an excitation wavelength of 560 nm and emission wavelength of 585 nm. Virus was allowed to fuse with vesicles for 30 min, after which SDS detergent was added to a final concentration of 2% to completely randomize the distribution of

R18 and to get maximum dequenching (Hoekstra et al., 1984). The extent of fusion was estimated as percentage dequenched (DQ) calculated as

$$DQ = \frac{F - F_0}{F_{det} - F_0}$$

where F is R18 fluorescence measured at the 30 min time point, F_0 is initial fluorescence intensity just after addition of virus and F_{det} is fluorescence intensity after addition of detergent.

Normalized dequenching is the ratio of dequenching in the presence of inhibitor to that in the absence of inhibitor. In the continuous assay of the kinetics of fusion, fluorescence intensity was acquired at 1-min time intervals at excitation and emission wavelengths of 560 and 580 nm, respectively.

3. Results

3.1. Effect of temperature and time course of RSV fusion with LUV

The effect of temperature on the fusion of virus with vesicles was evaluated in a continuous fusion assay. LUV containing egg phosphatidylcholine and cholesterol, (3:1 molar ratio) were prepared and added to R18 labeled virus at 37 °C to initiate fusion. Fig. 2 shows the dequenching of R18 fluorescence resulting from membrane merger of virus with the vesicles during fusion. The gradual increase in fluorescence with time is due to dye distribution that occurs during the lipid mixing stage. Both *cp-52*, the cold-passaged virus strain, and wild type A2 virus fuse with vesicles at comparable efficiency. While a quantitative comparison of the virus fusion with vesicles (Fig. 2) and Vero cells (Razinkov et al., 2001) is not appropriate due to differences in the composition and relative dimensions of these membranes, it is noteworthy that the time course of fusion is qualitatively similar. The time course of dequenching of R18 fluorescence due to fusion of *cp-52* virus with LUV's was evaluated at 10, 25 and 37 °C. This experiment showed that optimal dequenching occurred at 37 °C although there is some residual fusion activity at 10 °C. The virus

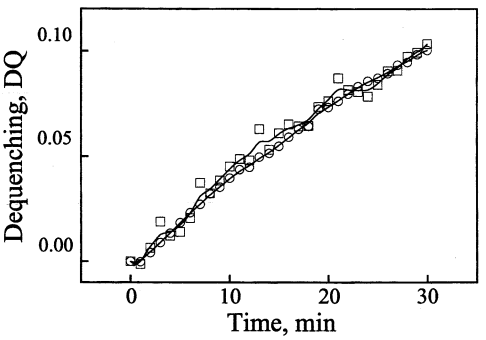

Fig. 2. Kinetics of fusion of R18 labeled RSV with LUV. □, A2 strain; ○, *cp-52* strain. Labeled virus (2 μg of total protein) was pre-incubated for 5 min at 37 °C and vesicles were added to a final concentration of 50 μM. Fluorescence of R18 was measured continuously at the excitation and emission wavelengths of 560 and 585 nm, respectively, in 1 min time intervals. Detergent was added to the solution at the end of 30 min incubation to a final concentration of 2%. Dequenching (DQ) was calculated as described in Section 2. The fitted curves connecting the data points were obtained using a smoothing function in the Kaleidagraph software package from Synergy Software (Reading, PA).

fusion, and the resulting dye spread, at 10 and 25 °C are approximately 25 and 50%, respectively, of the fusion activity observed at 37 °C (data not shown).

3.2. Effect of target membrane composition on RSV fusion

To determine the effect of lipid composition of vesicles on RSV fusion activity, large unilamellar vesicles were prepared with egg phosphatidyl choline (egg PC) in the absence and presence of cholesterol (Chol) and asialo (aGM1) or sialylated gangliosides (GM1) or sphingomyelin (SM). In these experiments the basic lipid composition of the vesicles is a 3:1 molar ratio of egg PC to cholesterol. The composition of lipids tested for their effect of viral fusion is as follows: egg PC with cholesterol and sphingomyelin (3:1:0.4 molar ratio), egg PC, cholesterol and sialoganglioside (3:1:0.2); and egg PC, cholesterol and asialoganglioside (3:1:0.2). The effect of lipid composition on viral fusion was evaluated in an end-point assay format. In this assay, based upon the results of the temperature and time course experiments,

virus was incubated with LUV's for 30 min at 37 °C. The results of virus–vesicle fusion as a function of lipid composition are shown in Fig. 3. The presence of cholesterol in vesicles increases fusion by about 20% compared with vesicles prepared from egg PC lipids alone. The gangliosides, in both forms, do not significantly affect RSV fusion. Since the interactions of cholesterol with sphingomyelin have been shown to alter the effect of cholesterol on fusion of influenza (Razinkov and Cohen, 2000) and sendai virus, vesicles were prepared with cholesterol/SM mixture to determine how this mixture can alter RSV fusion. As shown in Fig. 3, the eggPC/SM/cholesterol composition did not alter fusion compared with vesicles containing egg PC/Chol or egg PC alone. Since these results suggest that the optimum lipid composition should include cholesterol, we have used this composition to determine the effect of inhibitors on fusion of *cp-52* with the inhibitors.

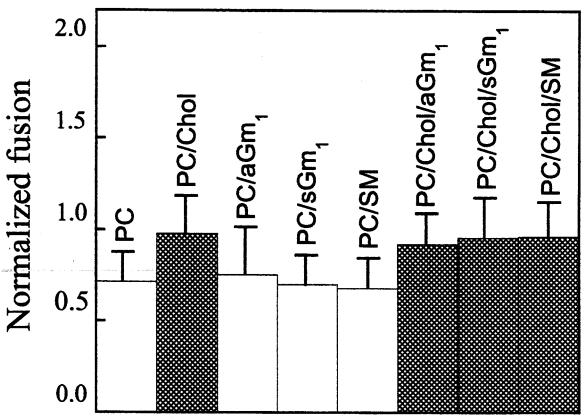

Fig. 3. Effect of lipid composition on *cp-52* fusion with LUV. The effect of lipid composition was tested at a final total concentration of 50 μM at 37 °C after 30 min incubation in all experiments. The basic composition of vesicles is egg PC and cholesterol at 3:1 molar ratio or egg PC alone at a total lipid composition of 50 μM. The concentration of sialo and asialogangliosides in the vesicles corresponds to a molar ratio of 3:1:0.2 of egg PC to cholesterol to gangliosides. In the case of vesicles containing sphingomyelin, the ratio is 3:1:0.4 of egg PC to cholesterol to sphingomyelin. In the vesicles prepared from egg PC alone (absence of cholesterol) the molar ratio of egg PC to sphingomyelin is 1:0.1 or egg PC to gangliosides is 1: 0.05. The dequenching of R18 fluorescence due to fusion was estimated as described in materials and methods and in legend to Fig. 2. The extent of fusion at various lipid compositions was normalized relative to the extent of fusion of vesicles consisting of egg PC and cholesterol. The error bars were calculated based upon an average of two experiments.

3.3. Inhibition of virus–vesicle fusion by RFI-641 and CL-309623

Previous spectrofluorimetry and fluorescence microscopy experiments had shown that RSV fusion with Vero cells can be inhibited by RFI-641 at low efficacious ($IC_{50} = 50$ nM) concentrations (Razinkov et al., 2001). Using the R18 fluorescence dequenching assays of fusion, we had previously shown that both RFI-641 and CL-309623 inhibit virus-cell fusion while an inactive analog of RFI-641, WAY-158830, did not inhibit fusion (see Fig. 1 for structures). We have, therefore, used these same inhibitors in the virus–vesicle fusion assay to determine if they would similarly inhibit fusion. The results of fusion of *cp-52* with vesicles in the presence of RFI-641 and WAY-158830 and the effect of CL-309623 on the fusion of either *cp-52* or A2 virus with vesicles are shown in Fig. 4. WAY-158830 has a negligible effect on virus-liposome fusion, similar to the fusion results with Vero cells reported earlier. Structurally, the only difference between WAY-158830 and the active analogs is the presence of the methyl group in the peripheral benzenesulfonamides (see Fig. 1). The IC_{50} of this analog in the virus growth assays was higher, $IC_{50} = 25$ μM, compared with the IC_{50} of 50 nM for RFI-641 in plaque formation assay of the A2 strain (Ding et al., 1998).) The inhibitory concentrations of RFI-641 and CL-309623 are comparable in the virus–vesicle fusion assay. The effect of CL-309623 on *cp-52* virus was also evaluated in a yield reduction assay. Both CL-309623 and RFI-641 inhibited *cp-52* mutant virus to a similar extent (Fig. 5).

3.4. CL-309623 binding interactions with purified RSV fusion (F) and attachment (G) proteins

Since we had previously shown that RSV inhibitors interact with purified preparations of F-protein (Ding et al., 1998), and also with virus (Razinkov et al., 2001) we have further explored the binding properties of the stilbene analog, CL-309623, with respect to G- and F-proteins of

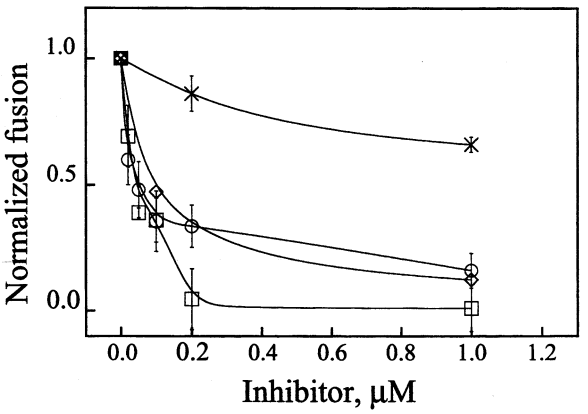

Fig. 4. Virus fusion with vesicles in the presence of RFI-641, CL-309623 and WAY-158830. *cp-52* and A2 strains were fused with egg PC/Chol (3:1 molar ratio) vesicles in the presence of inhibitor at 37 °C. Aliquots of virus were injected into a cuvette containing the egg PC/Chol vesicles. The inhibitor was added immediately before the addition of virus and incubated for 30 min at 37 °C, prior to the addition of detergent. The extent of fusion in the absence and presence of the inhibitor was estimated as described in the legend to Fig. 2. The extent of fusion in the presence of inhibitor was normalized relative to the fusion in the absence of inhibitor for each of the two virus strains. The normalized fusion values for *cp-52* strain in the presence WAY-158830 (X), RFI-641 (□), CL-309623 (○) and for A2 strain in the presence of CL-309623 (◇) are shown.

RSV. We have taken advantage of the fluorescence properties of CL-309623 to determine the binding efficiency and target selectivity to F- and G-proteins of RSV. The fusion proteins purified from A2 wild type strain and the *cp-52* cold-passaged mutant strain were used in the binding experiments. The wild type and the mutant fusion protein preparations are denoted as PFP2 and PFP3, respectively. The two fusion proteins differ in four amino acid substitutions, namely, K66E, Q101P, E218A and T523I. The carbohydrate content of PFP2 and the mutant virus are as follows: sialic acid 9.4 and 4.7%, mannose, 54.4 and 53.2% and glucosamine 36.1 and 32,1%, respectively. Both PFP2 and PFP3 fusion proteins were purified using identical protocols (Ding et al., 1998).

The fluorescence emission of CL-309623 is centered at 450 nm, a wavelength maximum that is significantly different from the intrinsic fluorescence of the tryptophan residues of the protein. We had previously shown that interactions of the biphenyl analog, RFI-641, with fusion protein are accompanied by an increase in quantum yield of fluorescence after incubation with F-protein (Ding et al., 1998). The binding of CL-309623 with fusion protein, PFP2, is similarly accompanied by a blue shift in the fluorescence emission maximum (Fig. 6A). Since the fusion protein is prepared in a buffer containing the detergent TRITON X-100, the effect of the detergent on the intrinsic fluorescence of the inhibitor was also determined in a control experiment. As expected from previous binding experiments, the detergent did not alter the fluorescence of the inhibitor in the same manner as the protein. Binding interactions of CL-309623 with PFP3 or PFP2 preparations of fusion protein enhanced the intrinsic fluorescence of the inhibitor as evidenced by a blue shift in the emission wavelength. The saturable binding and the observed affinity of CL-309623 to fusion protein purified from the wild type A2 strain, PFP2, is nearly identical to the affinity to PFP3 fusion

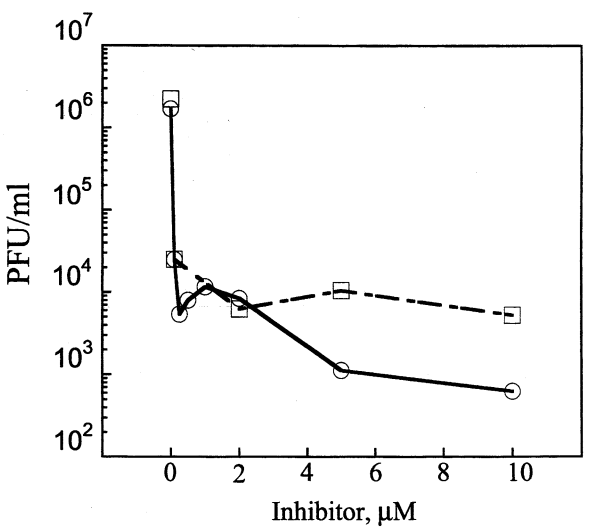

Fig. 5. CL-309623 and RFI-641 inhibit RSV replication in Vero cells. Vero cells were infected with the RSV deletion strain *cp-52* in media containing either CL-309623 (□) or RFI-641 (○) at the indicated concentrations in a yield reduction assay according to the procedure described in the materials and methods section. Viral progeny were visualized and quantified by serial dilution and immunoperoxidase staining.

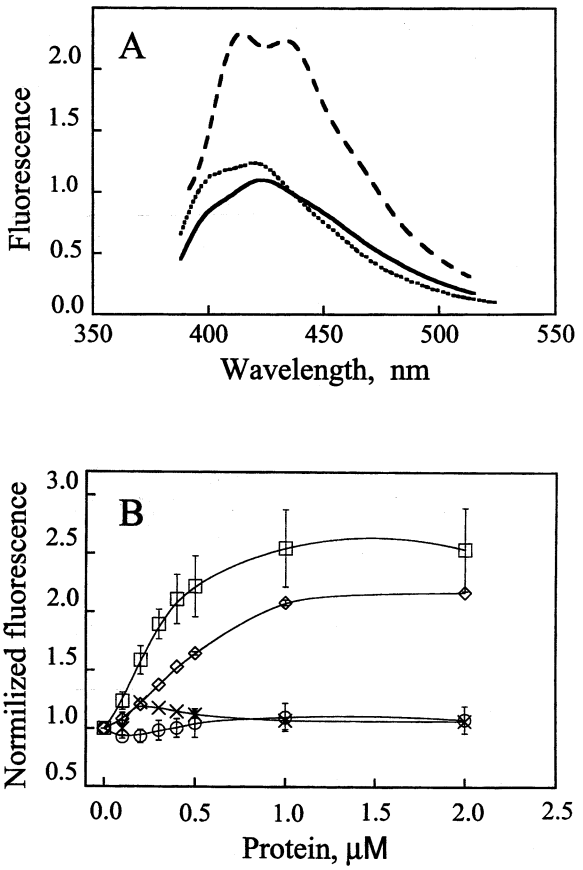

Fig. 6. (A) Interactions of CL-309623 compound with F- and G-proteins of RSV. The fluorescence emission of CL-309623 was recorded in the absence and presence of the proteins at excitation wavelength of 335 nm according to procedure described in materials and methods section. Fluorescence spectra of 0.2 μM CL-309623 in the absence (solid line), or the presence of F-protein at 0.5 μM (dashed line) and G-protein at 1 μM (dotted line). All fluorescence spectra of inhibitor were acquired in PBS buffer containing 0.001% TRITON X-100. Stock solutions of F- and G-proteins contained 0.01% of the detergent in PBS buffer. (B) Binding affinity of CL-309623 to G- and F-proteins. CL-309623 (0.2 μM) was combined with increasing concentrations of either the F- or G-proteins in a cuvette containing 0.5 ml of PBS buffer. The control experiment in the absence of protein was done in PBS buffer with the addition of 0.01% of the detergent. The fluorescence intensity at excitation and emission maxima of 335 nm and 450 nm was monitored and the results are shown as binding curves in the absence (○), and in the presence of G-protein (X), PFP2, F-protein from A2 strain (◇) and PFP3, F-protein from *cp-52* strain (□). Error bars are standard errors obtained from at least two experiments for each data point.

protein from *cp-52* virus, with an apparent dissociation constant (K_D) of 0.1 μM. The above-mentioned fluorescence properties of the inhibitor in the presence of G-protein were also evaluated to determine if such binding characteristics are common for both glycoproteins of RSV. The results of these binding experiments show that CL-309623 binds F-protein tightly while it does not interact with G-protein (Fig. 6A and B). The changes in the fluorescence properties of CL-309623 are consistent with the removal of the inhibitor from a hydrophilic environment in the free state to a more hydrophobic environment in the protein-bound state. To simulate this binding environment, the stilbene analog was placed in aqueous solutions containing dioxane, a solvent that lowers the dielectric constant of the medium. In these experiments the fluorescence emission similarly shifted to lower wavelengths with a concomitant increase in quantum yield. These results suggest that the bound environment of the inhibitor is hydrophobic despite the presence of ionized sulfonic acid groups probably due to local conformational changes or domain movements in the protein, charge neutralization and/or due to removal of water at binding site. This result is somewhat surprising given that a des-sulfonic acid analog of stilbene (Ding et al., 1998) does not specifically bind fusion protein and there is an absolute requirement for the negative charge at the stated positions for binding and activity (unpublished observations). These results are consistent with the interpretation that hydrophobic, electrostatic and hydrogen bonding interactions are necessary for specific interactions with fusion protein.

3.5. Lipid vesicles do not alter binding affinity of CL-309623 to fusion protein

To further address the question of specific inhibition of fusion protein, direct binding of CL-309623 to vesicles and competition experiments in the presence of fusion protein were also evaluated. Increasing concentrations of the vesicles (lipid concentration of 0–150 μM) were added to the inhibitor and potential interactions were evaluated both in the absence and presence of fusion

protein as a competing agent. The results in Fig. 7 show that the inhibitor does not bind to lipid vesicles significantly even at concentrations as high as 150 μM. 0.5 μM of fusion protein was sufficient to compete out the inhibitor in the presence of the large excess concentration of lipids. The results demonstrate specific binding to fusion protein and the lack of it to the cell membrane, further attesting that the inhibition is mediated via F-protein.

4. Discussion

The mechanism of activation of Sendai virus and its fusion with target cell membrane has been extensively characterized in the recent past (Wharton et al., 2000). Glycosaminoglycans, GAGs, have been shown to affect the fusion of both wild type RSV (Krusat and Streckert, 1997; Martinez and Melero, 2000) and *cp-52* viruses (Feldman et al., 2000). The retroviruses such as HIV that fuse at neutral pH are activated via interactions of fusion protein with the receptor and co-receptor on the cell surface (Wyatt and Sodoroski, 1998). Unlike the HIV and influenza viruses, specific protein receptors have not been identified for RSV. While the role of such receptors has been intensely pursued by other investigators, we have attempted to determine if membranes devoid of cellular receptors could mediate membrane fusion. For instance if the fusion protein is inhibited as a ternary complex with a potential receptor at the cell membrane, then using a simple liposome system would further address the question of target selectivity of the inhibitors. We have therefore used vesicles of known lipid composition, combining polysaccharides and cholesterol with phospholipids to investigate the inhibition of virus-induced membrane fusion. It has been shown that influenza and sendai viruses fuse with vesicles even though they bind specific sialo and asialoganglioside receptors on the cell surface. In this work we have used a mutant strain, *cp-52*, which consists of only the F-protein on its envelope and found that RSV efficiently fuses with vesicles in the absence of GAGs.

The kinetics of RSV fusion with vesicles is slower than that of influenza but appears to be comparable to that of sendai virus. The differ-

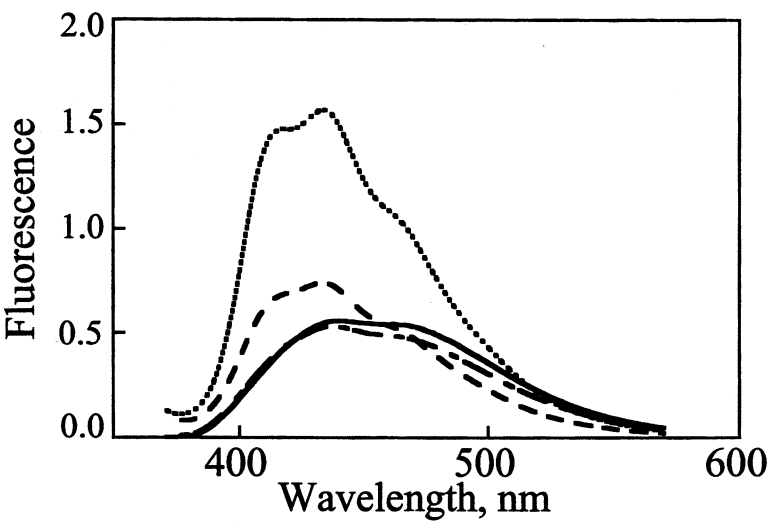

Fig. 7. CL-309623 interactions with PC/Chol vesicles. CL-309623(0.2 μM) was added to 0.5 ml of PBS buffer. Egg PC/Chol vesicles were added to a final concentration of 15 or 150 μM and the fluorescence spectra were recorded after 5 min incubation at 37 °C, followed by the addition of F-protein to a final concentration of 0.5 μM. The fluorescence spectra of CL-309623 in the absence of vesicles and protein (solid line), in the presence of 15 μM (dotted dashed line), 150 μM (dashed line) of vesicles, and in the presence of 150 μM of vesicles and 0.5 μM of F-protein (dotted line) are shown.

ences are possibly due to pH-induced changes in the conformation of hemagglutinin, which triggers fusion of influenza with endosomes (Wiley and Skehel, 1987). In RSV, activation and conformational changes (Gonzalez-Reyes et al., 2001) appear to occur upon an increase in temperature (Srinivasakumar et al., 1991). It is known that in sendai and influenza viruses, increased temperature is also a trigger for fusion. We have recently shown that virus (*cp-52* and A2) binding to Vero cells occurs at low temperatures while fusion does not (Razinkov et al., 2001). The temperature effects on RSV–vesicle fusion are qualitatively similar to those of RSV fusion with cells. The results are consistent with specific activation of fusion in the presence of the LUVs. Furthermore, our RSV–vesicle fusion results show that the kinetics of fusion of *cp-52* mutant is the same as that of A2 wild type. Since G-protein did not alter significantly RSV fusion, its interaction with GAGs (Martinez and Melero, 2000) perhaps decreases the dimensionality of F-protein diffusion from three- to two-dimensional in the extra cellular space (Schlessinger et al., 1995). Such a restriction of the bound virus can increase the encounter of fusion protein with membrane, thereby, aiding fusion.

It has been shown in certain viruses such as Semiliki forest, sendai and influenza viruses, that cholesterol plays a decisive role in virus entry into the cell. Our results with RSV are similar. Cholesterol has been shown to be organized in membranes as 'lipid rafts' (Varma and Mayor, 1998) disruption of which is thought to interfere with virus entry and fusion (Gower and Graham, 2001). Cholesterol possibly plays a similar role in RSV entry into cells. Except for cholesterol, the other lipids, and GAGs did not significantly effect virus–vesicle fusion. In the vesicle fusion experiments the concentration of lipids and the membrane surface are quite different than those observed with cells (Aloia et al., 1993). Despite these differences, the inhibitory effects of RFI-641 and CL-309623 on RSV fusion are comparable in the two assays. The effective concentration of RFI-641 necessary to reduce fusion activity in vesicles by about 50% is nearly identical to the inhibitory concentration required for Vero cells as shown in our previous work (Razinkov et al., 2001). These results, when taken together with the specificity of inhibitor binding to F- and not G-protein, are consistent with a mechanism where the inhibition is mediated primarily by an interaction with fusion protein in its native state on the virus. If the inhibitory mechanism were primarily due to the involvement of F-protein in a binary interaction with inhibitor, then the inhibition would not be altered significantly by interactions of fusion protein with potential receptors or GAGs. The inhibition of RSV entry into cells is not due to the formation of an inactive ternary complex of the protein-inhibitor complex with a putative receptor on the cell surface. HIV inhibition of fusion by T-20 peptides apparently occurs via interactions with fusion protein upon activating conformational changes that expose the HR1/HR2 heptad repeat domains of the fusion protein due to interactions with receptor co-receptor (Derdeyn et al., 2000; Furuta et al., 1998). The RSV inhibitors, on the other hand, block early events of virus entry including attachment and, more significantly, fusion of virus with cell (Razinkov et al., 2001). We have observed that RSV inhibition also occurs with several major cell types such as HeLa and fibroblast cells. Furthermore, RFI-641 inhibits RSV growth with IC_{50} values similar to those observed in the virus-cell fusion assay or the virus–vesicle fusion assay. If the primary inhibitory steps involved receptor-activated fusion or a late event in virus entry, then inhibition of the virus–vesicle fusion step should be significantly weaker than that of virus–cell fusion. Moreover, the lack of inhibition of both virus–cell and virus–vesicle in the presence of the inactive analog, WAY-158830, further supports the argument that the virus–vesicle fusion is relevant for deducing the mechanism of inhibition of RSV. The results described here, however, do not eliminate receptor involvement in RSV entry, although they show that if such a receptor exists it does not play a major role in the inhibitory mechanism of the compounds.

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