Modification of the trypsin cleavage site of rotavirus VP4 to a furin-sensitive form does not enhance replication efficiency

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The infectivity of rotavirus (RV) is dependent on an activation process triggered by the proteolytic cleavage of its spike protein VP4. This activation cleavage is performed by exogenous trypsin in the lumen of the intestines in vivo. Here, we report the generation and characterization of a recombinant RV expressing cDNA-derived VP4 with a modified cleavage site (arginine at position 247) recognized by endogenous furin as well as exogenous trypsin. Unexpectedly, the mutant virus (KU/rVP4-R247Furin) was incapable of plaque formation without an exogenous protease, although the mutant VP4s on virions were efficiently cleaved by endogenous furin. Furthermore, KU/rVP4-R247Furin showed impaired infectivity in MA104 and CV-1 cells even in the presence of trypsin compared with the parental virus carrying authentic VP4 (KU/rVP4). Although the total titre of KU/rVP4-R247Furin was comparable to that of KU/rVP4, the extracellular titre of KU/rVP4-R247Furin was markedly lower than its cell-associated titre in comparison with that of KU/rVP4. In contrast, the two viruses showed similar growth in a furin-defective LoVo cell line. These results suggest that intracellular cleavage of VP4 by furin may be disadvantageous for RV infectivity, possibly due to an inefficient virus release process.

INTRODUCTION

Rotavirus (RV), a member of the family Reoviridae, is the leading aetiological agent causing severe gastroenteritis in infants and young children worldwide, being responsible for an estimated 500 000 deaths annually (Parashar et al., 2003, 2006). RV is a non-enveloped virus comprising three concentric layers enclosing an 11-segmented genome of dsRNA (Estes & Kapikian, 2007).

Remarkable progress as to the understanding of various aspects of viruses has been made through reverse genetics technology that allows one to generate viruses possessing gene(s) derived from artificially manipulated cDNA. For the members of the family Reoviridae that possess 9–12 segmented genomes, it has been difficult to develop a reverse genetics system, mainly owing to their genomic complexity. We have established a reverse genetics system for RV (Komoto et al., 2006), which is a partially plasmid-based system that permits replacement of a particular viral gene with the aid of a helper virus. This approach has been used to generate recombinant RVs with cDNA-derived VP4 (Komoto et al., 2006, 2008), NSP2 (Trask et al., 2010) and NSP3 (Troupin et al., 2010) genes. Although this helper virus-dependent system is technically limited and gives low rates of recovery of recombinant viruses, it allows alteration of the RV genome, thus contributing to our understanding of this virus. On the other hand, recent advances in helper virus-free reverse genetics for reovirus (Kobayashi et al., 2007), bluetongue virus (Boyce et al., 2008) and African horse sickness virus (Matsuo et al., 2010) have indicated that it is possible to initiate cycles of replication of members of the family Reoviridae entirely from cDNA. However, the application of this to RV has been unsuccessful so far despite extensive experiments. This failure may be partially due to the fastidious conditions required for RV growth: cultivation of RV strictly requires trypsin added exogenously to the culture medium because this ensures activation of viral infectivity. Furthermore, FCS, which is essential for maintaining cultured cells, must be eliminated from the culture medium for the catalytic activity of trypsin during RV growth. Therefore, these constraints may partially explain the difficulty in rescuing a viable RV entirely from cDNA since the cells that are employed for transfection are damaged under these conditions.

The spike proteins of many enveloped viruses are initially synthesized as inactive precursors, but while they have the ability to bind to their cellular receptors, they are unable to...
mediate fusion, resulting in viral entry. Therefore, proteolytic cleavage of the precursor spike proteins at mono- or multi-basic cleavage site(s) by cellular proteases is absolutely required to convert them into an active state and to render the virus particles infectious (Klenk & Garten, 1994). The mono-basic cleavage sites are readily cleaved by exogenous trypsin-like proteases. In the multi-basic cleavage sites, the RXR/KR sequence with R at the carboxyl-terminal is readily cleaved by ubiquitous and endogenous furin-like proteases (Duckert et al., 2004).

VP4, a spike protein of non-enveloped RV, resembles the precursor spike proteins of enveloped viruses (so-called class I fusion proteins) because they exhibit substantial structural and functional similarity (Dormitzer et al., 2004). RV VP4, as an inactive precursor (88 kDa), is cleaved at the mono-basic cleavage sites by trypsin into VP5* (60 kDa) and VP8* (28 kDa) as active states, resulting in activation of RV infectivity (Clark et al., 1981; Estes et al., 1981). These observations raised the idea that modification of the VP4 trypsin cleavage site to a furin-sensitive state might allow engineering of a viable RV that can perform multicycle replication without trypsin. It was conceivable that this approach might make it easier to develop a versatile reverse genetics system for RV.

In order to examine this possibility, we generated and characterized a recombinant RV expressing cDNA-derived VP4 with a modified cleavage site recognized by furin as well as trypsin. Unexpectedly, the VP4 cleavage site mutant virus could not undergo multicycle replication without an exogenous protease, although the mutant VP4s on virions were efficiently cleaved by furin-like proteases. Furthermore, the mutant virus showed lower infectivity even in the presence of trypsin compared with the parental virus carrying authentic VP4. These results suggest that intracellular cleavage of VP4 by furin may be disadvantageous for RV infectivity.

RESULTS AND DISCUSSION
Generation of a VP4 cleavage site mutant by reverse genetics

First, we tried to obtain a recombinant RV susceptible to an endogenous protease at the VP4 trypsin cleavage site.
for activation of infectivity. VP4 contains three conserved mono-basic residues at its trypsin cleavage site (R231, R241 and R247) (Arias et al., 1996), which are recognized by trypsin with different susceptibilities. Among them, only R247 is supposed to be required for enhancement of infectivity (Arias et al., 1996; Gilbert & Greenberg, 1998). Therefore, to examine the potential use of an endogenous protease for VP4 cleavage, we introduced the multi-basic furin consensus sequence at the R247 position by substituting the sequence 244RHRR247 for 244IHYR247 in a pX8dT-based (Schnell et al., 1994) T7 RNA polymerase-driven plasmid, pT7/VP4(SA11), encoding the full-length VP4 gene of the SA11 virus (Fig. 1a). The mutated pT7/VP4(SA11)-R247Furin plasmid (Fig. 1a) was subjected to a reverse genetics system for RV (Komoto et al., 2006) to generate a recombinant RV expressing mutant VP4. The rescue procedure was performed using culture medium containing trypsin to preclude selective pressure for cleavability. As seen in Fig. 1(b), a viable mutant virus was successfully rescued. The rescued virus, named KU//rVP4-R247Furin, was plaque-purified three times. Virion dsRNAs from KU//rVP4-R247Furin were extracted and then subjected to PAGE analysis (Fig. 1b). As expected, the VP4 dsRNA of KU//rVP4-R247Furin (Fig. 1b, lane 3) migrated to almost the same position as the corresponding segments of the SA11 virus (lane 3) and recombinant KU//rVP4 virus possessing the cDNA-derived authentic SA11 VP4 gene with a KU backbone (lane 2) (Komoto et al., 2006), the mobility being slower than that of the VP4 segment of the KU helper virus (lanes 1 and 4). Sequencing of the VP4 gene from rescued KU//rVP4-R247Furin confirmed that the VP4 gene contained no unexpected mutations (data not shown).

**Proteolytic cleavage of mutant VP4**

To determine whether or not mutant VP4 of KU//rVP4-R247Furin could be recognized by furin-like proteases, the proteolytic cleavage of VP4 was examined by Western blotting using purified virions and anti-RV polyclonal antibody. MA104 cells infected with recombinant RVs were incubated in the presence of a protease inhibitor cocktail instead of trypsin to prevent non-specific digestion of VP4. It has been reported that the catalytic activity of endogenous furin is not inhibited under these conditions (Molloy et al., 1992). As shown in Fig. 2(a), whereas wild-type VP4 of KU//rVP4 remained to be detected, mutant VP4 of KU//rVP4-R247Furin mostly disappeared. This finding implies that mutant VP4 might have been successfully cleaved by furin-like proteases. The virus proteins of KU//rVP4-R247Furin were indistinguishable from those of parental KU//rVP4 in protein composition except for the manipulated VP4. Because our anti-RV antiserum fails to detect the cleaved products, VP5* and VP8* (data not shown), we raised a new polyclonal antibody against VP5* by immunizing rabbits with synthetic peptides. This anti-VP5* antiserum made it possible to detect the VP5* on virions (Fig. 2b). As anticipated, wild-type VP4s on virions remained mostly uncleaved because the virus was grown without trypsin. Very few wild-type VP4s appear to have been cleaved to the position similar to VP5*. Although the identity of this band has not been investigated, this may represent a product resulting from non-specific digestion, which has been reported not to be associated with the activation of infectivity (Gilbert & Greenberg, 1998). In sharp contrast, most, although not all, mutant VP4s on virions were cleaved, VP5* being generated. These results showed that mutant VP4 containing the furin cleavage site acts as a good substrate for furin-like proteases in the context of virions without the expression of other viral proteins being affected.
cells may contain partially cleaved VP4 that is not sufficient for the activation of infectivity because incubation with trypsin or soluble furin on plaque formation partially restores the plaque phenotype of this virus. The fact that incubation with trypsin partially restores the infectivity might also indicate that other proteolytic cleavage site(s) of VP4 (R231 or/and R241) may be required for efficient infectivity of this virus. Thus, introduction of the furin cleavage site into the VP4 trypsin cleavage site did not result in multiple-cycle virus growth without an exogenous protease.

Endogenous furin is responsible for impaired virus growth of KU/rVP4-R247Furin virus

The plaque phenotype shown in Fig. 3 implied that the infectivity of KU/rVP4-R247Furin is lower even in the presence of trypsin compared with that of parental KU/rVP4. Therefore, we further assessed their infectivities by determining their single-step growth curves. MA104 cells were infected with each of these viruses at a high m.o.i. of 5 and then incubated without an exogenous protease. As shown in Fig. 3(b) (left panel), KU/rVP4-R247Furin exhibited lower growth (<13-fold lower titre) compared with KU/rVP4, consistent with the results from plaque assays. Thus, it was demonstrated that the infectivity of KU/rVP4-R247Furin is impaired in MA104 and CV-1 cells compared with that of parental KU/rVP4.

Next, to examine the potential involvement of furin in the impaired infectivity of KU/rVP4-R247Furin, the virus was subjected to a single-round infection experiment in MA104 cells that were treated with the furin-specific inhibitor Dec-RVKR-CMK, prior and during virus infection. This furin blockage experiment showed that inhibition of furin restored the infectivity of KU/rVP4-R247Furin to almost the same level as that of KU/rVP4, whereas the titres of KU/rVP4 were unaffected under these conditions (Fig. 3b, middle panel). To further substantiate the role of furin in the impaired infectivity of KU/rVP4-R247Furin, we then examined single-round virus growth in LoVo cells because this cell line uniquely lacks active furin as a genetic defect (Takahashi et al., 1993). LoVo cells are about 10-fold less susceptible to RV infection than MA104 cells (Ciarlet et al., 2002). As shown in Fig. 3(b) (right panel), the absence of active furin abrogated the impairment of KU/rVP4-R247Furin infectivity, demonstrating that the catalytic activity of furin is responsible for the lower virus growth
of KU//rVP4-R247Furin observed in MA104 and CV-1 cells.

**Accumulation of infectious virus particles within cells infected with KU//rVP4-R247Furin virus**

VP4 cleavage by trypsin in natural infection occurs after the release of virus particles from the intestinal cells into the intestinal lumen (Ludert et al., 1996). Therefore, it was of interest to examine the competency of KU//rVP4-R247Furin to be released from infected cells. To address this, virus titres within infected cells (cell-associated) and those that were released into the culture medium (cell-free) were compared by single-round infection experiments in MA104 cells. As shown in Fig. 4(a), virus titres in cell-associated and cell-free fractions were comparable for infection with KU//rVP4. In sharp contrast, KU//rVP4-R247Furin exhibited distinctly different kinetics: the virus titres in cell-free fractions were markedly lower (<20-fold lower) than those in cell-associated fractions at all the time points examined, although the total titre of KU//rVP4-R247Furin was comparable to that of KU//rVP4. We also compared the cleavage states of VP4 on the viruses retained in infected cells and the viruses released into the culture medium. The virions in cell-associated and cell-free fractions were collected and purified and then subjected to Western blotting using anti-VP5* polyclonal antibody (Fig. 4b). As expected, wild-type VP4s on virions from both fractions remained mostly uncleaved because the virus was grown without trypsin. In contrast, the majority of mutant VP4s on the virions in the cell-associated fraction were cleaved to generate VP5*, confirming that the intracellular viruses of KU//VP4-R247Furin contain cleaved VP4s. On the other hand, mutant VP4s on the virions in the cell-free fraction showed a lower proportion of cleaved form than those in the cell-associated fraction as observed by the results from plaque assays (Fig. 3a).

Accordingly, these results suggest that intracellular cleavage of mutant VP4 by furin may induce the retention of virions within cells, leading to the impairment of KU//rVP4-

![Fig. 4. Comparison of cell-associated and cell-free viruses during single-step replication. (a) Single-step growth curves of recombinant RVs in cell-associated and cell-free fractions. MA104 cells were infected with KU//rVP4-R247Furin or KU//rVP4 at an m.o.i. of 5 and then cultured without an exogenous protease. The cell-associated (cell pellet) and cell-free (supernatant) viruses were collected at the indicated times post-infection. The virus titre of each fraction was determined by plaque assay. The data are shown as the mean viral titres and SD for three independent cell cultures. (b) Comparison of the cleavage states of VP4 on the viruses retained in infected cells and the viruses released into the culture medium. MA104 cells were infected with KU//rVP4-R247Furin or KU//rVP4 at an m.o.i. of 5 and then cultured for 16 h in the presence of a protease inhibitor cocktail instead of trypsin. Purified virions from cell-associated and cell-free fractions were analysed by SDS-PAGE and Western blotting using anti-VP5* polyclonal antibody. The KU//rVP4-R247Furin virions from cell-free fraction applied to the gel were 10-fold more concentrated (×10) to detect bands.](image-url)
R247Furin infectivity. Because the identification of determinants for RV release from infected cells has been hampered by the lack of effective strategies for experimental mutagenesis in the context of infectious virus particles, it was not expected that intracellular cleavability of VP4 would be associated with retardation of virus release. For many enveloped viruses including members of the families Orthomyxoviridae and Paramyxoviridae, intracellular cleavability at the cleavage site is frequently associated with increased virus infectivity (Klenk & Garten, 1994; Klenk et al., 2008). On the other hand, for dengue virus, a member of the family Flaviviridae, intracellular cleavability is associated with impaired virus infectivity accompanied by a decreased extracellular virus titre and intracellular accumulation of virions (Keelapang et al., 2004), suggesting an analogous consequence for KU//rVP4-R247Furin, as observed in this study. One possible explanation for the impaired infectivity of KU//rVP4-R247Furin is that furin may form a stable complex with mutant VP4, resulting in the retention of virions within cells (Keelapang et al., 2004). Another possibility is that intracellular cleavage of mutant VP4 may cause premature expression of activated virions, which may result in a strong interaction between virions and intracellular membranes, leading to impaired virus release (Keelapang et al., 2004). In any case, our results indicate the negative effect of the intracellular cleavability of VP4 on virus infectivity.

In the present study, we explored the possibility that intracellular cleavage of VP4 by endogenous furin supports multicycle RV replication in the absence of exogenous trypsin. For this purpose, we generated and characterized a recombinant RV expressing a mutant VP4 that can be cleaved by furin-like proteases as well as trypsin. The rescued KU//rVP4-R247Furin was viable and genetically stable. Unexpectedly, however, this VP4 cleavage site mutant was incapable of plaque formation without an exogenous protease. Since nascent viruses containing already cleaved VP4s in cells were significantly constrained within the cells, it is conceivable that the full-length VP4 and its stability may be required for efficient virus release from infected cells. Further studies regarding the linkage between the cleavability of VP4 and impaired virus release may provide new insights into the molecular basis of RV pathogenesis.

METHODS

Construction of a T7 plasmid encoding the VP4 gene. To modify the VP4 trypsin cleavage site at the R247 position into a furin-sensitive form, site-directed mutagenesis was carried out on a T7 RNA polymerase-driven plasmid, pT7/V4P(SA11), encoding the full-length VP4 gene of the SA11 virus (simian RV strain) (Komoto et al., 2006). The mutated plasmid, pT7/V4P(SA11)-R247Furin, was generated by using a QuikChange II site-directed mutagenesis kit (Stratagene) with primers (+) 5'-AGAGATGTGTAAGACAGCTAG-AGCGCAAGCTAATGAAGAG-3' and (-) 5'-ATTAGCTTGGCCTCTA- cggGTGTCTTACATCTTAGCAGTC-3'. The nucleotides in lower case were mutated to modify the amino acid sequence of the VP4 cleavage site.

Cells and viruses. Monkey kidney cell lines COS-7, MA104 and CV-1 were cultured in Eagle’s minimum essential medium (MEM) supplemented with 5% FCS (complete medium). Human colon adenocarcinoma cell line LoVo (IF050067; Health Science Research Resources Bank) was cultured in Ham’s F12 medium with 10% FCS.

To generate a recombinant RV expressing mutated VP4, a reverse genetics system for RV (Komoto et al., 2006) was applied. Briefly, a monolayer of COS-7 cells, which had been infected with a recombinant vaccinia virus expressing T7 RNA polymerase (rT7pol.) (Ishii et al., 2002), was transfected with pT7/V4P(SA11)-R247Furin and then infected with a KU helper virus (human RV strain) pretreated with trypsin (10 μg ml⁻¹; Sigma) for 30 min at 37°C. When cultures of transfected cells were passaged in MA104 cells with trypsin (1 μg ml⁻¹) and neutralizing mAbs, YO-2C2 (Taniguchi et al., 1985) and ST-1F2 (Taniguchi et al., 1987), that specifically neutralize the KU helper virus, an RV-induced cytopathic effect was observed. The generated virus, named KU//rVP4-R247Furin, was plaque-purified three times. Because the risk potential of KU//rVP4-R247Furin was unknown prior to the start of the experiments, all procedures with live viruses were performed using stringent biosafety precautions at Fujita Health University, as approved for work with these viruses by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Virion dsRNAs were extracted and then analysed by PAGE (Taniguchi et al., 1994).

Propagation of recombinant RVs. To prepare RVs possessing trypsin-untreated VP4, MA104 cells grown in 20×150 mm dishes were inoculated with viruses pretreated with trypsin (10 μg ml⁻¹) for 30 min at 37°C. After adsorption at an m.o.i. of 5 for 1 h at 37°C, the infected cells were washed twice with MEM without FCS (incomplete medium) and then cultured at 37°C for 16 h in incomplete medium containing a protease inhibitor cocktail [2 μg ml⁻¹ each of aprotinin (Roche), leupeptin (Roche) and pepstatin A (Peptide Institute)] instead of trypsin. The cultures were frozen and thawed three times, and then centrifuged at low speed to remove cell debris. Virions were pelleted from the supernatant by ultracentrifugation at 100 000 g for 3 h. Pelleted virions were resuspended in 4 ml PBS, pH 7.5, containing 0.5 mM MgCl₂ and 1 mM CaCl₂ (PBS + ). After fluorocarbon treatment, virions were repelleted by ultracentrifugation at 100000 g for 3 h. Purified virion pellets were resuspended in 150 μl PBS + .

Generation of RV-specific antibodies. A polyclonal antibody recognizing RV proteins was generated in our laboratory using standard procedures. Guinea pigs were immunized with CsCl-embedded purified virions (three-layered particles) of strain SA11. A polyclonal antibody recognizing VP5* was generated by Medical and Biological Laboratories. Rabbits were immunized with mixed synthetic peptides (corresponding to 296FKPANYQYTTRDGEVTI33 and 441DLRY GLPAADPNNGKE46O of SA11 VP4) conjugated to keyhole limpet haemocyanin.

Western blotting. Monolayers of MA104 cells in 150 mm dishes were infected with RVs as described above. For Western blotting, purified virions were subjected to SDS-PAGE and then transferred to PVDF membranes (GE Healthcare) (Komoto et al., 2002). Viral proteins were detected using an enhanced chemiluminescence system (GE Healthcare) following incubation with anti-RV or anti-VP5* polyclonal antibodies and secondary antibodies, horseradish peroxidase-conjugated donkey anti-guinea pig IgG or anti-rabbit IgG (Jackson ImmunoResearch).
Plaque assay. The plaque assay was performed as described previously (Urasawa et al., 1982). Briefly, monolayers of CV-1 cells were infected with trypsin-pretreated RVs, washed twice with incomplete medium and then cultured without an exogenous protease, or with either trypsin (1 \( \mu \text{g} \cdot \text{ml}^{-1} \)) or recombinant soluble furin (15 U ml\(^{-1} \)) in primary overlay medium (0.7% agarose). Soluble furin is a recombinant form of active furin from which the transmembrane and cytoplasmic domains have been removed. Since soluble furin requires Ca\(^{2+} \) for its catalytic activity (Rich et al., 1998), plaques were formed in primary overlay medium containing 12 mM CaCl\(_2\). After 3 days, infected cells were stained with secondary overlay medium containing 0.7% agarose and 0.005% neutral red. Plaque sizes were determined by measuring the mean diameters of 25 plaques in two independent assays.

Single-step virus replication. MA104 and LoVo cells in six-well plates were infected in triplicate with trypsin-pretreated RVs at an m.o.i. of 5, washed twice with incomplete medium and then incubated for various times in incomplete medium without an exogenous protease. In the furin blockage experiment, MA104 cells were treated with 81 \( \mu \text{M} \) of the furin-specific inhibitor, decanoyl-L-arginyl-L-vanyl-l-lysyl-l-arginyl-chloromethylketone (Dec-RVKR-CMK; Calbiochem), 5 h prior and during virus infection. Infected cells were frozen and thawed three times before determination of virus titres by plaque assay.

Comparison of cell-free and cell-associated viruses. Monolayers of MA104 cells in six-well plates were infected in triplicate with trypsin-pretreated RVs and then cultured as described above. After incubation for various times, infected cells and supernatants (2 ml) were transferred to microcentrifuge tubes. The cell pellets were resuspended in incomplete medium (2 ml) and then subjected to three freeze-thaw cycles. Viral titres in the supernatants (cell-free) and cell pellets (cell-associated) were determined by plaque assay.

To compare the cleavage states of VP4 on the viruses retained in infected cells and the viruses released into the culture medium, MA104 cells grown in 40 \( \times \) 150 mm dishes were inoculated with trypsin-pretreated RVs and then cultured for 16 h in the presence of a protease inhibitor cocktail instead of trypsin. Infected cells and supernatants (20 ml) were transferred to centrifuge tubes. The cell pellets were resuspended in incomplete medium (20 ml). Both preparations were frozen and thawed three times, and then subjected to purification of virions as described above. The resultant virion pellets were dissolved in 150 \( \mu \text{l} \) PBS +.

ACKNOWLEDGEMENTS

We thank Akiko Yui and Yohei Kawamoto for their technical assistance. This study was supported in part by Grants-in-Aid for Scientific Research on Priority Areas (Matrix of Infection Phenomena) (K.T.) and for Young Scientists (B) (S.K.) from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

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On: Mon, 10 Dec 2018 21:35:25


