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The Fusion Protein of Peste des Petits Ruminants Virus Mediates Biological Fusion in the Absence of Hemagglutinin–Neuraminidase Protein

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To study the process of membrane fusion in Morbilliviruses, the fusion (F) glycoproteins of Peste des petits ruminants virus (PPRV) and Rinderpest virus (RPV) were expressed transiently in mammalian cells. The recombinant F proteins were found to be localized at the surface of transfected cells. The fusion activity, as evident from cell fusion assays and lysis of chicken erythrocytes, documented that transiently expressed PPRV F glycoprotein induces cell fusion in the absence of homotypic hemagglutinin–neuraminidase (HN) attachment glycoprotein. The coexpression of homotypic HN increased the extent of fusion by twofold, while the efficiency of fusion was found to be substantially enhanced. In contrast, in RPV F-expressing cells, fusion was detected only when homotypic hemagglutinin (H) or heterotypic HN protein was coexpressed. This differs from the strict type-specific requirement for the attachment protein as in the fusion process of most of the paramyxoviruses. Further, we demonstrate by fluorescence transfer experiments that while PPRV F brings about both hemifusion and complete fusion on its own, RPV F induces only hemifusion while it brings about complete fusion in the presence of homotypic or heterotypic attachment protein.

INTRODUCTION

Viruses belonging to the Paramyxoviridae family contain two integral membrane glycoproteins, the hemagglutinin (H)/hemagglutinin–neuraminidase (HN) protein and the fusion (F) protein. The H/HN glycoprotein is responsible for attaching the virion to the target cell, while the F protein is believed to disrupt the target cell membrane, hence inducing the virus–cell and cell–cell fusion. The F protein is synthesized as an inactive precursor, F_0, which is subsequently cleaved by a cellular trypsin-like protease into F_1 and F_2 subunits that are held together by a disulfide bond on the surface of the cells. The requirements for virus-induced membrane fusion have been extensively studied in paramyxoviruses (Stegmann *et al.,* 1989; White, 1990). In most of the paramyxoviruses, coexpression of homotypic H/HN and F proteins is required to induce cell–cell fusion (Ebata *et al.,* 1991; Hu *et al.,* 1992; Sergel *et al.,* 1993; Wild *et al.,* 1991), whereas SV5 F protein is able to bring about cell fusion in the absence of homotypic HN protein (Paterson *et al.,* 1985; Horvath *et al.,* 1992; Bagai and Lamb, 1995). In the Morbillivirus genus, as demonstrated in measles virus (MV) and canine distemper virus (CDV), cell fusion requires the coexpression of F and H proteins (Wild *et al.,* 1991; Cattaneo and Rose, 1993; Bar-Lev Stern *et al.,* 1995). However, unlike the majority of paramyxoviruses, where there is a strict requirement for homotypic F and H/HN proteins to bring about cell-to-cell fusion, MV and CDV F and H proteins bring about heterotypic interactions as well leading to cell fusion (Bar-Lev Stern *et al.,* 1995).

The other two closely related members of the morbillivirus genus are Rinderpest virus (RPV) and Peste des petits ruminants virus (PPRV). The requirements for fusion function mediated by the F glycoprotein of either of the two viruses have not been defined so far. The F protein of PPRV purified from virus-infected cells has been shown to bring about hemolysis, which is an attribute of fusion function (Devireddy *et al.,* 1999). Recently we have shown that the attachment protein of PPRV is a HN and is biologically active when expressed in eukaryotic cells (Shaguna Seth and Shaila, 2001). We present evidence in this study that the F protein of PPRV when expressed transiently in eukaryotic cells is capable of causing cell–cell fusion in the absence of PPRV HN protein. Coexpression of HN protein in PPRV F protein expressing cells leads to an increase in syncytia formation by twofold, while there is a substantial increase in the size of syncytia (efficiency of fusion). In contrast, RPV F protein can bring about fusion only when homotypic H or heterotypic HN (PPRV HN) was coexpressed. However, RPV F when expressed alone was found to bring about hemifusion in the absence of H/HN protein.

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RESULTS

Transiently expressed F proteins are localized at the cell surface

Fusion proteins of both PPRV Nigeria 75/1 strain and RPV (RBOK) strain were expressed transiently using either a CMV-driven expression vector or a recombinant vaccinia virus-T7-based expression system in CV-1 cells. Flow cytometric analysis and cell surface immunofluorescence performed 24 h posttransfection revealed that the recombinant F proteins of PPRV and RPV were transported to the cell surface (Figs. 1A and 1B).

PPRV F protein induces cell fusion

The biological activity of the recombinant proteins expressed at the cell surface of transfected CV-1 cells was determined by examining the cells for syncytium formation. CV-1 cells were transfected with constructs coding for the glycoproteins in various combinations. At 24 h posttransfection, acetone-fixed cells were processed for immunoperoxidase staining and observed for the presence of syncytia. Syncytia were observed in CV-1 cells expressing PPRV F protein alone as compared to RPV F protein, which showed syncytia formation only in the presence of either homotypic H or heterotypic HN (Fig. 2A). To further confirm the observation that PPRV fusion protein was able to bring about biological fusion alone, hemolysis assay was performed using mock transfected cells, PPRV-infected cells, and cells transfected with either pSS97–2 (which codes for PPRV F) or pCMX-EF13 (which codes for RPV F). There was significant hemolysis in PPRV-infected and PPRV F-expressing cells as compared to the mock transfected and RPV F-expressing cells which did not show hemolysis (Fig. 2B).

Coexpression of PPRV HN protein and its effect on cell fusion

To ascertain whether the coexpression of PPRV HN increases the extent of fusion process induced by F protein, a content mixing assay (Nussbaum *et al.,* 1994) was performed. One set of CV-1 cells were infected with vTF7-3 and transfected with various constructs harboring the glycoprotein genes under T7 promoter in different combinations, while the other set of cells were transfected with pGINβ-gal reporter construct. These two populations were mixed, and to measure fusion, a colorimetric assay was performed as described under Materials and Methods. The results showed that fusion was brought about by PPRV F alone and was promoted twofold in the presence of homotypic HN. RPV F protein was able to induce fusion only in the presence of homotypic H or heterotypic HN protein (Fig. 3).

Effect of HN/H coexpression on efficiency of fusion

To examine the efficiency of fusion when PPRV F was coexpressed with homotypic HN or heterotypic H protein and RPV F was coexpressed with homotypic H or heterotypic HN protein, fusion or syncytium formation assays were performed. At 24 h posttransfection, F protein expressing cells were immunoperoxidase stained as described under Materials and Methods and observed under a light microscope. About 50 syncytia having two or more nuclei were counted and the efficiency of fusion was assessed. The results of such a fusion assay showed that the presence of syncytia with five or more nuclei was enhanced only when PPRV F was coexpressed with homotypic HN and in RPV F, efficiency of fusion was enhanced in the presence of either homotypic H or heterotypic HN (Fig. 4).

PPRV F brings about complete fusion while RPV F induces hemifusion

To analyze different stages of the fusion reaction, hemifusion and fusion pore building assays (Kemble *et al.,* 1993) were performed. Hemifusion was analyzed with R18-labeled erythrocytes. When hemifusion occurs, R18 is transferred from the outer layer of F-expressing cell membranes, without an exchange of cytoplasmic contents (Kemble *et al.,* 1994; Melikyan *et al.,* 1995). Fusion pore building can be monitored by the transfer of hydrophilic fluorescent calcein from the calcein-filled erythrocytes to the F-expressing cells. As shown in Fig. 5A, transfer of fluorescence from R18-labeled erythrocytes to cells expressing RPV F and PPRV F was clearly observed. On the other hand, as shown in Fig. 5B, calcein was readily transferred from erythrocytes to cells expressing PPRV F, whereas transfer to RPV F-expressing cells was not detected. However, transfer of calcein was restored in RPV F-expressing cells when homotypic H or heterotypic HN was coexpressed. As expected, cells expressing only the attachment proteins PPRV HN and RPV H did not show any dye transfer. These results indicate that PPRV F can bring about hemifusion and complete fusion on its own while RPV F can bring about only hemifusion in the absence of the attachment protein.

DISCUSSION

To study the fusion process in PPRV and RPV viruses, we expressed the F glycoproteins of these viruses transiently in eukaryotic cells. Transiently expressed proteins were detected at the cell surface. PPRV F brought about syncytium formation when expressed transiently in CV-1 cells as compared to RPV F, which did not show fusion on its own. This was confirmed by performing a hemolysis assay using cells expressing PPRV F and RPV F. It has been shown earlier that immunoaffinity-purified

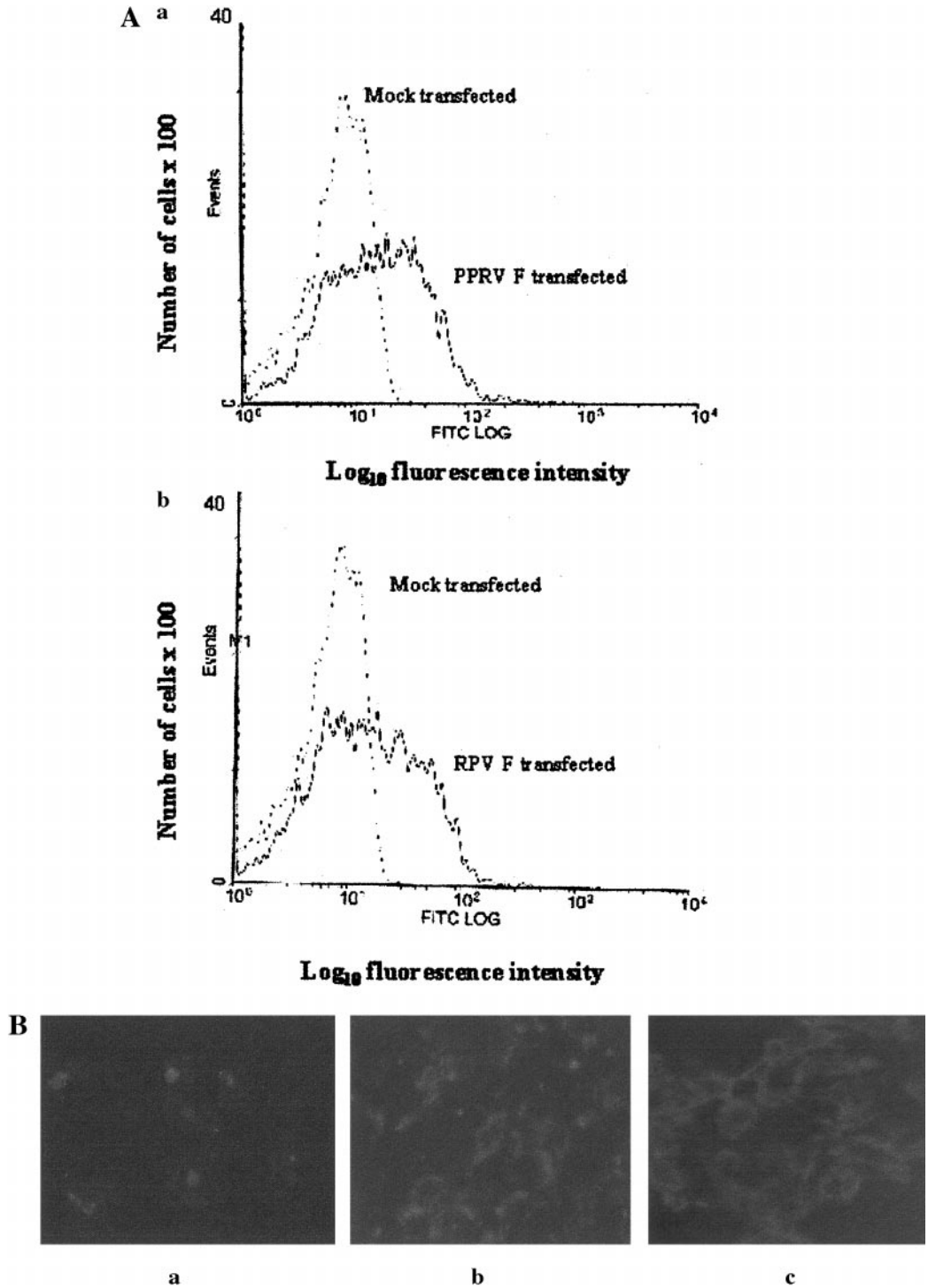

FIG. 1. Transient expression of PPRV and RPV F proteins. CV-1 cells were transfected with pSS97-2 (PPRV F) or pCMX-EF13 (RPV F). At 24 h posttransfection, cells were trypsinized and protein expression was analyzed. (A) Flow cytometric analysis of (a) pSS97-2-transfected cells and (b) pCMX-EF13-transfected cells. Cells were fixed with 2% paraformaldehyde, treated with rabbit-raised α-PPRV F, or α-RPV F antibody followed by FITC-conjugated goat α-rabbit IgG and subjected to flow cytometric analysis. Mock transfected cells which were treated with primary as well as secondary antibodies served as negative controls. (B) Cell surface immunofluorescence analysis of transfected CV-1 cells. Transfected CV-1 cells were treated with specific antibodies as mentioned above and cell surface immunofluorescence was detected microscopically. (a) Mock transfected, (b) pSS97-2-transfected, (c) pCMX-EF13-transfected CV-1 cells. [Magnification, 100×].

PPRV F protein is biologically active as it is able to bring about hemolysis and antibodies against PPRV F protein not only inhibited hemolysis but also inhibited PPRV-induced cell fusion (Devireddy *et al.,* 1999). The content mixing assay further substantiated that PPRV F was able to bring about fusion when expressed alone and it in-

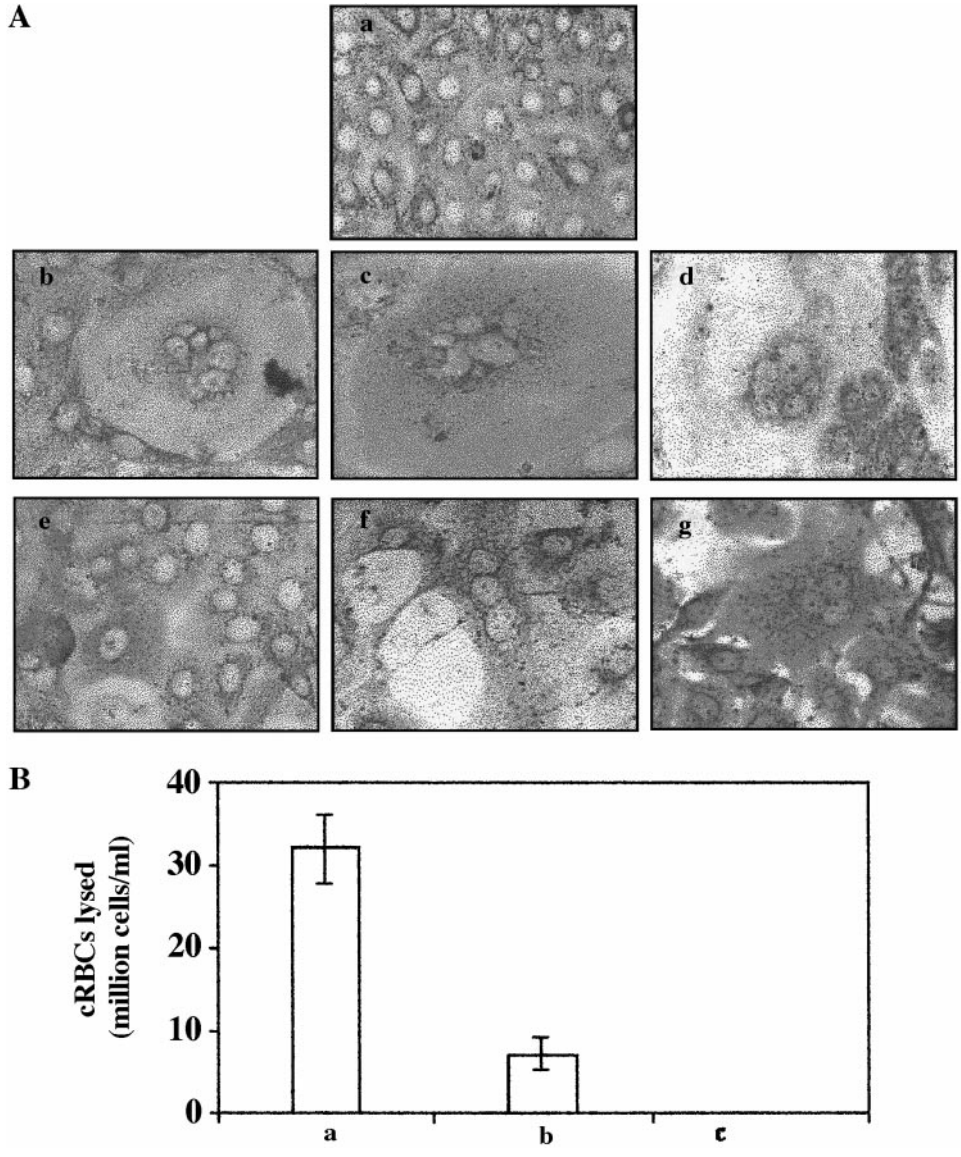

FIG. 2. Biological activities of PPRV and RPV F proteins. (A) Syncytium formation assay was performed with (a) mock transfected cells and cells expressing (b) PPRV F alone, (c) PPRV F with homotypic HN, (d) PPRV F with heterotypic H, (e) RPV F alone, (f) RPV F with homotypic H, and (g) RPV F with heterotypic HN. At 24 h posttransfection, cells were fixed, treated with PPRV and RPV F specific antibodies and horseradish peroxidase secondary antibody, stained, and observed microscopically. (B) CV-1 cells infected with (a) PPRV Nigeria 75/1 strain or transfected with (b) pSS97-2 (PPRV F) and (c) pCMX-EF13 (RPV F) constructs were subjected to hemolysis assay. Cells were lysed by freeze thawing thrice and incubated with 10% cRBCs for 1 h at 37°C. Absorbance of the supernatant at 540 nm was determined. A standard curve was generated by incubating a known amount of RBCs with the ammonium chloride buffer (0.14 M ammonium chloride, 0.016 M Tris, pH 7.2) to lyse RBCs and the absorbance taken at 540 nm. The *y* axis represents the number of RBCs bound and lysed in the PPRV-infected, PPRV F-transfected, and RPV F-transfected samples. (Results represent three independent experiments ± standard deviation.)

creased the extent of fusion by twofold with the coexpressed homotypic HN but not with heterotypic H. This is the first demonstration of a morbillivirus F protein inducing cell-to-cell fusion when expressed alone. The efficiency of fusion in cells coexpressing PPRV F and homotypic HN was also enhanced but there was no effect on the efficiency of fusion promotion when it was coexpressed with heterotypic H.

In RPV F, fusion was detected only in the presence of homotypic H or heterotypic HN. This observation is distinct from the fusion process of most other paramyxoviruses where there is a strict type-specific requirement for H/HN protein. However, the heterotypic interactions between the hemagglutinin/hemagglutinin–neuraminidase and fusion proteins of RPV and PPRV are only one way as RPV H does not promote the extent or efficiency of fusion by PPRV F. The SV5 fusion protein was also shown to cause syncytia when it was coexpressed with influenza virus hemagglutinin or hPIV-3 or NDV HN proteins (Bagai and Lamb, 1995). The hPIV-1 HN, but not Sendai

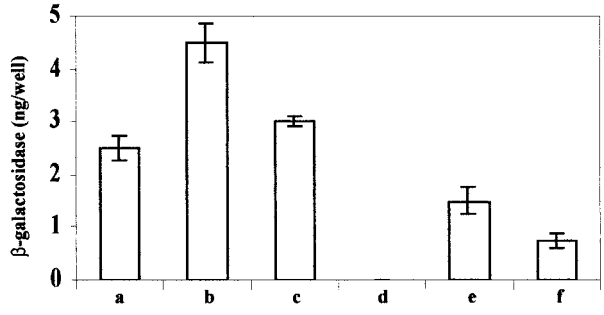

FIG. 3. Content mixing assay. One culture of CV-1 cells was infected with recombinant vaccinia virus VTF7-3 which encodes T7 RNA polymerase and transfected with (a) pGEM-PPRV F, (b) pGEM-PPRV F + pGEM-HN, (c) pGEM-PPRV F + RBH3.4 (pBS-RPV H), and (d) EF13 (pBS-RPV F), (e) EF13 + RBH3.4, and (f) EF13 + pGEM-HN. Cells were then treated with *Vibrio cholerae* neuraminidase. A second culture of CV-1 cells was transfected with β-galactosidase-expressing plasmid pGINT7 β-gal. The two cell populations were mixed and incubated for 4 h at 37°C. Cell fusion was measured by colorimetric assay (Nussbaum *et al.,* 1994) (Results represent three independent experiments ± standard deviation.)

virus HN, was shown to promote fusion function of either hPIV-1 or Sendai virus F proteins (Bousse *et al.,* 1994). Furthermore, studies on hemagglutinin proteins of MV and CDV have shown that cotransfecting F and H genes of MV or those of CDV resulted in extensive syncytium formation in permissive cells while transfecting either F or H alone did not. Similar experiments with heterologous pairs of proteins, CDV F with MV H or MV F with CDV H, caused significant cell fusion in both cases (Bar-Lev Stern *et al.,* 1995). Thus, PPRV F protein is unusual among morbilliviruses in bringing about biological fusion by itself and PPRV HN protein promotes the fusion function of both PPRV and RPV F proteins. When different stages of fusion process was analyzed using R18 and calcein-labeling experiments, RPV F protein showed hemifusion but failed to show exchange of cytoplasmic contents (complete fusion) when expressed alone, unlike PPRV F protein, which showed both in the absence of the attachment protein. In this study, the hemifusion has been shown in the cells expressing PPRV F and RPV F alone without the coexpression of HN or H proteins, which suggests that F protein may be interacting with a yet unrecognized receptor on RBCs. It is also possible that the close proximity of F protein expressing cells in confluent monolayers with RBCs trigger a fusion process in the absence of any attachment protein. The observation that some viral fusion proteins require the coexpression of homotypic HN while others like SV5 and PPRV fusion proteins bring about fusion by themselves shows the complexity of mechanism of fusion process between virus and host cell membranes. For those paramyxoviruses requiring their homotypic HN for fusion activity, binding of HN molecule to a sialic acid moiety on the target cell causes a conformational change in HN which in turn triggers the putative conformational change in the F protein that is necessary to initiate fusion (Lamb, 1993; Sergel *et al.,* 1993). For SV5, which does not require its homotypic HN for fusion activity, it has been hypothesized that either close contact with the target membrane or binding to an as yet unidentified receptor may induce the putative conformational change required to initiate fusion (Lamb, 1993). We speculate that this explanation could be extended to PPRV F protein as well. The results of this study would further strengthen our existing knowledge on the fusion activity of Morbillivirus fusion glycoproteins and the intricate mechanisms involved in the process of fusion.

MATERIALS AND METHODS

Cells and viruses

Vero and CV-1 cells were obtained from National Centre for Cell Science, Pune, India and were maintained in DMEM supplemented with 5% fetal calf serum (Gibco-BRL, USA) at 37°C. Vaccine strain of PPRV (Nigeria 75/1), kindly provided by Dr. A. Diallo, CIRAD-EMVT, France, and a field isolate of PPRV (Ind/TN87/1) (Shaila *et al.,* 1989) were employed in this study. A tissue culture adapted vaccine strain of RPV (RBOK) (Plowright and Ferris, 1957) was obtained from the Institute of Animal Health and Veterinary Biologicals, Bangalore, India. All these viruses were amplified in Vero cells.

Plasmid constructs

The following constructs were made in the eukaryotic expression vector, pCMX, driven by a strong cytomegalovirus (CMV) promoter (a kind gift from Dr. P. N. Rangarajan, Department of Biochemistry, Indian Institute of

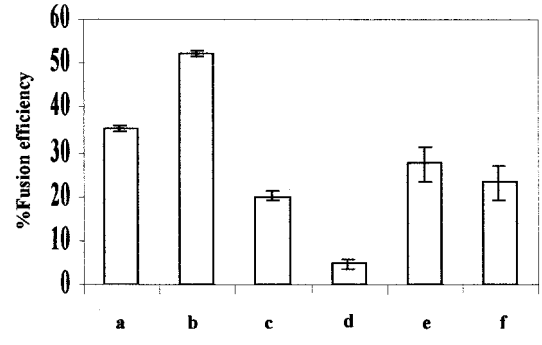

FIG. 4. Efficiency of fusion. Cell fusion assays were performed with CV-1 cells transfected with the following constructs: (a) pSS97-2 (PPRV F), (b) pSS97-2 + pSSHNCMX (PPRV HN), (c) pSS97-2 + pCMV-RBH 3.4 (RPV H), (d) pCMX-EF13 (RPV F), (e) pCMX-EF13 + pCMV-RBH3.4, and (f) pCMX-EF13 + pSSHNCMX. At 24 h posttransfection, cells were fixed, treated with PPRV and RPV F-specific antibodies and horseradish peroxidase conjugated secondary antibody, stained, and observed microscopically. Fifty syncytia having five or more nuclei were counted to measure efficiency of fusion and the results were expressed as % fusion efficiency, which is (number of syncytia with five or more nuclei/total no. of syncytia) × 100. (Results represent three independent experiments ± standard deviation.)

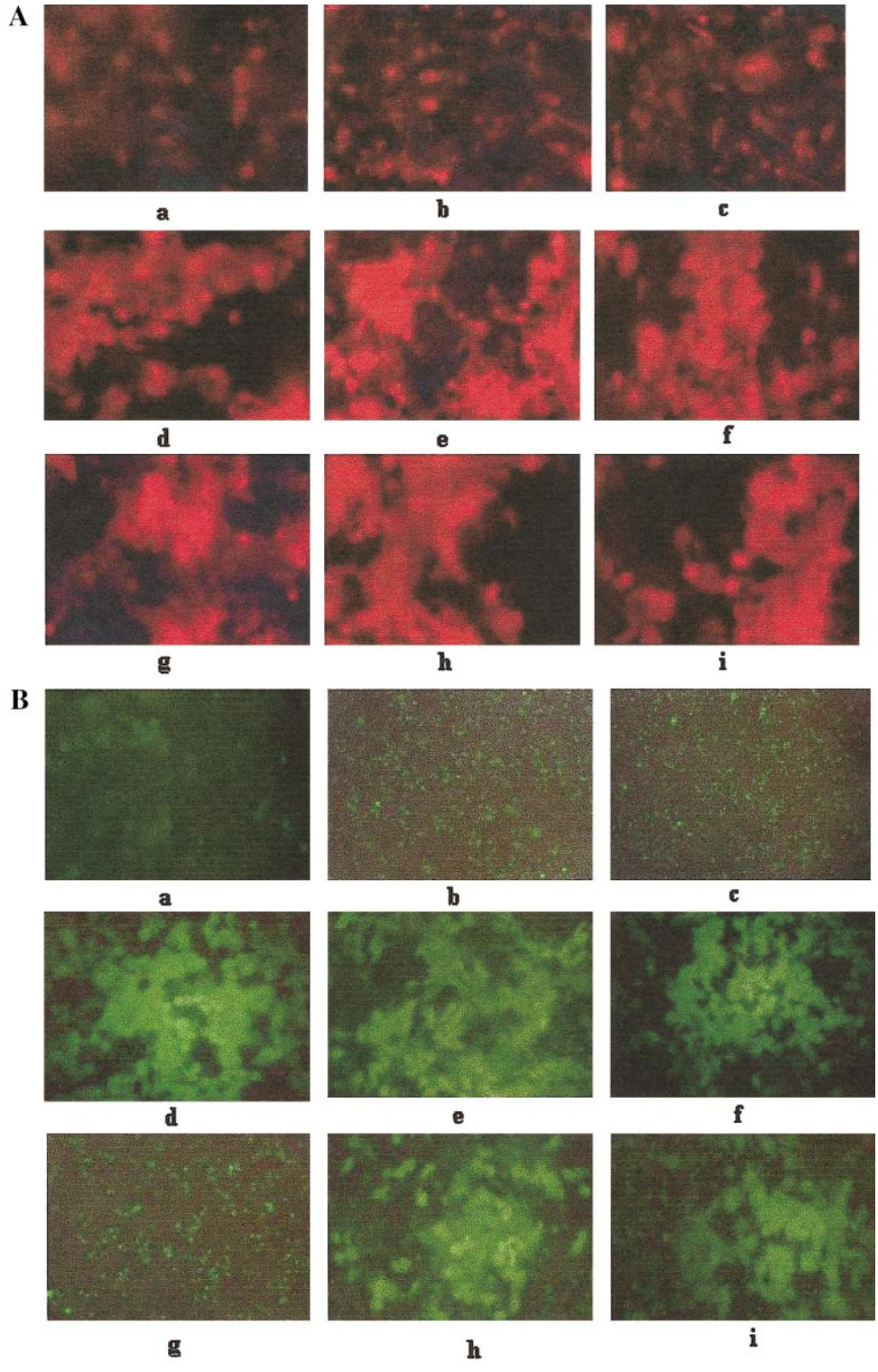

FIG. 5. Fusion assays. (A) Hemifusion assay. Transiently transfected CV-1 cells were incubated with R18-labeled erythrocytes and the fluorescence transfer from erythrocyte membranes to (a) mock transfected cells or cells expressing (b) PPRV HN, (c) RPV H, (d) PPRV F, (e) PPRV F + PPRV HN, (f) PPRV F + RPV H, (g) RPV F, (h) RPV F + RPV H, and (i) RPV F + PPRV HN was examined microscopically as described under Materials and Methods. (B) Complete fusion assay. Transiently transfected CV-1 cells were incubated with calcein-labeled erythrocytes and fluorescence transfer from calcein-filled erythrocytes to (a) mock transfected cells or cells expressing (b) PPRV HN, (c) RPV H, (d) PPRV F, (e) PPRV F + PPRV HN, (f) PPRV F + RPV H, (g) RPV F, (h) RPV F + RPV H, and (i) RPV F + PPRV HN was examined microscopically as described under Materials and Methods.

Science, Bangalore) and also in pGEM-7Zf vector under T7 promoter. pGEM-HN, PPRV HN gene of PPRV Nigeria 75/1 strain was subcloned from pBACPAK vector (carrying the full-length clone of PPRV HN gene of PPRV Nigeria 75/1 kindly provided by Dr. A. Diallo, CIRAD-EMVT, France) as a *Not*I/*Bam*HI, end-filled fragment in

*Sma*I-digested pGEM-7Zf. RBH3.4 (pBS-RPV H), this was a kind gift from Dr. T. Barrett, Institute for Animal Health, Pirbright, U.K. pGEM-F, PPRV F was released from a clone of full-length F gene of PPRV-FNZY12 in pT7T3D (Meyer and Diallo, 1995) (a gift from Dr. A. Diallo, CIRAD-EMVT, France) as a *Xho*I/*Bam*HI fragment and end-filled using Klenow polymerase and was ligated to a *Sma*I-digested, dephosphorylated pGEM-7Zf vector. EF13 is a pBluescript clone of RPVF (the cDNA for the fusion protein of RPV lacking 500 nucleotides from the 5′UTR cloned in pBluescript), a kind gift from Dr. T. Barrett, Institute for Animal Health, Pirbright, U.K. pSS97–2, the F gene of PPRV lacking the 5′UTR 300 bp was released from FNZY12 (pT7T3D-F), as a *Xho*I/*Bam*HI fragment and was directionally subcloned in *Xho*I/*Bam*HI-digested pCMX.PL1. pCMX-EF13 (RPV F), RPV EF13 was released from pBS-KS(+) EF 13 using *Eco*RI/*Sac*I, blunt ended using Klenow polymerase, and ligated to an *Eco*RV-digested, dephosphorylated pCMX.PL2. pSSHNCMX, the full-length PPRV HN was released from pGEM-HN plasmid (carrying the full-length clone of PPRV HN gene of PPRV Nigeria 75/1) as a *Kpn*I/*Bam*HI fragment and was directionally subcloned in *Kpn*I/*Bam*HI-digested pCMX.PL2. pCMV-RBH3.4, this construct was made by releasing the H gene from RBH 3.4 as *Pst*I and *Xho*I fragment and cloned in *Pst*I and *Sal*I site of pCMVintBL vector.

Transfection of CV-1 cells

CV-1 cells were plated in 35-mm tissue culture dishes at a density of 1×10^6 cells in 2 ml DMEM supplemented with 5% FCS (Gibco-BRL). When the cells were 70% confluent, medium was removed and cells were washed with phosphate-buffered saline (PBS). Five microliters of Lipofectamine (2 mg/ml) and 7 μg DNA were mixed in 500 μl OPTI-MEM medium (Gibco-BRL) and incubated for 30 min at room temperature. This mix was then added to the cells and the dishes were incubated at 37°C with 5% CO_2 in a humidified incubator for 4 h. The transfection mix was then removed and 2 ml of complete DMEM was added to the dishes and left for different time points at 37°C.

Transient expression using vTF7-3 infection/transfection system

For transient expression using vTF7-3/T7 infection/transfection, the seed virus stock of vTF7-3 (kindly provided by Dr. B. Moss, NIH, USA) was vortexed to disperse clumps and a sample was removed to a sterile microfuge tube, to which an equal volume of 0.25 mg/ml trypsin was added and incubated at 37°C for 30 min with vortexing every 10 min. This was then used to infect CV-1 cells in a six-well plate at a multiplicity of infection of 10 PFU/cell in 0.5 ml of 2.5% FCS containing DMEM. The cells were incubated for 1 h at 37°C after which the virus inoculum was aspirated and transfection performed as described above.

Cell-surface immunofluorescence

At 24 h posttransfection, cells were washed with PBS containing 0.1% sodium azide and processed for cell-surface immunofluorescence. Cells were incubated with 1% BSA in PBS for 1 h at 37°C. This was followed by incubation with 100 μl of 1:100 diluted rabbit hyperimmune serum raised against PPRV F or RPV F (Devireddy *et al.,* 1998) for 1 h at 37°C. The dishes were washed thrice with PBS and then 100 μl of 1:100 diluted goat anti-rabbit IgG–FITC conjugate was added and incubated at 37°C for 1 h. Cells were washed with PBS thrice and mounted on slides in 50% glycerol and the slides were viewed under a fluorescence microscope (Olympus).

Flow cytometric analysis

CV-1 cells were plated in 60-mm tissue culture dishes at a density of 2×10^6 in 3 ml complete DMEM. After 24 h of transfection at 37°C, cells were trypsinized and washed once with PBS. Cells were then fixed in 2% paraformaldehyde at 4°C for 1 h. Cells were washed with PBS and incubated with 1% BSA for 1 h at 37°C. After washing thrice in PBS, cells were treated with 1:100 dilution of anti PPRV F or RPV F polyclonal antibody in PBS at 37°C for 1 h. Cells were washed three times in PBS before 1:100 diluted goat anti-rabbit IgG–FITC conjugate was added. After 1 h incubation at 37°C, cells were washed thrice in PBS and resuspended gently in 500 μl PBS and were processed for flow cytometric analysis. Mock transfected cells, which were treated with both primary and secondary antibody, served as controls. Approximately 10,000 cells were used for each analysis.

Hemolysis

Hemolysis was measured according to Ebata *et al.* (1991). Briefly, cells transfected with the plasmid constructs were trypsinized 24 h after transfection, resuspended in 800 μl PBS, and lysed by freezing and thawing thrice to increase the hemolytic activity. Cells were then incubated with 200 μl of 10% solution of chicken erythrocytes for 1 h at 37°C. Debris were spun down and the absorbance at 540 nm was determined. A standard curve was generated after lysing a known amount of RBCs in ammonium chloride buffer (0.14 M ammonium chloride, 0.016 M Tris, pH 7.2) recording the absorbance values at 540 nm. In the figure, *y* axis represents the number of RBCs bound and lysed by PPRV-infected, PPRV F-transfected, or RPV F-transfected samples.

Syncytium formation assay

At 24 h posttransfection, CV-1 cells were washed with PBS and fixed in 50% acetone–50% methanol at −20°C for 1 h. Cells were then incubated with 10% goat serum in PBS for 1 h at 37°C. This was followed by incubation with 100 μl of 1:100 diluted rabbit hyperimmune serum raised against PPRV F or RPV F for 1 h at 37°C. The dishes were washed thrice with PBS and then 100 μl of 1:100 diluted goat anti-rabbit IgG–HRP conjugate was added and incubated at 37°C for 1 h. Cells were washed with PBS thrice and incubated with 1 ml of substrate solution for 5 min at room temperature (substrate: 6 mg diaminobenzidine tetrahydrochloride in 10 ml of 50 mM Tris–Cl pH 7.6 with 10 μl of H_2O_2). The reaction was stopped by washing thrice in water. Coverslips were mounted on slides in 50% glycerol prior to microscopic examination for syncytium formation. The extent of fusion was ascertained by visualizing approximately 1500 cells for the presence of syncytia and the efficiency is assessed by the number of syncytia having five or more than five nuclei out of 50 syncytia observed. The content mixing assay was performed as described by Nussbaum *et al.* (1994).

Labeling of erythrocyte membranes with R18

Freshly prepared guinea pig erythrocytes (0.5 ml) were suspended in 10 ml PBS. The suspension was vigorously mixed with 20 μl R18 (Molecular Probes, Eugene, OR) solution (2 mM in ethanol), and the mixture was incubated at 37°C for 15 min as described previously (Morris *et al.,* 1989). The erythrocytes were again pelleted, resuspended in 10 ml of PBS containing 0.4% bovine serum albumin, and incubated for 15 min at 37°C. R18-labeled erythrocytes were washed by centrifugation until the supernatant became clear and then used for hemadsorption and fusion testing.

Labeling of erythrocytes with calcein

Calcein AM (10 mM; 5 μl; Molecular Probes) in dimethyl sulfoxide was added to 10 ml of a guinea pig erythrocyte suspension. The mixture was vortexed and incubated for 30 min at 37°C as described (Kemble *et al.,* 1993). After the removal of excess calcein by centrifugation, the erythrocytes were incubated in 10 ml of PBS containing 0.4% bovine serum albumin for 15 min at 37°C. Calcein-filled erythrocytes were washed twice and used for further experiments.

The labeled erythrocytes were incubated with CV-1 cells transiently expressing the viral glycoproteins in different combinations for 1 h at 4°C for binding followed by incubation at 37°C for 30 min. The cells were washed thrice with PBS and viewed under a fluorescence microscope (Olympus).

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