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Analysis of the pH Requirement for Membrane Fusion of Different Isolates of the Paramyxovirus Parainfluenza Virus 5

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Paramyxoviruses enter cells by fusing their envelopes with the plasma membrane, a process that occurs at neutral pH. Recently, it has been found that there is an exception to this dogma in that a porcine isolate of the paramyxovirus parainfluenza virus 5 (PIV5), known as SER, requires a low-pH step for fusion (S. Seth, A. Vincent, and R. W. Compans, J. Virol. 77: 6520-6527, 2003). As a low-pH activation mechanism for fusion would greatly facilitate biophysical studies of paramyxovirus-mediated membrane fusion, we have reexamined the triggering of the PIV5 SER fusion protein. Using multiple assays, we could not find a requirement for low-pH triggering of PIV5 SER fusion. The challenge of discovering how the paramyxovirus receptor binding protein (HN, H, or G) activates the metastable fusion protein to cause membrane fusion at neutral pH remains.

Enveloped viruses gain entry into cells by fusing their lipid bilayer with a membrane of the host cell. The viral proteins that mediate membrane fusion may fold into a metastable state that requires activation to undergo a protein refolding event to bring about the coalescence of the viral and cellular membranes. Such a mechanism is known to occur for influenza virus hemagglutinin (HA), paramyxovirus fusion protein (F), human immunodeficiency virus (HIV-1) envelope glycoprotein (gp120/41), and alphavirus E1 glycoprotein (4, 6, 9–11). Until recently, it was thought that activation of viral fusion occurred through one of two routes (7). The first route is activation at the plasma membrane, and this route is used by paramyxoviruses, HIV-1, and herpesviruses, among others. The second route involves internalization of the virion and activation of fusion by the low-pH environment found in endosomal compartments. Fusion between the viral and intracellular membranes releases the viral genome into the cytoplasm. This pathway is used by influenza viruses, alphaviruses, and the rhabdoviruses vesicular stomatitis virus and rabies virus, among others. Recently, it has been recognized that there are variations of the two major themes, with some viruses beginning their entry activation process by receptor binding at the plasma membrane but also requiring internalization and the low-pH environment of the endosomal lumen to complete the activation process. The avian sarcosis/leukosis virus envelope glycoprotein is an example (1, 12, 13).

The porcine isolate of the paramyxovirus parainfluenza virus 5 (PIV5), known as SER, unlike the W3A isolate (also called SV5), does not induce readily detectable syncytium formation (25). The SER and W3A F proteins differ by only nine amino acids in their ectodomains, but SER has a 22-residue-longer cytoplasmic tail than W3A F due to the substitution of a translational stop codon for a serine residue (25). Mutagenesis of specific residues in the SER cytoplasmic tail significantly enhances the ability of SER to cause syncytia (23), and it has been suggested that the SER cytoplasmic tail forms a specific protein structure that inhibits the F protein conformational changes required for fusion activation (26). Recently, it has been reported that SER entry into cells occurs by a low-pH-dependent process, suggesting that the conversion to the fusion-active state for SER F protein is triggered by exposure to reduced pH (22). PIV5 isolate SER would be the first paramyxovirus to require a low-pH step for fusion. Thus, the fusion requirements of the PIV5 isolate SER required further investigation.

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Madin-Darby bovine kidney (MDBK), CV-1, Vero, BHK-21F, and BSR T7/5 cells were grown as previously described (16). The PIV5 isolate W3A and the PIV5 porcine isolate SER (22, 23, 25) (provided by Richard W. Compans, Emory University School of Medicine, Atlanta, Ga.) were grown in MDBK cells. pCAGGS plasmids encoding W3A F, W3A hemagglutinin-neuraminidase (HN), and influenza virus HA (A/Udorn/72) have been described previously (18). The cDNAs encoding SER F and SER HN were synthesized by reverse transcription-PCR, using RNA isolated from SER virus-in-
fected cells, and cloned into the eukaryotic expression vector pCAGGS. The nucleotide sequence of the F and HN cDNAs was determined and found to be identical to that obtained previously (25).

The infectious titers of PIV5 isolates W3A and SER were determined by plaque assay on CV-1 cells, and monolayers were immunostained, using vacF and vacHN rabbit sera (17), as previously described (3). Virions were purified on 15% to 60% sucrose gradients as previously described (16).

To examine syncytium formation, BHK-21F cells were transfected with 1.0 μg pCAGGS expressing W3A F, SER F, W3A HN, SER HN, or influenza virus (A/Udorn/72) HA (20). Also, BHK-21F cells were infected with W3A, SER, or influenza virus at a multiplicity of infection (MOI) of 10 PFU/cell. At 16 h posttransfection (p.t.) or postinfection (p.i.), cells were fixed in phosphate-buffered saline (PBS; buffered with 10 mM HEPES and 10 mM MES), pH 7.0 or pH 5.3, was added to the cells for 2 min at 37°C, and the cells were further incubated in Dulbecco’s modified Eagle’s medium (DMEM) at neutral pH. For influenza virus-infected cells and HA-expressing cells, HA was cleaved by the addition of N-acetyl trypsin (10 μg/ml; 10 min; 37°C) prior to low-pH treatment. After 4 h of incubation, the cells were fixed, stained, and photographed as previously described (26).

For quantification of fusion, a luciferase reporter assay was used (21). A real-time fusion assay was used to measure virus-cell fusion. Human erythrocyte (RBC) ghosts (2.5 mg/ml protein) were prepared as previously described (24). For NA-treated RBC ghosts, Vibrio cholerae neuraminidase (Sigma Aldrich, St. Louis, Mo.) was added at 100 mU/ml for 1 h at 37°C. Purified virus (20 μg per sample) was labeled with octadecyl rhodamine B (R18) (Invitrogen, Carlsbad, Calif.), and virus-gHOST fusion assays were performed essentially as previously described (24).

Metabolic labeling, immunoprecipitation, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were performed as previously described (16). To detect virus-specific polypeptides synthesized in direct lysates of virus-infected cells, actinomycin D (5 μg/ml) was also added to the cultures from the start of infection (14). Bafilomycin A1 (BFLA1) was obtained from Calbiochem (EMD Biosciences, La Jolla, Calif.).

Immunofluorescent staining of W3A-, SER-, or influenza virus-infected CV-1 cells was done using P/V protein-specific monoclonal antibody (MAb) P/k (19) or the influenza virus M2 protein-specific MAb 14C2 (29) and an Alexa-488-conjugated goat anti-mouse secondary antibody. Cells were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) and fluorescence visualized using a deconvoluting (ApoTome) Axiovert 200 microscope (Zeiss, Thornwood, N.Y.).

**PIV5 SER infectious centers detected by immunostaining.** The lack of syncytium formation caused by PIV5 SER has made it difficult to quantify virus titers. However, we observed that infectious titers of PIV5 SER could be readily determined by immunostaining of infectious centers (Fig. 1). The small size of the stained infectious centers does suggest limited cell-to-cell spread of virus, but PIV5 SER grew to an infectious titer similar to that of PIV5 W3A (1 × 10⁸ PFU/ml).

**Syncytium formation caused by PIV5 isolates.** BHK-21F cells were transfected with plasmids expressing the SER and W3A F protein together with their homotypic and heterotypic receptor binding protein HN. Cell surface expression levels of the glycoproteins were examined by cell surface biotinylation and found to be essentially equivalent for each protein and to be approximately equivalent to the cell surface expression levels observed in W3A and SER virus-infected cells (data not shown). Transfected cells were treated either with or without a 2-min low-pH incubation (pH 5.3). As a control for low-pH-induced fusion, cells expressing influenza virus HA were used (Fig. 2A). In a related experiment, BHK-21F cells infected with PIV5 isolates SER or W3A or with influenza virus were examined for syncytium formation, either with or without a low-pH treatment (Fig. 2B). In the cells expressing W3A F and HN proteins, extensive syncytium formation (Fig. 2A) was observed, as expected (8, 15). In cells expressing SER F and HN, small foci of syncytia of up to 10 to 14 nuclei could be detected (Fig. 2A), but no syncytia were detected in SER virus-infected cells (Fig. 2B). SER HN supported extensive syncytium formation by the W3A F protein, whereas W3A HN did not enhance the limited syncytium formation detected with coexpression of SER F and SER HN. Low-pH treatment of cells expressing HA induced syncytium formation. However,
low-pH treatment of either cells expressing SER F and HN or SER virus-infected cells did not induce an increase of syncytium formation over that observed at pH 7.0 (Fig. 2A and B). The data shown here are in contrast to data obtained by Seth and coworkers (22), who observed extensive syncytium formation after low-pH treatment of cells expressing SER F and HN.

Detection of PIV5 SER and W3A cell-cell fusion using luciferase reporter assays. Vero cells expressing F and HN and, as controls, cells expressing HA were incubated with BSR T7/5 cells either with or without low-pH treatment. Coexpression of SER F and HN resulted in fusion that was detectable by luciferase reporter assays (Fig. 2C), and both W3A and SER
fusion levels were very similar after pH 7.0 or pH 5.3 treatment. In contrast, influenza virus HA showed detectable fusion only after low-pH treatment, as expected.

**Real-time kinetics of virus-erythrocyte ghost fusion.** To examine the kinetics of fusion of PIV5 W3A and SER to target membranes, a real-time fluorescence-dequenching fusion assay was used. Purified W3A, SER, and influenza viruses were labeled with R18 and incubated with RBC ghosts at 4°C. Fusion was initiated by injection of the virus bound to ghosts into prewarmed (37°C) PBS, and fluorescence recording was begun. After ~60 seconds, the pH was lowered to pH 5 and fluorescence recording was continued. As controls, RBC ghosts were treated with *Vibrio cholerae* neuraminidase to block virus binding. Dequenching of R18 (an increase in fluorescence at 590 nm) indicates fusion of the labeled virions to the RBC ghosts. As shown in Fig. 2D, influenza virions showed fusion only after the pH was lowered to 5.0. In contrast, fusion of W3A virions occurred immediately after injection of the virus/ghosts into the prewarmed PBS and continued for ~130 seconds before reaching a plateau (Fig. 2D). Fusion was only minimally affected by changing the pH to 5 ~60 seconds after initiation of the fusion reaction. SER virions also caused detectable fusion in the real-time fluorescence-dequenching assay (Fig. 2D). Lowering the pH on SER virus/ghosts to pH 5.0 did not cause a change in the kinetics of fusion from that observed at pH 7.0. The maximum extent of fusion of the pH 5.0-treated sample is slightly higher than that of the pH 7.0-treated sample, but the increased extent was evident prior to the change of pH. For all three virus/ghost preparations, the large spikes in optical density at 590 nm at ~20 seconds after recording began is due to light scatter from injection of the virus/ghosts into the cuvette.

**Bafilomycin A1 treatment does not affect PIV5 SER replication.** Another approach to studying a possible low-pH requirement for SER fusion activation is using the inhibitor of the vacuolar type H⁺ ATPase, bafilomycin A1. This drug blocks the lowering of pH within acidic compartments in the cell, including the endosomal lumen. CV-1 cells were infected with W3A, SER, or influenza virus. The cells were either untreated; treated with BFLA1 (1.0 μM) before, during, and after infection; or treated with BFLA1 20 min after infection, with drug levels maintained throughout further incubations. At 16 h p.i. (for W3A and SER) or 10 h p.i. (for influenza virus), the cells were metabolically labeled and either the proteins were analyzed directly by SDS-PAGE or the HN, P, M, and V proteins were immunoprecipitated with specific MAbs and
then the proteins were analyzed by SDS-PAGE. As shown in Fig. 3A and B, BFLA1 was highly effective at preventing influenza virus polypeptide synthesis if added to the cells prior to infection. However, if BFLA1 was added to the influenza virus-infected cells 20 min after infection, then, due to the blocking of virus fusion activation, the window of opportunity for prevention of influenza virus-specific protein synthesis was lost. In contrast, BFLA1 treatment before, during, and after W3A or SER infection did not block W3A or SER protein synthesis. Decreased protein synthesis was observed for W3A and SER in cells treated with BFLA1 compared to that in untreated control cells, but a nonspecific toxic effect of the drug can be anticipated, given that the drug was present on cells for 18 h.

To further examine the effect of BFLA1 on PIV5 protein synthesis, MDBK cells were infected with W3A or SER and BFLA1 was added either with the virus inoculum or 0.5 PFU/cell) with the PIV5 isolates W3A, SER, Mil, Mel, LN, BFLA1, and Vero cells were infected at a low MOI (0.005 to 0.5 of PIV5. Thus, to test if any of these isolates were sensitive to drug treatment, the cells were washed extensively and incubated in DMEM for a further 16 h prior to being metabolically labeled. The P and V proteins were immunoprecipitated, and the proteins were analyzed by SDS-PAGE. As shown in Fig. 3C, the P and V proteins were readily detected at all times, whether the drug was added at the time of infection or up to 2 h p.i.

Another method for examining viral protein synthesis in the continuous presence of BFLA1 is immunostaining the cells for specific viral antigens. MDBK cells were infected with W3A, SER, or influenza virus in the presence or absence of BFLA1 and maintained with or without the drug for 10 h prior to being immunostained with antisera specific for the P/V proteins (W3A and SER) or the M2 protein (influenza virus). As shown in Fig. 4A, no difference in staining pattern could be observed for untreated or BFLA1-treated W3A- or SER-infected cells. In contrast, for influenza virus-infected cells, no M2 protein could be detected in BFLA1-treated cells.

In addition to W3A and SER, there are many other isolates of PIV5. Thus, to test if any of these isolates were sensitive to BFLA1, Vero cells were infected at a low MOI (0.005 to 0.5 PFU/cell) with the PIV5 isolates W3A, SER, Mil, Mel, LN, Den, and RO (2) and, as a control, influenza virus. The cells were either untreated or pretreated with 0.5 μM BFLA1, and treated cells remained in the presence of the BFLA1. At 18 h p.i., the treated cells were immunostained for the P/V protein (for influenza virus, antiserum specific for HA was used). As shown in Fig. 4B, the P/V proteins could be stained in both untreated and BFLA1-treated W3A- or SER-infected cells. Decreased protein synthesis was observed for W3A and SER in cells treated with BFLA1 compared to that in untreated control cells, but a nonspecific toxic effect of the drug can be anticipated, given that the drug was present on cells for 18 h.

In summary, using three different assays for fusion (syncytium formation, a luciferase reporter assay for cell-cell fusion, and real-time virus-red blood cell ghost fusion) and lowering of pH, no evidence for low-pH-induced fusion by PIV5 SER could be obtained. For each assay, influenza virus was used as a control for low-pH-induced fusion. Furthermore, no effect of the drug on PIV5 SER replication was observed when using various protocols for BFLA1 treatment of PIV5 SER-infected cells. For each assay, influenza virus was used as a positive control, and in each case, influenza virus-specific protein synthesis was inhibited by the drug. Thus, the data could not provide any evidence for a requirement of a low-pH step during PIV5 SER entry into cells. We have no explanation for the data obtained by Seth and colleagues (22), who observed low-pH-dependent activation of fusion by PIV5 SER. Indeed, such an activation mechanism would have greatly facilitated studies of paramyxovirus-mediated membrane fusion, as currently temperature shifts have to be used to activate the F protein. Now that the atomic structures of both the metastable pre-fusion form of F and the postfusigenic form of F have been obtained (27, 28), revealing the major F protein refolding event that takes place during fusion, one of the big challenges remaining is discovering how the paramyxovirus receptor binding protein (HN, H, or G) activates the metastable fusion protein to cause membrane fusion at neutral pH.

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