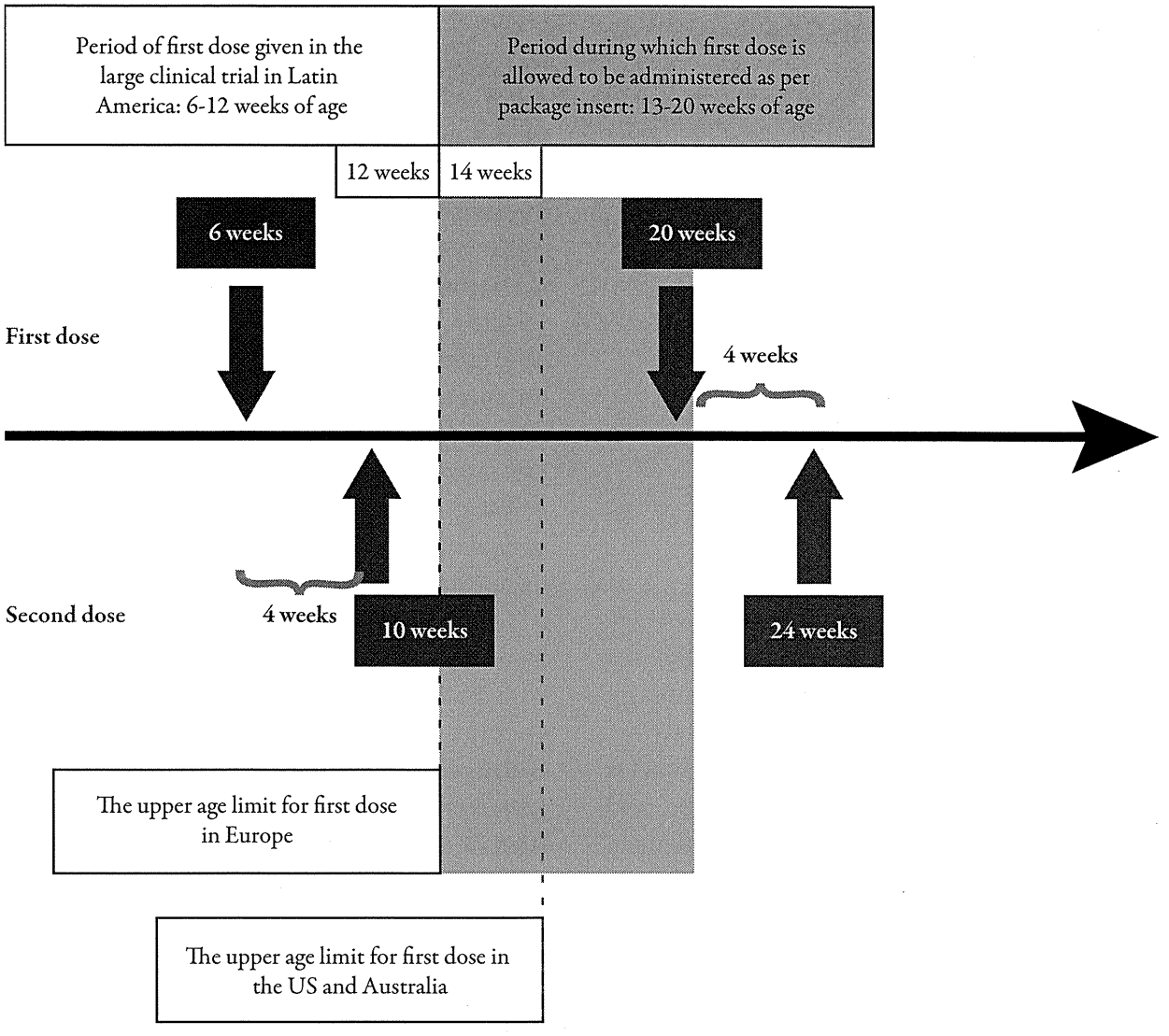
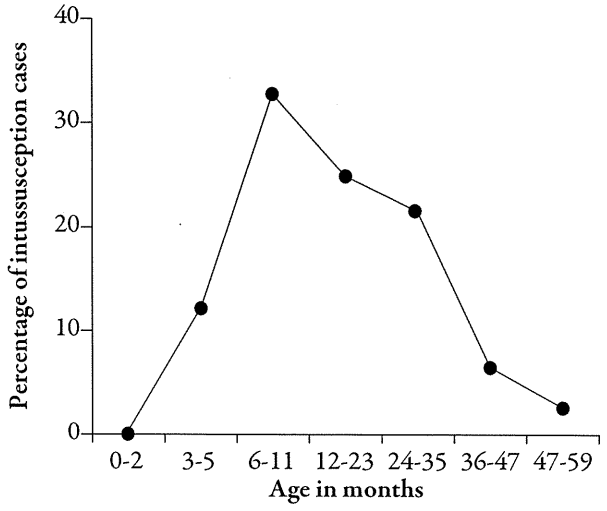
Figure 1. The age period during which the first and the second dose of Rotarix are to be administered according to the package insert distributed in Japan. The first dose may start at 6 weeks of age but before 20 weeks of age, and the second dose may start at 10 weeks of age and end before 24 weeks of age. In Europe, the upper limit of the first dose is 12 weeks, while in the US and Australia it is 14 weeks. As the first dose of Rotarix is allowed to be given to infants between 13-20 weeks of age in Japan (the area highlighted in green), a concern arises that there will be an increased number of Rotarix recipients who develop intussusception by chance alone in the first week after the first dose.



but it increases rapidly thereafter[38,39] (Figure 2[40]). The WHO recommends that the first dose should be administered at age 6-15 weeks.[41] In Australia, the first dose of Rotarix is scheduled to be given to infants between 6-14 weeks of age (Figure 1).[42] In the US, the Advisory Committee on Immunization Practices (ACIP) revised its recommendation in 2009 to extend the maximum age for the first dose to be 14 weeks and 6 days,[43] but it clearly stated that vaccination should not be initiated for infants after 15 weeks and 0 days (Figure 1). In Europe, the European Medicines Agency[44] states that both doses should preferably be administered before 16 weeks of age, meaning that the first dose is to be given to infants at

Figure 2. Age distribution of patients with naturally-occurring intussusception in a sentinel hospital in northern Japan. Note that there was no case of intussusception during the first 3 months of life (0-2 months of age). *Adapted from the figure published in Nakagomi T, Takahashi Y, Arisawa K, Nakagomi O. A high incidence of intussusception in Japan as studied in a sentinel hospital over a 25-year period (1978-2002). Epidemiol Infect. 2006;134:57-61.*[40]



6 weeks and no later than 12 weeks of age (a minimum interval of 4 weeks between the doses being taken into account; Figure 1). The expert group of the European Society for Paediatric Diseases and the European Society for Paediatric Gastroenterology, Hepatology, and Nutrition (ESPID/ESPGHAN) also recommended that the first dose of rotavirus vaccine should be given between 6-12 weeks of age, and did not recommend catch-up vaccination with the first dose in infants older than 3 months of age.[45]

Failed Administration (Regurgitation)

The Rotarix package insert states that a single replacement dose may be given at the same vaccination visit in the event that the infant spits out or regurgitates most of the vaccine dose.[35] The ACIP does not recommend re-dosing after regurgitation of any amount on the basis of the absence of data on the benefits or risks with re-dosing.[43] From a practical perspective, who should pay the cost of re-dosing may also be an issue.

Concomitant Vaccine Administration

In countries where Rotarix is given as part of the routine immunization schedule, such as in the US, Rotarix is administered together with other childhood vaccines, including diphtheria-tetanus-acellular pertussis (DTaP), inactivated poliovirus, hepatitis B, *Haemophilus influenzae* type B conjugate vaccine, and heptavalent pneumococcal conjugate vaccine.[43] Thus, Rotarix may be conveniently administered at the time of the first and the second visits of DTaP immunization which, in the US, are scheduled to be given to infants at 2 and 4 months of age. Unfortunately, in Japan, the DTaP immunization does not start until the infant is 3 months of age. However, Rotarix can also be administered at 2 months of age together with *Haemophilus influenzae* type b conjugate vaccine or heptavalent pneumococcal conjugate vaccine. It may appear strange, however, to find in the package insert that such concomitant administration is allowed only when the immunization practitioner judges it necessary.[35]

With regard to the oral polio vaccine, the expert group of ESPID/ESPGHAN discouraged co-administration with Rotarix in its guidelines[45] due to the insufficient clinical efficacy and safety data available to support it. However, there are data available that failed to detect any difference in efficacies[46] or rotavirus Ig (immunoglobulin) A seroconversion rates[47,48] between those who were co-administered Rotarix with oral polio vaccine and those who were not. Another issue specific to Japan may be the arrangement of the scheduled BCG (Bacillus Calmette-Guérin

tuberculosis vaccine) immunization, which is administered between 3-6 months of age.

Contraindications

Contraindications include uncorrected congenital malformation of the gastrointestinal tract, such as Meckel's diverticulum, because such malformation would predispose the infant to intussusception.[35] The Centers for Disease Control and Prevention (CDC) in the US has recently updated the contraindications for rotavirus vaccines (Rotarix and RotaTeq) to include a history of intussusception.[49] It has been reported that patients with severe combined immunodeficiency never eliminate the vaccine strains.[50] Therefore, current contraindications are: (a) infants with a history of severe allergic reaction after a previous dose; (b) infants diagnosed with severe combined immunodeficiency; and (c) infants with a history of intussusception.

THE RESULTS OF CLINICAL TRIAL OF ROTARIX IN JAPAN

Prior to filing an application for licensure in Japan, a phase 3, randomized (vaccine: placebo = 2:1 ratio), double-blind, placebo-controlled clinical trial was conducted to evaluate the efficacy, reactogenicity, safety, and immunogenicity of Rotarix in Japanese infants when it was administered as two doses at 2 and 3 months of age.[51] More specifically, 765 infants aged 6-14 weeks were enrolled, of whom 750 were estimated to be administered the first dose (either the vaccine or the placebo) before 12 weeks of age. Efficacy against any severe rotavirus gastroenteritis leading to medical intervention, caused by circulating wild-type rotavirus, from 2 weeks after the second dose until 2 years of age was 79.3% (95% CI: 60.5-89.8%) and 91.6% (95% CI: 62.4-99.1%), respectively. Seroconversion at the time of 1 month after the second dose was 85% (95% CI: 68.9-95%) in the Rotarix group, whereas only one in 20 infants (5%) in the placebo group seroconverted (due to wild-type rotavirus infection).

TWO KEY ISSUES RELATING TO THE ROTARIX INTRODUCTION IN JAPAN

Lastly, the authors will conclude this review by addressing the two important issues. These issues fall on the specific area of rotavirus research where the authors believe that they have expert views; whether Rotarix is effective in preventing severe diarrhea caused by fully heterotypic G2P[4] rotavirus strains and whether Rotarix is safe with respect to intussusception.

The first issue is about a persisting concern derived from the fact that Rotarix is a vaccine containing only a single human G1P[8] strain, RIX4414. The guiding principle for the development of a single-strain human rotavirus vaccine was that the induction of protective immunity does not entirely depend on neutralizing antibodies specific to either G or P serotype, but more on factors that mediate heterotypic immunity.[52-55] In a large clinical trial in Latin America, and later in Europe, Rotarix was shown to be effective in preventing severe rotavirus diarrhea caused by partially heterotypic G3P[8], G4P[8], and G9P[8] strains, as well as fully-homotypic G1P[8] strains.[56,57] Since fully-heterotypic G2P[4] strains were infrequent during these clinical trials, protective efficacy appeared less convincing, with wide 95% CIs. Soon after the introduction of Rotarix into the universal immunization schedule in Brazil, Gurgel et al.[58] and Nakagomi et al.[59] noticed a high predominance of G2P[4] strains,

although there was also a simultaneous marked decrease in the detection rate of rotavirus.[59] While these studies suggested a shift in the predominantly circulating strains to G2P[4] in highly vaccinated regions, it remained unclear whether such phenomena resulted from Rotarix use or were a simple reflection of natural variation.[60-62] Thus, the key issue here is whether Rotarix would be effective in preventing severe rotavirus diarrhea caused by fully-heterotypic G2P[4] strains. Three case-control studies to quantitatively measure the effectiveness of Rotarix against G2P[4] strains were carried out in different locations in Brazil.[63-65] In the areas of Brazil where these studies were conducted the proportion of G2P[4] genotypes were high (82%-100%), and the effectiveness of Rotarix against severe acute diarrhea or hospitalization due to G2P[4] strains were 79% (95% CI: 74-82) in Aracaju,[63] 85% (95% CI: 54-95) in Recife,[64] and 75% (95% CI: 57-86) in Belém.[65] These data provide strong evidence for the protective efficacy of Rotarix even against rotaviruses fully heterotypic to the vaccine strain. This has important implications because Rotarix would also be expected to provide a reasonable level of protection against fully heterotypic strains other than G2P[4]; these include G12P[6], prevalent in Nepal,[14] and G8P[6], prevalent in Malawi.[16] However, a recent study that analyzed an outbreak of gastroenteritis hospitalizations caused by G2P[4] rotavirus in an impoverished region in Australia failed to provide evidence of effectiveness against G2P[4] rotavirus strains.[66] Thus, the issue of protection against fully-heterotypic G2P[4] strains still warrants closer attention, especially in poorer regions.

The second issue concerns the safety of Rotarix with respect to intussusception. This safety was established in a large-scale clinical trial involving more than 63,000 infants from 11 countries in Latin America and Finland. This trial revealed no statistically significant increases in the risk of Rotarix causing intussusception when it was administered to infants aged between 6-12 weeks in 10 Latin American countries (Figure 1), between 6-13 weeks in Chile, and 6-14 weeks in Finland.[7] The study was powered to detect as small a risk as approximately six cases per 10,000 vaccine recipients; it was, thus, concluded that Rotarix was not considered to cause intussuseption as frequently as Rotashield® (Wyeth-Lederle Vaccines, Radnor, PA, USA) did. However, two postlicensure studies conducted in Mexico and Brazil detected a small, yet statistically significant, relative risk of 5.3 (95% CI: 3.9-9.3), translating into an excess risk of intussuseption of one in 51,000 vaccinees in Mexico in the first week after the first dose; a relative risk of 2.6 (95% CI: 1.3-5.2), in Brazil, translating into an excess risk of one in 68,000 vaccinees in the first week after the second dose.[67] These risks were smaller than those that prelicensure clinical trials were powered to detect. Another study conducted in Australia, where either Rotarix or RotaTeq were introduced into the universal immunization program depending on the Australian state, detected a relative risk of 3.5 (95% CI: 0.7-10.1) in the first week after the first dose of Rotarix, and a relative risk of 5.3 (95% CI: 1.1-15.4) in the first week after the first dose of RotaTeq.[68] The implications from these studies are clear; firstly, the hypothesis that intussusception is unique to the Rotashield vaccine and that other rotavirus vaccines are inherently free from the risk of intussusception has clearly been questioned. Secondly, since RIX4414 was originally derived from a virulent human rotavirus, natural infection with wild-type rotavirus can also cause intussusceptions; a hypothesis that the authors have previously maintained.[69,70]

Reviewing these emerging data and recognizing a very small, yet finite, level of risk associated with the Rotarix vaccination, the authors conclude that Rotarix is safe to administer to infants at the globally-recommended age period, such as between 6-12 weeks of age, when naturally occurring intussusception is rare (Figures 1 and 2). On the other hand, the authors see no reason to be bold enough to recommend the Rotarix immunization beyond 13-15 weeks of age, as is allowed according to the package insert of Rotarix in Japan[35] and promotional pamphlets.[37] The benefits that will be gained by expanding the immunization period for the first dose to 20 weeks of age very unlikely outweighs the risk of an increased number of intussusception cases, whose causal relationship with Rotarix will never be ruled out at the individual case level. If one were to suppose that the attributable risk is 5%, there would be no way to tell which one of 20 patients (5%) with intussusception that occurred in the first week after the first dose was due to the vaccine. A greater number of such temporally associated cases of intussusception would be expected in Japan than in countries such as Australia and the US. This is due to the greater incidence of intussusception in the first year of life in Japan (185 per 100,000 people/year [95% CI: 133-250])[40] than in the US (30-50 per 100,000 people/year)[71] or Australia (71 per 100,000 people/year [95% CI: 52-97]).[72]

CONCLUSION

A live-attenuated, orally-administered, monovalent rotavirus vaccine, Rotarix, has been launched on the Japanese market. Rotarix has, thus far, shown a remarkable track record in the reduction of morbidity and mortality primarily in Latin American countries, purging itself of the skepticism regarding its effectiveness against serologically unrelated G2P[4] strains. While recent postlicensure surveillance detected a small, yet finite, risk of Rotarix causing intussusception, the authors conclude that Rotarix is safe to administer to infants between 6-12 weeks of age for the first dose and by 24 weeks of age for the second dose. However, the authors strongly discourage the delayed administration of the first dose between 13-20 weeks of age, which is allowed without any warning. Given the high incidence of naturally occurring intussusception in Japan, this should prevent pediatricians and parents from having ill-perceptions of Rotarix being associated with an increased number of temporally associated intussusception, and fully appreciate the benefit of the rotavirus vaccine.

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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; Espejo *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

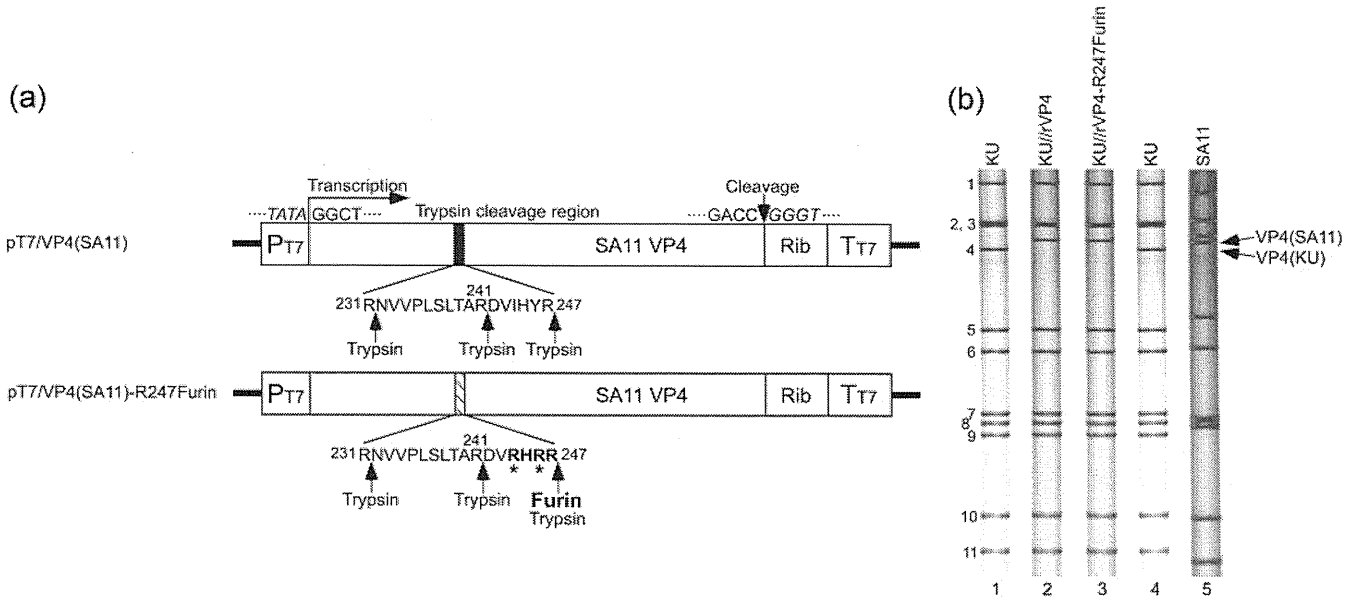

Fig. 1. Generation of a recombinant RV expressing a cDNA-derived VP4 with a modified cleavage site. (a) Schematic representation of the SA11 virus-based transcription plasmids encoding the full-length VP4 gene. Plasmid pT7/VP4(SA11) encodes the authentic VP4 gene of SA11, flanked by the T7 RNA polymerase promoter and the hepatitis delta virus (HDV) ribozyme, followed by the T7 RNA polymerase terminator. Manipulation of the VP4 gene by means of amino acid mutations (positions are indicated by asterisks below the sequence) was carried out in pT7/VP4(SA11); the mutant plasmid, pT7/VP4(SA11)-R247Furin, contains two amino acid mutations within the trypsin cleavage site that create a furin cleavage site at the R247 position. The numbers indicate the amino acid positions in the SA11 VP4 sequence. P_{T7}, Rib and T_{T7} denote the T7 RNA polymerase promoter, HDV ribozyme and T7 RNA polymerase terminator, respectively. (b) PAGE of dsRNAs extracted from the rescued VP4 cleavage site mutant. Lanes 1 and 4, dsRNAs from KU helper virus; lanes 2 and 3, dsRNAs from parental KU//rVP4 virus containing the authentic VP4 gene (lane 2) and rescued KU//rVP4-R247Furin virus (lane 3); and lane 5, dsRNAs of the SA11 virus used for VP4 gene cloning. The numbers on the left indicate the order of the genomic dsRNA segments of the KU helper virus.

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 $_{244}$RHRR$_{247}$ for $_{244}$IHYR$_{247}$ 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 5) 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.

Plaque formation of KU//rVP4-R247Furin virus

Multicycle replication and plaque formation require the proteolytic cleavage of VP4. To examine the replication potential of KU//rVP4-R247Furin in the absence of trypsin, we performed plaque assays on CV-1 cells without an exogenous protease or with either trypsin or soluble furin as controls (Fig. 3a). The KU//rVP4 plaques were exclusively visible in the presence of trypsin. On the other hand, unexpectedly, KU//rVP4-R247Furin was incapable of plaque formation in the absence of an exogenous protease. This finding indicated that KU//rVP4-R247Furin still requires an exogenous protease for plaque formation. Furthermore, the KU//rVP4-R247Furin plaques were smaller in the presence of either trypsin or soluble furin (diameters 1.80 ± 0.30 and 1.68 ± 0.43, respectively) compared with those of KU//rVP4 in the presence of trypsin (diameter 4.92 ± 0.66) (Fig. 3a). The virions released from KU//rVP4-R247Furin-infected

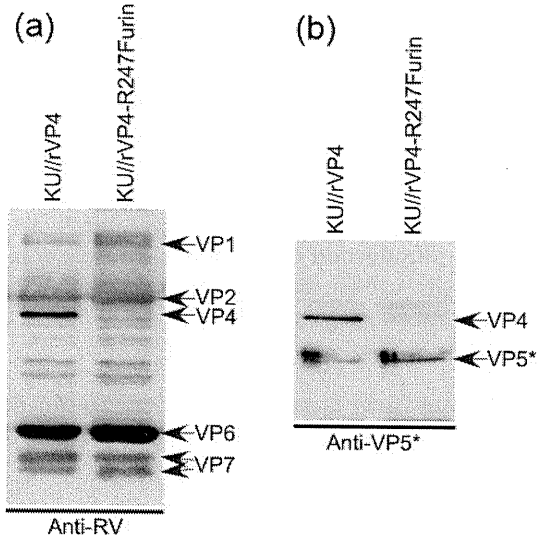

Fig. 2. Proteolytic cleavage of the mutant VP4. MA104 cells were infected with KU//rVP4-R247Furin or KU//rVP4 at an m.o.i. of 5 and then cultured for 16 h in the presence of a protease inhibitor cocktail instead of trypsin. Purified virions were analysed by SDS-PAGE and Western blotting using anti-RV polyclonal antibody (a) and anti-VP5* polyclonal antibody (b).

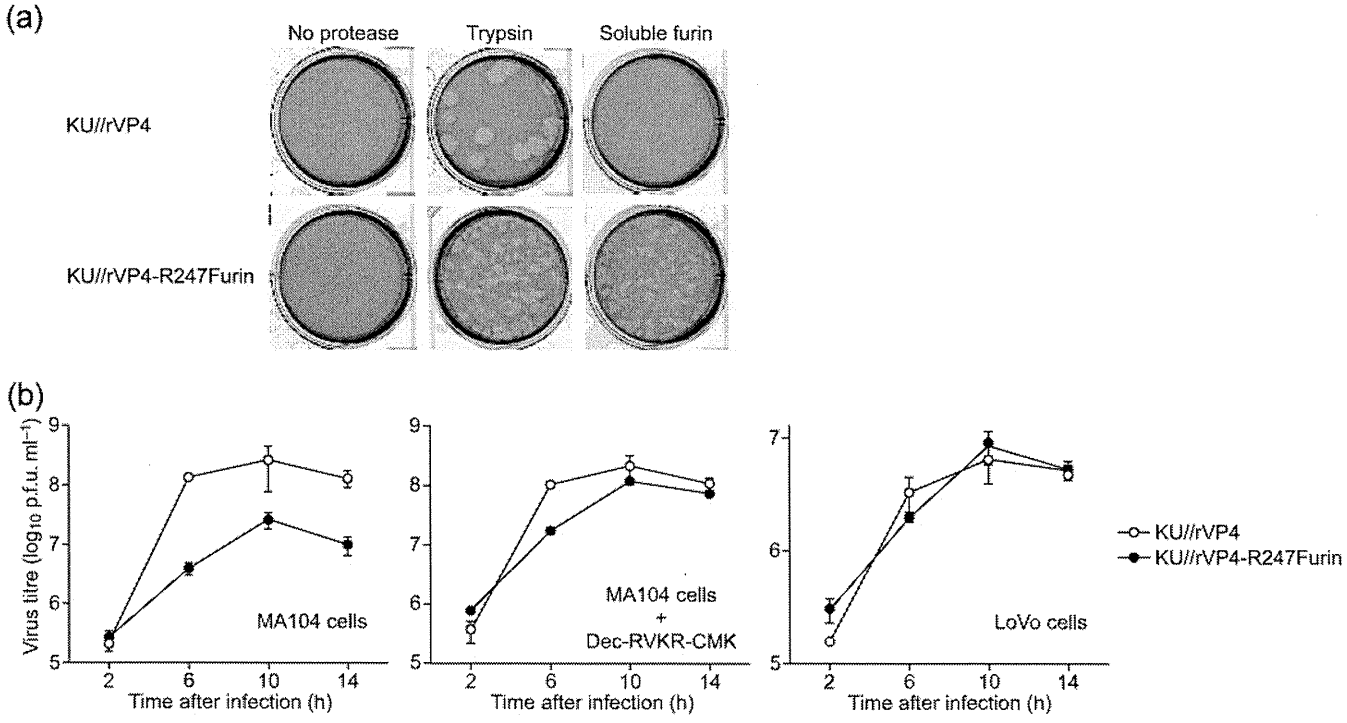

Fig. 3. Multicycle replication and virus infectivity of the mutant with VP4 having a furin cleavage site. (a) Plaque formation by recombinant RVs. KU//rVP4-R247Furin or KU//rVP4 was directly plated onto CV-1 cells for plaque formation and then incubated without an exogenous protease in the overlay medium or with either trypsin or soluble furin as controls. (b) Single-step growth curves of recombinant RVs. MA104 (left and middle panels) and LoVo cells (right panel) were infected with KU//rVP4-R247Furin or KU//rVP4 at an m.o.i. of 5 and then incubated for various times without an exogenous protease. (Middle panel) In a furin blockage experiment, MA104 cells were treated with the furin-specific inhibitor Dec-RVKR-CMK prior and during virus infection. Virus titres in the cultures were determined by plaque assay. The data are shown as the mean viral titres and SD for three independent cell cultures.

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-

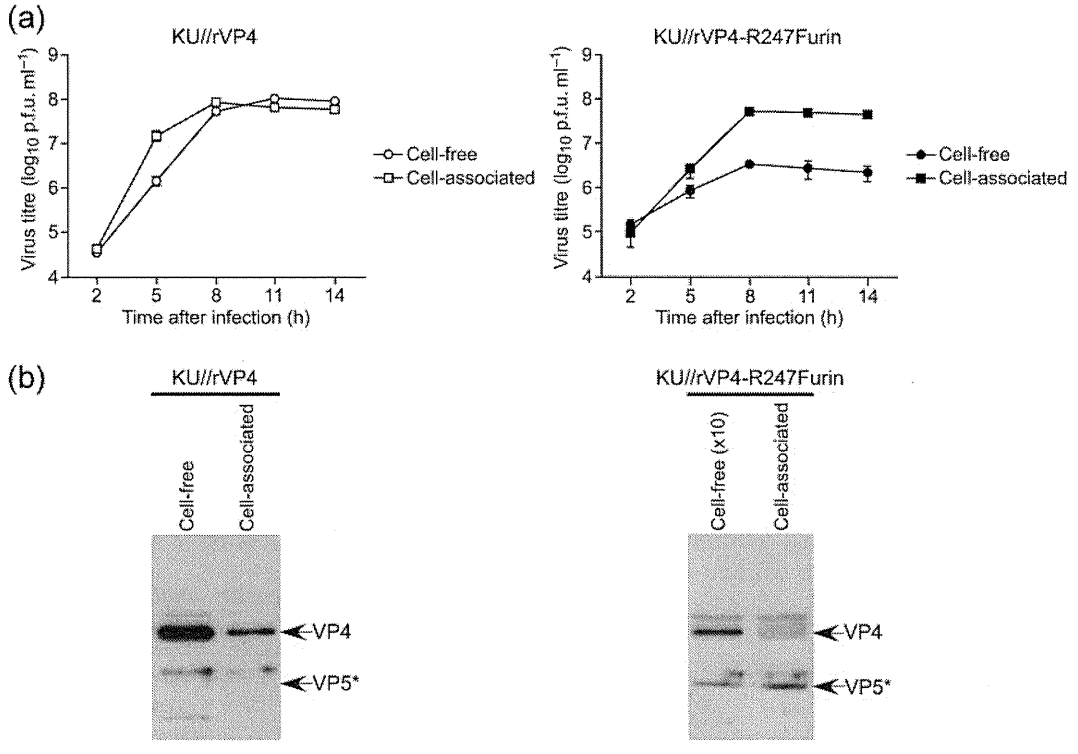

Fig. 4. Comparison of cell-associated and cell-free viruses during single-step replication. (a) Single-step growth curves of recombinant RVs in cell-associated and cell-free fractions. MA104 cells were infected with KU//rVP4-R247Furin or KU//rVP4 at an m.o.i. of 5 and then cultured without an exogenous 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.

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/VP4(SA11), encoding the full-length VP4 gene of the SA11 virus (simian RV strain) (Komoto *et al.*, 2006). The mutated plasmid, pT7/VP4(SA11)-R247Furin, was generated by using a QuikChange II site-directed mutagenesis kit (Stratagene) with primers (+) 5′-AGAGATGTAAgACACcgTAG-AGCGCAAGCTAATGAAG-3′ and (−) 5′-ATTAGCTTGCGCTCTA-cgGTGTcTTACATCTCTAGCAGTC-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 (rDIs-T7pol.) (Ishii *et al.*, 2002), was transfected with pT7/VP4(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 $MgCl_2$ and 1 mM $CaCl_2$ (PBS+). After fluorocarbon treatment, virions were repelleted by ultracentrifugation at 100 000 *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-banded purified virions (triple-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 $_{296}$FKPANYQYTYTRDGEEVT$_{313}$ and $_{444}$LDRLY GLPAADPNNGKE$_{460}$ 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 μg ml^{-1}) or recombinant soluble furin (15 U ml^{-1}; New England BioLabs) 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 (Richt *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 μM 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 × 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 μl PBS+.

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Crystal Structures of GII.10 and GII.12 Norovirus Protruding Domains in Complex with Histo-Blood Group Antigens Reveal Details for a Potential Site of Vulnerability▽†‡

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Noroviruses are the dominant cause of outbreaks of gastroenteritis worldwide, and interactions with human histo-blood group antigens (HBGAs) are thought to play a critical role in their entry mechanism. Structures of noroviruses from genogroups GI and GII in complex with HBGAs, however, reveal different modes of interaction. To gain insight into norovirus recognition of HBGAs, we determined crystal structures of norovirus protruding domains from two rarely detected GII genotypes, GII.10 and GII.12, alone and in complex with a panel of HBGAs, and analyzed structure-function implications related to conservation of the HBGA binding pocket. The GII.10- and GII.12-apo structures as well as the previously solved GII.4-apo structure resembled each other more closely than the GI.1-derived structure, and all three GII structures showed similar modes of HBGA recognition. The primary GII norovirus-HBGA interaction involved six hydrogen bonds between a terminal αfucose1-2 of the HBGAs and a dimeric capsid interface, which was composed of elements from two protruding subdomains. Norovirus interactions with other saccharide units of the HBGAs were variable and involved fewer hydrogen bonds. Sequence analysis revealed a site of GII norovirus sequence conservation to reside under the critical αfucose1-2 and to be one of the few patches of conserved residues on the outer virion-capsid surface. The site was smaller than that involved in full HBGA recognition, a consequence of variable recognition of peripheral saccharides. Despite this evasion tactic, the HBGA site of viral vulnerability may provide a viable target for small molecule- and antibody-mediated neutralization of GII norovirus.

Human noroviruses are an important etiological agent of sporadic gastroenteritis and the dominant cause of outbreaks of gastroenteritis around the world (21, 35). Although the disease is self-limiting, symptoms can persist for days or even weeks, and transmission from person to person is difficult to control once the outbreak has occurred. Cross-protection from future norovirus infections is uncertain, and it is not uncommon for reinfection with a genetically similar strain (20, 27, 46). Currently, there are no vaccines for noroviruses (14, 23). The norovirus positive-sense, single-stranded RNA genome has three open reading frames (ORF1 to ORF3), in which ORF1 encodes the nonstructural proteins, ORF2 encodes the capsid protein, and ORF3 encodes a small basic structural protein. Based on complete capsid gene sequences, human noroviruses can be divided into 2 main genogroups (GI and GII), which can be further subdivided into at least 25 different genotypes (GI.1 to -8 and GII.1 to -17) (18, 47).

Human noroviruses are uncultivable, but expression of the capsid protein in a baculovirus expression system results in the self-assembly of virus-like particles (VLPs) that are morphologically and antigenically similar to the native virion (16). The X-ray crystal structure of the VLP from the prototypic GI.1 Norwalk virus (genus, *Norovirus*; type species, *Norwalk virus*) identifies two domains, the shell and protruding (P) domains (30). The P domain is further divided into P1 and P2 subdomains, with the P1 subdomain interacting with the shell and the P2 subdomain residing on the outer surface of the capsid and likely containing the determinants for antigenicity and receptor binding (16, 37). The P domain can be crystallized separately, and structures of P domains for GI.1 and GII.4 have been determined (3, 4). These replicate many of the structural details of the VLP, including a P domain dimer interface.

The human histo-blood group antigens (HBGAs) have been identified as potential receptors of norovirus (15). The HBGAs are complex carbohydrates linked to proteins or lipids present on epithelial cells and other cells in the body or found as free antigens. At least nine different HBGAs that can bind to norovirus have been described (13, 19, 31, 34, 37, 40, 41), although relatively weak interactions, differential quality of reagents, pH, binding time, and other experimental variables have led to conflicting results concerning the specifics of HBGA binding to different noroviruses (reviewed in reference 39). Defined interactions from crystal structures, however, have been determined for three HBGAs in complex with P

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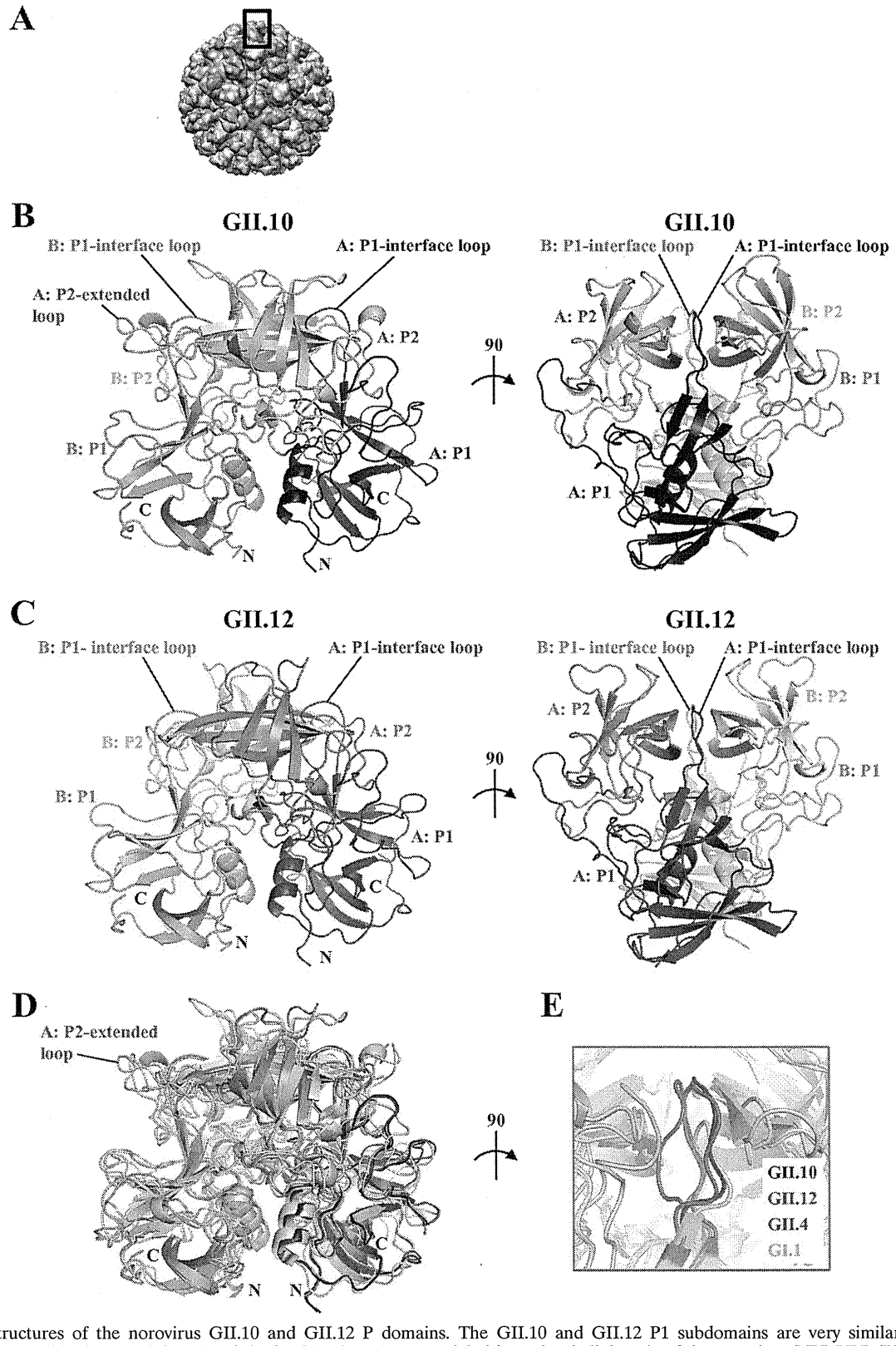

FIG. 1. Structures of the norovirus GII.10 and GII.12 P domains. The GII.10 and GII.12 P1 subdomains are very similar, with greater differences observed in the P2 subdomains. (A) The GII.10 VLP was modeled from the shell domain of the norovirus (NV) VLP (PDB ID, 1IHM) and the unbound GII.10 P domain (PDB ID, 3ONU). The GII.10 VLP (T = 3) was modeled with different monomer interactions, A/B and C/C, where each A, B, and C monomer was colored light blue, salmon, and orange, respectively. The boxed region showed the location of the P domain capsid dimer. (B) The X-ray crystal structure of the unbound GII.10 P domain dimer was determined to have 1.4-Å resolution and colored according to monomers (chains A and B) and P1 and P2 subdomains, i.e., chain A P1 (blue), chain A P2 (light blue), chain B P1 (violet), and chain B P2

domains of two norovirus genotypes (Norwalk virus GI.1 and VA387 GII.4) (3, 4, 6). These studies identified a number of HBGA binding differences between the norovirus GI and GII genogroups. The GI.1 genotype bound HBGAs at the outer (P2) surface of the capsid with a monomeric interaction involving a single P2 subdomain. GII.4 also bound HBGAs at the top of the P2 subdomain but with a completely different set of residues, which spanned a P2 subdomain dimer interface. To better understand the molecular basis of HBGA binding and to examine the relationship between HBGA recognition and norovirus sequence conservation, we determined 11 different crystal structures of P domains from two rarely detected noroviruses, Vietnam026 (026) GII.10 and Hiro GII.12, alone and in complex with a panel of HBGAs. Structure-function relationships derived from analyses of the GII norovirus-HBGA structures were used to provide insight into both the sequence conservation and the potential vulnerability of the HBGA site of recognition to small molecule- or antibody-mediated neutralization.

MATERIALS AND METHODS

Sequence analysis. Amino acid sequences of the entire norovirus capsid were aligned with ClustalX, and the distances were calculated by Kimura's two-parameter method. Phylogenetic trees with 1,000 bootstrap replicates were generated using the neighbor-joining method with ClustalX. GenBank accession numbers were described elsewhere (10), with the addition of VA387 (GenBank accession number AAK84679) (see Fig. S1A in the supplemental material).

Protein expression, purification, and crystallization of the norovirus P domain. The norovirus Vietnam026 GII.10 strain (GenBank accession number AF504671) was isolated from a stool specimen obtained from a male infant under 12 months of age presenting acute sporadic gastroenteritis in December 1999 at the General Children's Hospital No. 1 in Ho Chi Minh City, Vietnam (9). The norovirus Hiro GII.12 strain (GenBank accession number AB044366) was isolated from an adult male in a small outbreak of gastroenteritis in November 1999 in Hiroshima, Japan (9). An amino acid alignment of Norwalk virus, VA387, Vietnam026, and Hiro was used to predict the N and C termini of the Vietnam026 and Hiro P domains. Because residues at the N and C termini of the VA387 P domain structure were disordered (4), we designed our constructs to omit these regions. The near-full-length GII.10 (residues 224 to 538) and GII.12 (residues 224 to 525) P domains (314 and 301 amino acids in length, respectively) were optimized for *Escherichia coli* expression, cloned in a modified pMal-c2x vector at BamHI and NotI (New England Biolabs), and transformed into BL21 cells (Invitrogen). Expression was induced with IPTG (isopropyl-β-D-thiogalactopyranoside; 1 mM) for 18 h at 22°C. A His-tagged fusion-P domain protein was purified from an Ni column (Qiagen) and digested with HRV-3C protease (Novagen) overnight at 4°C, and the P domain was separated on the Ni column. The P domain was further purified by size exclusion chromatography with a Superdex-200 column (GE), concentrated to 2 to 10 mg/ml, and stored in GFB (0.35 M NaCl, 2.5 mM Tris [pH 7.0], 0.02% NaN_3) before crystallization. Dynamic light scattering (DLS) of the P domains determined that the majority of the protein was dimeric (data not shown). Crystals of the P domain were obtained by the hanging-drop vapor diffusion method. The GII.10 P domain crystallized under different conditions using Hampton Research reagents, but for this study, we chose to use two similar crystallization conditions. The first condition contained ammonium citrate (0.66 M, pH 6.5) and isopropanol (1.65%, vol/vol). The second condition contained imidazole (0.1 M, pH 6.5), polyethylene glycol 8000 (PEG 8000) (4.95%, wt/vol), and isopropanol (13.2%, vol/vol). The GII.12 P domain crystals were grown in PEG 1500 (30%, wt/vol), magnesium sulfate hydrate (0.2 M), sodium acetate anhydrous (0.1 M, pH 5.5), and 2-methyl-2,4-pentanediol (3%, vol/vol). Crystals were grown in a 1:1 mixture of the protein sample and mother liquor at 25°C for 2 to 6 days. For the P domain and HBGA complexes, we either soaked a 60 molar excess of HBGA into premade crystals and/or cocrystallized the HBGA and P domain. Prior to data collection, crystals were transferred to a cryoprotectant containing the mother liquor in 30% ethylene glycol, and those bound to HBGAs also contained 30 to 60 molar excess of HBGA.

Data collection, structure solution, and refinement. X-ray diffraction data were collected at the Southeast Regional Collaborative Access Team (SER-CAT) beamlines 22-ID and 22-BM at the Advanced Photon Source, Argonne National Laboratory, Argonne, IL, and processed with HKL2000 (26) or XDS (17). Structures were solved by molecular replacement in PHASER (24) using Protein Data Bank (PDB) identifier (ID) 2OBR as a search model. Structures were refined in multiple rounds of manual model building in COOT (8) and refined with TLS in REFMAC (7) and PHENIX (1). Parameters for the stereochemistry of saccharide residues were taken from a new monomer library (version 5.21) incorporated in REFMAC/CCP4 (G. Murshudov, unpublished data). Glycosidic bonds for di-, tri-, and tetrasaccharides were defined in PHENIX during refinement.

Structure analysis and figures. Superpositions and root mean square deviation (RMSD) calculations were made with CCP4 (7), and figures were rendered using PyMOL (version 1.2r3; Schrödinger, LLC). CHIMERA (29) and VIPER (5) were used to generate the virion structure for Fig. 1A.

Analysis of sequence conservation. Sequence conservation was analyzed separately for GI and GII strains. An alignment of a representative set of GII sequences was used to compute residue conservation scores. Residue conservation was computed using the AL2CO server with a Henikoff-Henikoff sequence weighting scheme, normalized conservation values, and entropy-based conservation calculation (28). The computed residue conservation scores were mapped onto the surface of the unbound GII.10 structure using AL2CO (28) and a PyMOL script. An analogous procedure for the GI conservation analysis was applied, and the results were mapped onto a previously determined bound Norwalk virus structure (PDB ID 2ZL5 [4]). A model of the GII.10 capsid colored by residue conservation was built using the unbound GII.10 structure, with the S domains and capsid symmetry modeled based on the Norwalk virus capsid structure (PDB ID 1IHM [30]). Sequences used for analysis included those found in reference 10 with GenBank accession numbers AAL12980, AF414423, Q68291, Q913B6, Q913B7, Q915C6, Q915D2, Q916E4, Q916E5, Q916E6, Q91H09, Q91I15, Q9IV39, Q9IV40, Q9IV46, Q9PYA7, U46039, U70059, U02030, AB220921, AB220923, AB291542, AB303930, AB303931, AB303938, AF080550, AF080552, AF080553, AF080554, AF080558, AF425763, AF425764, AF425765, AF427114, AF427115, AF427117, AF427120, AF427122, AJ004864, AJ277613, AJ277619, AY032605, AY502019, AY532117, AY532118, AY532119, AY532120, AY532121, AY532122, AY532125, AY532128, AY532129, AY532133, AY532134, AY587988, AY588019, AY588029, AY741811, DQ078794, DQ078829, DQ364459, DQ419907, DQ975270, EF126962, EF126963, EF187592, EU078406, EU078407, EU078410, Q8QY55, Q915C2, Q915C4, Q915C9, BAG70515, and BAG70482.

RMSD analysis. The six structures of GII.10 bound to different HBGAs were analyzed for structural conservation. To be able to perform heavy-atom RMSD computation, only residues with identical atomic composition in all six structures were included in the analysis. For each such residue, the minimized heavy-atom RMSDs (after alignment) between all pairs of structures were computed and used to obtain the average per-residue RMSD. Residues were divided into the following two categories: binding site and nonbinding site. The set of binding site residues included Asn355, Arg356, Trp381, Glu382, Asp385, Ala400, Ser401, Lys449, Gly451, and Tyr452, and the set of nonbinding site residues included all

(salmon). The chain A P2-extended loop protruded out from the side of the P domain (the chain B extended loop was not fitted into the structure). The P1-interface loop was at the dimer interface and surface exposed. (C) The X-ray crystal structure of the unbound GII.12 P domain monomer determined to 1.6-Å resolution (shown here as a modeled dimer) was colored according to monomers and P1 and P2 subdomains, i.e., chain A P1 (hot pink), chain A P2 (orange), chain B P1 (green), and chain B P2 (cyan). As for the GII.10 P1-interface loop, the GII.12 P1-interface loop was at the dimer interface and surface exposed. (D) Superposition of GII.10 (PDB ID, 3ONU), GII.12 (PDB ID, 3R6J), GII.4 (PDB ID, 2OBR), and GI.1 (PDB ID, 2ZL5), colored as shown in panels B and C, dark gray, and light gray, respectively, indicated that the four structures were very similar, except for several differences, including the P1-interface loop and the P2-extended loop. (E) The P1-interface loop (a close-up 90° rotation of panel D) was located at a dimer interface for all four structures. The lengths of the P1-interface loops were the same for GII.10, GII.12, and GII.4 but shorter for GI.1 (residues 445 to 456, 432 to 443, 436 to 447, and 425 to 431, respectively).