Calcium-Dependent Rubella Virus Fusion Occurs in Early Endosomes

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ABSTRACT

The E1 membrane protein of rubella virus (RuV) is a class II membrane fusion protein structurally related to the fusion proteins of the alphaviruses, flaviviruses, and phleboviruses. Virus entry is mediated by a low pH-dependent fusion reaction through E1's insertion into the cell membrane and refolding to a stable homotrimer. Unlike the other described class II proteins, RuV E1 contains 2 fusion loops, which complex a metal ion between them by interactions with residues N88 and D136. Insertion of the E1 protein into the target membrane, fusion, and infection require calcium and are blocked by alanine substitution of N88 or D136. Here we addressed the requirements of E1 for calcium binding and the intracellular location of the calcium requirement during virus entry. Our results demonstrated that N88 and D136 are optimally configured to support RuV fusion and are strongly selected for during the virus life cycle. While E1 has some similarities with cellular proteins that bind calcium and anionic lipids, RuV binding to the membrane was independent of anionic lipids. Virus fusion occurred within early endosomes, and chelation of intracellular calcium showed that calcium within the early endosome was required for virus fusion and infection. Calcium triggered the reversible insertion of E1 into the target membrane at neutral pH, but E1 homotrimer formation and fusion required a low pH. Thus, RuV E1, unlike other known class II fusion proteins, has distinct triggers for membrane insertion and fusion protein refolding mediated, respectively, by endosomal calcium and low pH.

IMPORTANCE

Rubella virus causes a mild disease of childhood, but infection of pregnant women frequently results in miscarriage or severe birth defects. In spite of an effective vaccine, RuV disease remains a serious problem in many developing countries. RuV infection of host cells involves endocytic uptake and low pH-triggered membrane fusion and is unusual in its requirement for calcium binding by the membrane fusion protein. Here we addressed the mechanism of the calcium requirement and the required location of calcium during virus entry. Both calcium and low pH were essential during the virus fusion reaction, which was shown to occur in the early endosome compartment.

Rubella virus (RuV) is a small enveloped single-stranded RNA virus and the sole member of the *Rubivirus* genus. Rubivirus and alphaviruses together comprise the *Togaviridae* (for an overview, see reference 1). While alphaviruses are generally transmitted by mosquito vectors, RuV spreads by airborne transmission between humans, the only known host (2). RuV causes a mild childhood disease commonly referred to as German measles (for a review, see references 1 and 3). However, RuV is able to cross the placental barrier, and infection of pregnant women, particularly during the first trimester, can cause miscarriage, stillbirth, or severe fetal malformations known as congenital rubella syndrome (CRS) (1, 4). While vaccination has essentially eliminated RuV disease and CRS in the Americas (5), it remains a problem in countries without effective vaccination programs and for individuals who refuse vaccination (6). More than 100,000 babies are born with CRS each year (5).

Similar to the alphaviruses (7), RuV enters cells through clathrin-mediated endocytosis and low pH-triggered membrane fusion (8–10). The viral genomic RNA is translated to produce the nonstructural proteins, which mediate RNA replication and transcription (reviewed in reference 1). The structural proteins capsid, E2, and E1 are synthesized as a polyprotein and processed by signal peptidase. Capsid protein assembles with the RNA to form the viral nucleocapsid, while the E2 and E1 membrane glycoproteins are translocated into the endoplasmic reticulum and associate as heterodimers. RuV buds into the Golgi complex, and the virus particle is transported via the secretory pathway to the plasma membrane. Structural studies show that E1 and E2 form rows of

heterodimers on the surface of the viral particles (11, 12), while antibody studies suggest that the smaller E2 protein is masked or covered by E1 (13, 14). E1 is the membrane fusion protein and also appears to be responsible for receptor binding (10, 15, 16). Recent structural evidence demonstrates that E1 is a class II fusion protein with a structure similar to that of the fusion proteins of alphaviruses, flaviviruses, and phleboviruses (17). All of these proteins are composed of three β -sheet-rich domains: a central domain I (DI) connecting to an elongated DII on one side and to the Ig-like DIII on the other side, followed by the stem and C-terminal transmembrane (TM) regions (17). Upon low-pH exposure, the tip of RuV DII is predicted to insert in the target membrane. E1 then trimerizes, and DIII and the stem fold back along the central DI/DII trimer core to form a hairpin-like structure (10, 17, 18). These

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conformational changes in the class II proteins drive the merger of the virus and endosome membranes (reviewed in references 19 and 20).

Despite its similarities to other viral class II fusion proteins, RuV E1 displays a striking difference in the membrane-binding region at the tip of domain II (17). Instead of a single membrane-interacting fusion loop like in the alphavirus, flavivirus, and phlebovirus fusion proteins, RuV E1 contains two fusion loops (FLs; FL1 and FL2). The three-dimensional structure reveals the presence of a metal binding site between the fusion loops (17). In the structure, this site coordinates either Na^+ or Ca^{2+} through interactions with residues N88 and D136 on FL1 and FL2, respectively. While the class III fusion proteins of the baculoviruses, herpesviruses, and rhabdoviruses also contain two fusion loops, they do not contain metal binding sites (21–23), and no such sites have been detected in other viral fusion peptides or loops to date.

We previously investigated the role of the metal binding site in the fusion function of RuV E1 (18). Our results demonstrated that RuV fusion specifically requires Ca^{2+} and that other tested cations, including Na^+ , Mg^{2+} , Mn^{2+} , and Zn^{2+} , are inactive. In the absence of Ca^{2+} , RuV binds to the cell surface, is taken up by endocytosis, and undergoes the low-pH-triggered conformational change in the E1 fusion protein. However, stable insertion of RuV E1 into liposome target membranes is blocked in the absence of Ca^{2+} . Alanine substitution of either N88 or D136 does not affect virus particle production but completely blocks virus-target membrane interaction, fusion, and infection.

These studies raised a number of interesting questions on the fusion and entry mechanism of RuV. These include questions on the requirement for specific residues in the fusion loops, how calcium affects the arrangement of these residues, and how calcium promotes lipid binding during the E1-membrane interaction. Importantly, the intracellular site of RuV fusion is not known, and the role of calcium at this site is unclear (18).

Here we investigated the consequences of Ca^{2+} coordination for RuV fitness and entry. We found that the configuration of the Ca^{2+} -coordinating residues N88 and D136 was specifically optimal for RuV E1 function. We were unable to isolate any revertants of N88/D136 mutants other than true revertants, supporting a strong selective pressure to maintain RuV E1 Ca^{2+} dependence. Although the configuration of the RuV FLs and their calcium binding suggested similarities to proteins that interact with anionic lipids (17, 18), direct studies of virus-liposome interactions showed that E1 did not require anionic lipids, suggesting that RuV fusion would not require delivery to late endosomes that are enriched in them. Instead, we found that RuV fusion occurs in the early endosome compartment and requires intraendosomal calcium.

MATERIALS AND METHODS

Cells and transfections. Vero cells were maintained in growth medium, consisting of Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal bovine serum, 1% L-glutamine, 100 U penicillin/ml, and 100 μg streptomycin/ml, at 37°C (18). BHK-21 cells were maintained in growth medium plus 10% tryptose phosphate broth. BHK-21 and Vero cells were transfected where indicated using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions.

Viruses. All RuV experiments were based on the RuV M33 infectious clone (pBRM33) (24), kindly provided by Tom Hobman (University of Alberta, Edmonton, Alberta, Canada). Mutants of pBRM33 were engineered by circular site-directed mutagenesis of subgenomic constructs as

previously described (18). Mutations were confirmed by automated DNA sequencing. Wild-type (WT) and mutant RuV RNAs were transcribed *in vitro* from pBRM33 and electroporated into BHK-21 cells to generate RuV stocks (18). The titers of the stocks were determined by an assay for infectious centers (IC) (18). Radiolabeled WT or mutant RuV was produced as previously described (18). Briefly, viral RNAs were electroporated into Vero cells, cells were radiolabeled with [^{35}S]methionine/cysteine, and virus was purified by banding on sucrose gradients. Nonradiolabeled virus particles were similarly purified from culture supernatants harvested at 72 h postinfection.

Experiments with the alphavirus Semliki Forest virus (SFV) used either a well-defined plaque-purified SFV strain (25) or gradient-purified radiolabeled SFV derived from the pSP6-SFV4 infectious clone (26). The dengue virus (DENV) serotype 1 Western Pacific (WP) strain was propagated as previously described (27), using C6/36 mosquito cells, in DMEM containing 2% heat-inactivated fetal calf serum and 10 mM HEPES, pH 8.0.

Isolation of RuV revertants. Viral RNAs from the RuV mutants were electroporated into Vero cells and plated in 6-well plates. Cells were incubated for 10 days, after which supernatants were clarified by low-speed centrifugation and the presence of infectious virus in each individual culture was determined by assay of an aliquot of the supernatant for infectious centers. The remaining culture supernatant from each well was concentrated by ultracentrifugation at $210,000 \times g$ for 3 h at 4°C. RNA was isolated from pelleted particles using a MagMAX viral RNA isolation kit (Ambion). Viral RNA was reverse transcribed using Q5 High-Fidelity DNA polymerase from NEB BioLabs (Ipswich, MA), and the cDNA was amplified using a nested PCR strategy (for the outer PCR, sense primer 5'-CGAGGAAGCTTCAGACTCCCTACG and antisense primer 3'-TGCAGCAACAGGTGCGGGAA; for the inner PCR, sense primer 5'-ACTGCTGCGCATGCCAGTG and antisense primer 3'-GAATCTAGTGGGCTA GTGC). The entire E1-coding sequence from the amplified cDNA was sequenced using the following primers: sense primer 5'-GCGAGGAGGC TTTCACCTAC, sense primer 5'-CTTCCCCACCGCAACCGTGAT, and antisense primer 5'-TGGATCCACTCGGGCATT.

Virus assembly assays. BHK-21 cells were electroporated with RuV RNAs and cultured in growth medium for 48 h. The cells were then lysed, and virus particles were pelleted from the culture medium (18). Viral proteins were analyzed by SDS-PAGE and Western blotting using an anti-RuV serum (Meridian Life Science) and quantitated using an Odyssey infrared system (LI-COR). Virus release was calculated as the ratio between extracellular and cell-associated E1, E2, and capsid proteins, using the sum of their protein densities.

Immunofluorescence. As described in detail previously (18), cells were fixed in 4% paraformaldehyde for 20 min and permeabilized by treatment for 5 min in phosphate-buffered saline (PBS) containing 0.2% Triton X-100. Cells were labeled by incubation for 2 h at 37°C in PBS containing 5% bovine serum albumin (BSA) and an anti-RuV antiserum (LifeSpan BioSciences, Inc.), a rabbit antiserum to the SFV envelope proteins (28), or the mouse 4G2 monoclonal antibody, which recognizes the fusion loop of flavivirus E proteins (29), followed by staining with the appropriate secondary antibodies and 1 $\mu\text{g}/\text{ml}$ Hoechst stain (Invitrogen) to stain the nuclei. Cells were imaged (≥ 5 fields/condition) by epifluorescence microscopy, and infected cells versus total cells were quantitated using CellProfiler cell image analysis software (www.cellprofiler.org/).

Fusion infection assay. As previously described (18), WT RuV (multiplicity of infection [MOI], 2.5) or equal amounts of mutant RuV stocks were prebound to Vero cells for 1.5 h on ice in binding medium (RPMI without sodium bicarbonate plus 0.2% BSA and 10 mM HEPES, pH 7.0). The cells were washed and incubated at 37°C for 4 min in fusion medium (calcium-free minimal essential medium [MEM] without bicarbonate plus 0.2% BSA, 20 mM MES [morpholineethanesulfonic acid] at the pH indicated on Fig. 1D, and the indicated concentration of CaCl_2). 1,2-Bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid) acetoxymethyl ester (BAPTA-AM; 50 μM) was also added as indicated (see the legend to

Fig. 5B). Cells were then incubated at 37°C in growth medium containing 20 mM NH₄Cl, and infection was scored by determination of the immunofluorescence at 48 h postinfection.

E1 trypsin resistance. The low-pH-dependent conformational change in RuV E1 was quantitated by assessing E1's resistance to digestion with trypsin as previously described (18). In brief, purified radiolabeled RuV was treated with buffers at pH 8 or 5.5 and 2 mM CaCl₂ for 5 min at 37°C, adjusted to neutral pH, and digested for 30 min at 37°C with 125 µg trypsin/ml in 1% NP-40. The reaction was quenched by addition of 125 µg/ml soybean trypsin inhibitor, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 trypsin inhibitor unit (TIU) aprotinin/ml. Samples were immunoprecipitated with a MAb to RuV E1 (Meridian Life Science) and quantitated by SDS-PAGE and autoradiography.

Virus-liposome coflotation assay. Liposomes were prepared by freeze-thaw and extrusion through 400-nm-pore-size polycarbonate filters (30), using a 1:1:1:1.5 molar ratio of phosphatidylcholine (PC; egg yolk), phosphatidylethanolamine (PE; egg yolk), sphingomyelin (Sph; bovine brain), and cholesterol (Chol; Avanti Polar Lipids, Alabaster, AL); a 1:1:1:1:1.5 ratio of PC, PE, Sph, anionic lipid (phosphatidylserine [PS] or lysobisphosphatidic acid [LBPA], both from egg yolk), and Chol; a 1:1:1.5 molar ratio of PC, Sph, and Chol; or a 1:1:1 molar ratio of PC, PE, and Sph. Radiolabeled RuV or SFV was mixed with liposomes in buffers containing the concentrations of CaCl₂ indicated below, treated for 1 min at 37°C, and analyzed by the use of sucrose flotation gradients (18, 31). Coflotation was calculated as the percentage of the total virus radioactivity recovered in the top two out of seven collected fractions. Total recovery of virus radioactivity was ~95% under all conditions.

Entry and fusion assays. For entry and fusion assays, the viruses indicated below were bound to prechilled Vero cells on ice for 1.5 h in binding medium. The cells were washed and then shifted to 37°C in calcium-free MEM (Sigma-Aldrich) supplemented with 10 mM HEPES, pH 7, 0.2% BSA, and 2 mM CaCl₂ to permit entry for the times and conditions indicated below. To score the infection, cells were transferred to growth medium containing 20 mM NH₄Cl to prevent secondary infection and cultured for 48 h at 37°C (RuV) or for 24 h at 28°C (SFV). Infected cells were quantitated by immunofluorescence.

To determine the kinetics of escape from inhibition with BAPTA-AM, cells were shifted to 37°C in calcium-free MEM (Sigma-Aldrich) supplemented with 10 mM HEPES, pH 7, 0.2% BSA, and 2 mM CaCl₂. At the times indicated below, 50 µM BAPTA-AM (Cayman Chemical) was added in the same medium. After 35 min total, growth medium containing 20 mM NH₄Cl was added and infection was scored as described above.

To quantitate internalization, cells were incubated with radiolabeled RuV as described above. Noninternalized virus was removed by stripping it away with subtilisin A for 45 min at 4°C. The reaction was quenched with 1 mM PMSF and 0.2% BSA, and cell-associated radioactivity was quantitated.

To determine the kinetics of lipid mixing upon internalization, RuV was labeled with a self-quenching concentration of the lipophilic dye 1,1'-diiododecyl-3,3,3',3'-tetramethylindodicarbocyanine perchlorate (DiD), used as a probe, as previously described (18, 32). Labeled particles were prebound to Vero cells plated in 8-well Lab-Tek chambers (Thermo Scientific, Waltham, MA) and allowed to internalize for the period of time indicated below at 37°C. Cells were then fixed in 4% paraformaldehyde, counterstained with Hoechst stain, and analyzed using an SP5 Leica confocal microscope equipped with an acousto-optical beam splitter in the Einstein Analytical Imaging Facility. z-stacks of 0.5 µm from several fields were acquired using exactly the same settings for each field. A maximal z-stack projection was generated, and the DiD signal from the z-stack projection from each cell was quantified using ImageJ software (NIH; <http://imagej.nih.gov/ij>). The data are presented as the average amount of DiD signal calculated per nucleus enumerated; the signals from ≥200 cells were quantitated for each condition.

To evaluate colocalization between the RuV fusion and the Rab5/Rab7 endosomal markers, Vero cells were transfected with plasmids harboring

enhanced green fluorescent protein (EGFP)-tagged Rab5 (Rab5-EGFP) or Rab7-EGFP (kindly provided by Robert Lodge) and cultured for 36 h. DiD-labeled RuV (DiD-RuV) was then prebound to cells as described above and incubated at 37°C for 10 or 60 min. The cells were then fixed and their nuclei were stained as described above, and images were acquired using the SP5 Leica confocal microscope. The images shown in the figures are z-stack projections generated by ImageJ software. Mander's coefficient was determined for each cell by comparing the localization of DiD-RuV to that of Rab-EGFP using Volocity software (PerkinElmer).

Virus infection versus DN mutant Rab proteins. Plasmid pEGFP-Rab5 S34N expressing dominant negative (DN) mutant Rab5 was kindly provided by Robert Lodge. The T22N mutation conferring negative trans-dominance to Rab7 (33) was introduced by circular point mutagenesis of the pEGFP-Rab7 WT and was confirmed by automated DNA sequencing. To test the effects on virus entry, BHK-21 cells were transfected with WT or DN mutant Rab constructs and cultured for 36 h. BHK-21 cells were used for these experiments, as they proved amenable to transfection followed by infection. To synchronize entry, RuV, SFV, or DENV was bound to cells on ice as described above, shifted to 37°C for 1 h (SFV) or 3 h (RuV and DENV), and then cultured at 37°C in medium containing 20 mM NH₄Cl for 16 h (SFV) or 36 h (RuV and DENV). These times were chosen to optimize the detection of virus infection without cell loss. Infected cells were scored by determination of immunofluorescence, as described above.

Statistics. Statistical analysis was performed by a paired Student *t* test using Prism software (GraphPad Software, Inc.). Results were considered statistically significant at *P* values of <0.05.

RESULTS

The configuration of Ca²⁺-coordinating residues on E1 is optimal. Residues N88 and D136 in the two fusion loops of RuV E1 coordinate calcium (Fig. 1A), and alanine substitution of either or both residues produces fusion-inactive, nonviable virus (18). Since substitution by the hydrophobic alanine residue is predicted to greatly reduce the affinity for Ca²⁺ (34), we tested whether simply swapping the amino acids at N88 and D136 (N88D D136N double mutant) or swapping either amino acid alone (N88D or D136N single mutants) could produce functional E1 fusion activity. While cells electroporated with WT RuV RNA produced titers of ~10⁵ IC/ml by 24 h and maximal growth to titers of ~10⁷ IC/ml by ~72 h (Fig. 1B), the N88D D136N double mutant was completely nonviable. The N88D and D136N single mutants were undetectable at 24 h, but by 72 h their titers reached levels of ~10³ and 10¹ IC/ml, respectively. Equivalent expression of E1, E2, and capsid was detected in cells electroporated with WT or mutant virus RNA (data not shown), and there was no significant difference in the efficiency of particle production between WT virus and any of the mutants (Fig. 1C). However, the mutant virus particles were unable to fuse with cells at low pH even in the presence of elevated calcium levels (10 mM) (Fig. 1D). Radiolabeled WT and N88D D136N double mutant viruses were treated at low pH and digested with trypsin to assay for the pH-triggered E1 conformational change (10, 18). The neutral pH forms of WT and mutant E1 were efficiently digested by trypsin (Fig. 1E), suggesting similar prefusion E2-E1 heterodimer structures. Following low-pH treatment, both the WT and mutant E1 proteins were trypsin resistant (Fig. 1E), supporting formation of the postfusion E1 homotrimer by both viruses. However, unlike the WT virus, treatment at low pH in the presence of calcium did not promote N88D D136N double mutant virus-liposome interaction, as determined by a coflotation assay (Fig. 1F). Thus, similar to the alanine mutants (18), N88D D136N mutant E1 was assembled onto virus particles

FIG 1 Functional characterization of the RuV E1 N88D D136N double mutant. (A) View of the domain II tip of the RuV E1 structure (PDB accession number 4ADJ) (17). The two fusion loops (FL1, FL2) are shown in cyan, the coordinated Ca^{2+} ion is shown as an orange sphere, and the E1 Ca^{2+} -coordinating residues N88 and D136 are shown in red. The figure was prepared using the PyMOL program (57). (B) Growth kinetics. BHK-21 cells were electroporated with WT or mutant RuV RNA and incubated at 37°C for the indicated times, and the progeny virus present in the growth medium was quantitated by assay for infectious centers (IC). (C) Virus assembly assay. BHK-21 cells were electroporated with WT or mutant RuV RNA and incubated at 37°C for 48 h. The envelope proteins in the cell lysates and in virus particles in the medium were quantitated by Western blotting, and virus release was calculated. The graph shows the means and ranges from 2 independent experiments, with release being normalized to that of WT RuV. (D) Fusion infection assay. Equal volumes of WT RuV and mutant stocks were prebound to Vero cells on ice, treated for 4 min at 37°C with buffer of the indicated pH and calcium concentration, and then cultured for 48 h in growth medium supplemented with NH_4Cl to prevent further infection. Infected cells were scored by immunofluorescent staining, and the score was normalized to that of WT RuV treated at pH 6.0 with 2 mM CaCl_2 . The graph shows the means and ranges from 2 independent experiments. (E) E1 trypsin resistance assay. Purified radiolabeled RuV was treated at the indicated pH for 5 min at 37°C, adjusted to neutral pH, and digested with trypsin. Envelope proteins were quantitated by immunoprecipitation, SDS-PAGE, and autoradiography. The amount of trypsin-resistant E1 relative to that for parallel controls premixed with trypsin inhibitor was calculated. The graph shows the means and standard deviations from 3 independent experiments. (F) Virus-liposome coflotation assay. Radiolabeled WT or mutant RuV was mixed with liposomes, and the mixture was incubated at the indicated pH for 1 min at 37°C in the presence or absence of 2 mM CaCl_2 . Samples were adjusted to neutral pH, and the virus-liposome association was determined by coflotation on calcium-free sucrose gradients. The graph shows the means and standard deviations from 3 independent experiments.

and responded to low pH but was unable to stably insert into liposome target membranes.

Ca^{2+} dependence imposes a strong selection pressure on RuV. The gradual appearance of low levels of infectious virus in the N88D and D136N single mutant cultures (Fig. 1B) suggested the possible generation of revertant viruses. To isolate such revertants, we cultured Vero cells electroporated with RNA from a variety of RuV mutants in the E1-calcium interaction site, all of which had an initial lethal phenotype. Even after 10 days of culture, no infectious virus was recovered from cells electroporated with viral RNA requiring ≥ 2 nucleotide changes to regain the WT amino acid residue. These mutations included RuV E1 N88A (2 mutated nucleotides), D136A (2 mutated nucleotides), N88A and D136A (4 mutated nucleotides), or N88D and D136N (2 mutated nucleotides) (data not shown). Addition of 0.5 mM MnCl_2 during *in vitro* transcription to increase the error rate did not promote the recovery of revertants from the mutants with these mutations (data not shown). In contrast, infectious virus particles were re-

covered from independent cultures of cells electroporated with RuV E1 N88D or D136N mutant RNA, each of which required only one mutation to regain the WT amino acid. Sequence analysis showed that these were true revertants to the WT amino acid residue, although the nucleotide sequences of most revertants differed from the WT nucleotide sequence (Table 1). Multiple approaches thus proved unable to generate viable RuV lacking the WT calcium-binding residues. RuV calcium dependence therefore appears to be under very strong positive selection.

Lipid specificity of RuV E1-membrane interaction. The calcium dependence of the RuV E1-membrane interaction is, to date, a unique phenomenon among viral fusion proteins. Several cellular proteins have been described to be calcium-dependent lipid binding proteins, including the TIM family proteins and synaptotagmins (35, 36). Calcium binding enables these proteins to interact with anionic lipids, such as phosphatidylserine (PS). We used virus-liposome flotation assays to test for a role of PS in the RuV E1-membrane interaction. As shown in Fig. 2A, PS actually

TABLE 1 Revertants of RuV mutants in the E1 calcium-binding site

Revertant	Wild-type E1 sequence	Mutation introduced	Reverted sequence	Resulting amino acid
N88D#1	AAC	GAT	AAT	N
N88D#2	AAC	GAT	AAT/AAC ^a	N
N88D#3	AAC	GAT	AAT	N
N88D#4	AAC	GAT	AAT/AAC ^a	N
N88D#5	AAC	GAT	AAT	N
D136N#1	GAC	AAT	GAT	D
D136N#2	GAC	AAT	GAT	D
D136N#4	GAC	AAT	GAT	D

^a Both codons were detected in the indicated samples.

tended to decrease rather than increase the RuV-liposome association and did not enhance the RuV-liposome interaction even at suboptimal Ca^{2+} concentrations (Fig. 2B). RuV-liposome binding at pH 8 was also decreased in the presence of PS. PS did not affect the liposome binding of the Ca^{2+} -independent alphavirus SFV, ruling out the possibility of nonspecific electrostatic effects of this lipid (Fig. 2C).

We also tested the effects of lysobisphosphatidic acid (LBPA), an anionic lipid enriched in intraluminal vesicles of late endosomes (37). Similarly to PS, LBPA did not enhance the liposome binding of RuV or affect the binding of SFV (Fig. 3A). As previously observed (e.g., see reference 38), cholesterol enhanced the membrane interaction of SFV, and we found that it also enhanced the RuV-liposome interaction (Fig. 3B). In contrast, phosphatidylethanolamine (PE) had no detectable impact on the binding of either RuV or SFV to liposomes (Fig. 3C).

Ca^{2+} -dependent E1-membrane interaction at neutral pH.

We next addressed the role of calcium and low pH in the membrane insertion of RuV E1. To evaluate this, we mixed radiolabeled WT RuV with liposomes in the presence of 2 mM CaCl_2 , treated the mixture for 1 min at 37°C with buffer at pH 8.0 or 6.0, and adjusted the samples to neutral pH. We then added 3 mM EDTA to one set of samples and analyzed them by sucrose gradient flotation in the absence of calcium. A second set of samples was not treated with EDTA and was analyzed by sucrose gradient flotation in the continued presence of 2 mM CaCl_2 . As we observed in prior studies (18) and in Fig. 1F, 2, and 3, RuV was strongly associated with liposomes after low-pH treatment in the presence

of calcium and remained associated when calcium was removed by treatment with EDTA (Fig. 4, WT samples treated with EDTA), consistent with the occurrence of the virus-membrane fusion that is predicted under these conditions. In agreement with our previous results (18), no liposome association was observed when the samples were treated at neutral pH followed by flotation in the absence of calcium. However, we observed a strong virus-liposome interaction when the samples were maintained in the presence of 2 mM calcium throughout the experiment, whether or not they had also been exposed to low pH (Fig. 4). This calcium-dependent membrane interaction at neutral pH required the Ca^{2+} -coordinating residues in RuV E1 and was blocked in the N88D D136N double mutant (Fig. 4). Together, our current and prior (18) results suggest that after RuV fusion with liposomes at low pH, the virus-membrane association is calcium insensitive and irreversible. However, our data also identify a calcium-dependent E1 membrane insertion step that mediates a reversible virus-membrane interaction at neutral pH, prior to E1 refolding to the homotrimer and virus-membrane fusion. While E1 refolding to the homotrimer was independent of calcium coordination, as it occurred, for example, in the N88D D136N double mutant (Fig. 1E), E1 insertion into the target membrane at neutral pH required calcium coordination.

The role of endosomal calcium in RuV fusion. Our results suggested that RuV E1 might bind Ca^{2+} in the extracellular medium and insert into the plasma membrane at neutral pH, while fusion would subsequently occur within the low pH environment of the endocytic pathway, irrespective of the Ca^{2+} concentration. We previously showed that chelation of extracellular calcium by EDTA or removal of calcium from the medium during uptake blocked RuV-membrane fusion in the endocytic pathway (18). However, these results could not differentiate between effects on E1 membrane insertion at the cell surface and those within the endosome compartment. To address the location of calcium-dependent insertion, we took advantage of the membrane-permeant calcium chelator BAPTA-AM (39–41). This chelator is inactive until its acetoxymethyl group is removed by intracellular esterases, thus trapping charged active BAPTA within the cell, where it specifically chelates intracellular calcium. To determine if BAPTA-AM is able to inhibit intracellular RuV fusion and infection, we prebound virus to Vero cells on ice and then allowed the

FIG 2 Effect of PS on RuV-liposome interaction. (A) Radiolabeled RuV was mixed with liposomes with or without PS and incubated for 1 min at 37°C in buffer containing 2 mM CaCl_2 at pH 6 or 8. (B and C) Radiolabeled RuV (B) or SFV (C) was incubated with liposomes as described in the legend to panel A but with various CaCl_2 concentrations. In each case, the virus-liposome association was determined by cofloation on calcium-free sucrose gradients. The graphs show the means and standard deviations from 3 independent experiments (A) or the means and ranges from 2 independent experiments (B and C).

FIG 3 Effect of liposome composition on virus-liposome interaction. Radio-labeled RuV (left) or SFV (right) was incubated for 1 min at 37°C at pH 8 or 6 with liposomes with or without LBPA (A), cholesterol (B), or PE (C), using a CaCl_2 concentration of 2 mM. The virus-liposome association was determined by coflotation on calcium-free sucrose gradients. The graphs show the means and standard deviations from 3 independent experiments.

cells to internalize virus for 20 min at 37° in medium containing 2 mM CaCl_2 and a range of BAPTA-AM concentrations. The cells were then transferred to growth medium containing 20 mM NH_4Cl to prevent secondary infection, and infected cells were quantitated after 48 h (Fig. 5A). RuV infection was strongly inhibited by the presence of BAPTA-AM during virus uptake, with an ~85% decrease being observed with 50 μM BAPTA-AM. Under the same conditions, infection with SFV was unimpaired, excluding the possibility of an effect of BAPTA-AM on cell viability, endocytosis, or endosomal acidification. To rule out potential direct effects of BAPTA-AM on RuV fusion or infectivity, we evaluated low-pH-triggered RuV fusion at the plasma membrane in the presence of BAPTA-AM. RuV was prebound to Vero cells and treated at pH 6.2 for 4 min at 37°C in the pres-

FIG 4 Effect of calcium on RuV-liposome interaction at neutral pH. Radio-labeled WT or mutant RuV was mixed with liposomes in buffer containing 2 mM CaCl_2 , and the mixture was incubated at pH 8 or 6 for 1 min at 37°C. Samples were then adjusted to neutral pH, and 3 mM EDTA was added to one set of samples (+). The virus-liposome association was then determined by coflotation on calcium-free sucrose gradients for the samples with EDTA or on gradients containing 2 mM CaCl_2 for the samples without EDTA (–). The graph shows the means and standard deviations from 3 independent experiments.

ence or absence of 2 mM calcium and 50 μM BAPTA-AM. Virus infection due to fusion at low pH was scored by immunofluorescence (Fig. 5B). RuV fusion was strongly dependent on calcium but was unaffected by the presence of BAPTA-AM. Our results thus demonstrate that the requirement for calcium during RuV fusion occurs within the endocytic pathway and not at the plasma membrane.

Time course of RuV entry pathway. We then addressed the kinetics of the steps in RuV entry. RuV has been shown to enter

FIG 5 Effect of BAPTA-AM on RuV infection. (A) Endocytic infection. RuV or SFV was prebound to Vero cells on ice for 1.5 h in binding medium, shifted to 37°C for 20 min in medium containing 2 mM CaCl_2 and the indicated concentration of BAPTA-AM, and then cultured 48 h at 37°C in growth medium plus 20 mM NH_4Cl to prevent secondary infection. Infected cells were scored by immunofluorescent staining. Infectivity was normalized to that observed without BAPTA-AM, which was ~10% infected cells. (B) Fusion infection assay. Virus was prebound to Vero cells on ice as described in the legend to panel A. Cells were washed and incubated for 4 min at 37°C in pH 6.2 fusion medium containing 2 mM CaCl_2 and 50 μM BAPTA-AM, as indicated. Cells were then cultured, and infectivity was scored as described in the legend to panel A. Infectivity was normalized to that observed in the sample treated with 2 mM CaCl_2 in the absence of BAPTA-AM. The graphs in panels A and B show the means and standard deviations from 3 independent experiments.

FIG 6 Kinetics of RuV entry and fusion in Vero cells. (A) Internalization assay. Purified radiolabeled RuV was prebound to Vero cells for 1.5 h on ice, and the cells were shifted to 37°C for the indicated times. The remaining surface-bound virus was then removed by treatment with subtilisin A, and the cell-associated radioactivity was quantitated and normalized to that obtained after 30 min of incubation at 37°C. (B) NH₄Cl escape assay. Unlabeled RuV was prebound to Vero cells for 1.5 h on ice. Cells were shifted to 37°C and incubated for the indicated times. NH₄Cl (20 mM) was then added to block endosomal acidification, and the cells were cultured for 48 h, fixed, stained, and imaged. The number of infected cells was quantitated and normalized to that obtained after 30 min of incubation at 37°C. (C) BAPTA-AM escape assay. RuV was prebound to cells as described in the legend to panel B. Cells were shifted to 37°C and incubated for the indicated times before addition of 50 μ M BAPTA-AM. NH₄Cl (20 mM) was added 35 min after the shift to 37°C to prevent further endosomal acidification, and the cells were cultured for 48 h, fixed, stained, and imaged. The number of infected cells was quantitated and normalized to that obtained when BAPTA-AM was added after 30 min of incubation. (D) Lipid mixing assay. DiD-labeled RuV was prebound to Vero cells as described in the legend to panel B, and then the cells were incubated for up to 30 min at 37°C. The cells were then fixed, the nuclei were counterstained, and the cells were imaged by confocal microscopy. Virus lipid mixing was quantitated (see Materials and Methods), and the amount was normalized to that obtained after 20 min at 37°C. The graphs present the means and standard deviations from 5 (A), 3 (C), or 4 (B, D) independent experiments.

cells through clathrin-mediated endocytosis, but it can also be affected by treatment with 5-(*N*-ethyl-*N*-isopropyl) amiloride (EIPA), which inhibits macropinocytosis (8). In our hands, EIPA treatment inhibited RuV infection by only 20% under conditions that completely blocked macropinocytic entry of a control virus (data not shown), thus strongly supporting the suggestion that clathrin-mediated endocytosis is the major pathway of RuV entry.

Endocytic uptake was assayed by prebinding radiolabeled RuV to Vero cells on ice, incubating at 37°C to permit endocytosis for the times indicated below, and then removing nonendocytosed virus by stripping it away with subtilisin A (Fig. 6A). Similar to other viruses that are internalized by clathrin-mediated endocytosis (42), RuV uptake was very rapid, with a half-life ($t_{1/2}$) of ~ 3.4 min. We evaluated the time required for the low-pH-dependent step(s) in RuV entry by prebinding virus to Vero cells on ice, adding 20 mM NH₄Cl at various times during internalization at 37°C, and monitoring virus infection. The $t_{1/2}$ for escape from

NH₄Cl neutralization of endosomal acidity was ~ 10 min (Fig. 6B). The time required for the calcium-dependent step(s) in RuV entry was tested by assessing RuV escape from inhibition by 50 μ M BAPTA-AM. The $t_{1/2}$ for RuV escape from inhibition by BAPTA-AM was ~ 12.6 min (Fig. 6C). We labeled RuV with the lipophilic dye DiD at self-quenching concentrations to follow lipid mixing between the viral and endosome membranes (18). This assay reflects the initial mixing of the two membranes but not the final formation of the fusion pore that releases the viral nucleocapsid into the cytoplasm. Dequenching of DiD had a $t_{1/2}$ of ~ 8.7 min (Fig. 6D). Thus, lipid mixing occurred rapidly (~ 5 min) after endocytic uptake, while the data suggest that an additional time of >2 to 4 min was required to complete the low-pH and calcium-dependent entry steps.

RuV fuses in early endosomes. The kinetics of RuV entry and the observed lack of a requirement for anionic lipids in the RuV-membrane interaction suggested that RuV fusion occurs in the early endosome compartment. To address the intracellular site of fusion, we directly imaged RuV lipid mixing events in Vero cells expressing either an early (Rab5-EGFP) or a late (Rab7-EGFP) endosome marker. We quantitated the extent of colocalization of fused RuV with these markers after endocytic uptake for 10 min, a time at which about half of the internalized virus had fused (Fig. 6D). At this time point, a significantly stronger level of colocalization occurred with Rab5 than with Rab7 (Fig. 7A and B). When endocytic uptake was extended to 60 min, we observed that the fused virus DiD marker chased into Rab7-positive late endosomes (Fig. 7C and D).

These observations support the suggestion that the early endosome compartment is the site of RuV fusion. To directly address the requirement for RuV delivery to early endosomes in virus infection, we transiently expressed WT or DN mutant Rab5 and Rab7 in BHK-21 cells. The DN mutants inhibit delivery to the early (DN mutant Rab5) or late (DN mutant Rab7) endosome compartments (33, 43). Cells were infected with RuV or with SFV or DENV, which were used as controls for early or late endosome entry, respectively (44, 45). Overexpression of DN mutant Rab5 significantly decreased the level of infection of BHK-21 cells by both SFV and RuV (Fig. 7E). In contrast, SFV infection was unaffected by expression of DN mutant Rab7, while DENV infection was strongly inhibited ($\sim 25\%$ of that for the control) (Fig. 7F). A modest decrease was observed for RuV infection, but it was much lower than that observed with DN mutant Rab5 ($\sim 79\%$ versus $\sim 27\%$ of that for the control). We do not know if this result reflects a true involvement of a Rab7-containing endosome or an indirect effect of DN mutant Rab7 expression, for example, on endosomal calcium content. Together, our data indicate that RuV fusion primarily occurs in early endosomes.

DISCUSSION

Here we have addressed the intracellular site of RuV fusion within the clathrin-mediated endocytic pathway. Both the RuV pH threshold for fusion ($\sim$ pH 6.2) (18) and the observed rapid time course of lipid mixing (~ 5 min after endocytosis) were in keeping with RuV fusion in the early endosome. Direct studies showed that RuV-lipid mixing occurred preferentially in Rab5-enriched early endosomes and that infection was blocked by DN mutant Rab5.

Our prior data showed that lipid mixing is dependent on both low pH and calcium (18), and thus, the results of our current study

FIG 7 Fusion of RuV occurs in early endosomes. (A to D) DiD-labeled RuV was prebound on ice to Vero cells transiently expressing WT Rab5- or Rab7-EGFP. The cells were shifted to 37°C for 10 min (A) or 60 min (C) to allow internalization and then immediately fixed and counterstained for nuclei (blue) and imaged by confocal microscopy to detect the fused intracellular RuV (red) and GFP-labeled Rab protein (green). Bars, 10 μ m. (B and D) Mander's coefficient was used to compare the extent of colocalization of DiD-RuV in relation to Rab5 and Rab7 after a 10-min (B) or 60-min (D) internalization period. In panels B and D, each dot represents a single infected Rab-EGFP-expressing cell. (E and F) BHK-21 cells were transiently transfected with WT and DN mutant Rab5 (E) or Rab7 (F). At 36 h posttransfection, cells were synchronously infected with SFV (MOI = 0.1), RuV (MOI = 1), or DENV (MOI = 0.5). NH_4Cl (20 mM) was then added to block secondary infection. Cells were incubated for 16 h (SFV) or 36 h (RuV and DENV) to allow synthesis of viral proteins in primary infected cells. Cells were then fixed, and infection was scored by immunofluorescent staining. For each virus, the data are shown relative to those for the corresponding WT control. The graphs present the means and standard deviations from 4 (E) or 3 (F) independent experiments.

indicate that the early endosome provides both of these critical factors. The single published paper that we have identified that quantitated endosomal calcium suggested that it is present at a concentration of $<10 \mu\text{M}$ in early endosomes (46). However, in this study, calcium was measured in the endosome lumen, while

the relevant concentration for RuV fusion is presumably at the endosome membrane. On the other hand, our finding of calcium-triggered membrane insertion of RuV E1 at neutral pH suggested that the relevant calcium-requiring step could even be at the plasma membrane, before virus delivery to an acidified compart-

ment. To directly test the role of endosomal calcium in RuV fusion, we determined the ability of BAPTA-AM to block virus infection. BAPTA-AM diffuses freely across membranes but requires activation to BAPTA by intracellular esterases and thus chelates only intracellular calcium (39–41). BAPTA-AM has been found to strongly chelate intracellular calcium stores within 2 min of cell treatment (47). Our data showed that BAPTA-AM efficiently blocked RuV infection but not SFV infection and that endocytosed RuV became BAPTA-AM insensitive within ~9 min after uptake. Thus, the calcium-containing and acidic environment of the early endosome provides the trigger for RuV E1 membrane insertion and fusion. While the E1 protein may insert into the plasma membrane under high-calcium conditions, our data indicate that if such insertion occurs, it is insufficient to overcome a lack of calcium in the early endosome.

Proteins of the TIM family of lipid binding proteins display a preference for binding anionic lipids, particularly PS, and require calcium for this interaction (35, 36). This suggested an appealing model in which calcium-bound RuV E1 interacts with PS, thereby explaining the calcium dependence of the RuV E1-membrane association and fusion activity. However, extensive liposome cofloatation studies showed no enhancement of the RuV-liposome interaction by the anionic lipids PS and LBPA. This is in keeping with RuV fusion in early endosomes versus the enrichment of these anionic lipids in late endosomes and/or the inner leaflet of the plasma membrane (48, 49). We also tested PE, as a recent report showed that this phospholipid can efficiently bind to the TIM1 protein (50), but again, no enhancement of the RuV-membrane association was observed. Thus, our data argue that the lipid interactions of RuV E1 differ from those of proteins of the TIM family and synaptotagmin proteins and that RuV E1 does not have a strong specificity for particular phospholipids, including anionic lipids.

Given the importance of calcium for RuV E1 membrane insertion, it is perhaps surprising that the affinity of the protein for calcium appears to be relatively low, as shown by the high concentration of Ca^{2+} needed for maximal fusion *in vitro* and the ready replacement of Ca^{2+} by Na^+ when the Ca^+ concentration is reduced (17, 18). These observations are in keeping with calcium coordination by N88, since asparagine is predicted to confer a relatively low Ca^{2+} affinity compared to that of the coordinating residue D136 (34, 51). Replacement of N88 with aspartate produced virus that was noninfectious. It is possible that the cavity between the two FLs cannot accommodate two negatively charged aspartate residues without deleterious effects on the conformation of the FLs. It is also possible that the function of the fusion protein requires it to interact reversibly with both Na^+ and Ca^{2+} and that the affinity is optimized for this purpose. This agrees with our findings that all tested substitutions or swaps of N88 and D136 were lethal and that only true revertants of such mutations were isolated. We favor a model in which both optimal calcium binding and the correct conformation of the two fusion loops are required.

We show here that, as long as calcium is present, RuV E1 stably binds target membranes even at neutral pH. In contrast, on the alphavirus particle, E1 is prevented from interacting with membranes at neutral pH by its close association with the E2 protein, which caps the fusion loop (52, 53). Low pH triggers the initial uncapping of the alphavirus fusion loop to allow insertion and then induces the subsequent conformational changes in E1 that lead to the stable postfusion homotrimer (reviewed in reference

19). In the case of RuV, it is not known if the fusion loops are shielded in the virus particle or if they are accessible but inactive in membrane insertion in the absence of calcium. Our *in vitro* data suggested that RuV E1 membrane insertion and homotrimer formation require distinct triggers, with Ca^{2+} binding by the FLs promoting membrane interaction and a low pH being required for refolding to the postfusion conformation. The kinetics of cell entry showed that lipid mixing, which requires fusion loop insertion, occurs rapidly (~5 min) after uptake into an acidic and calcium-containing endosome. An additional period (>2 to 4 min) appeared to be necessary to complete the steps that are dependent on low pH and calcium. These steps presumably mediate the final formation of the fusion pore that releases the viral nucleocapsid into the cytoplasm and could include completion of E1 refolding to the final hairpin, cooperative interactions, or other steps.

While the entry pathways of several other viruses are known to be affected by calcium levels, the key feature in these cases is a reduction in the calcium concentration during entry. For example, the fusion reaction of murine leukemia virus at the cell surface is promoted by calcium removal, which enhances the isomerization of a critical disulfide bond between the SU (peripheral) and TM subunits and thus helps to trigger fusion (54). The nonenveloped polyomaviruses and rotaviruses are destabilized by the low calcium concentration of the cytoplasm, which promotes their uncoating during entry (55, 56). To date, RuV E1 remains the only virus fusion protein whose membrane interaction and fusion activity specifically require calcium binding. Future studies of viral fusion proteins may reveal the presence of additional metal binding motifs and the involvement of calcium or other metals in membrane fusion.

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