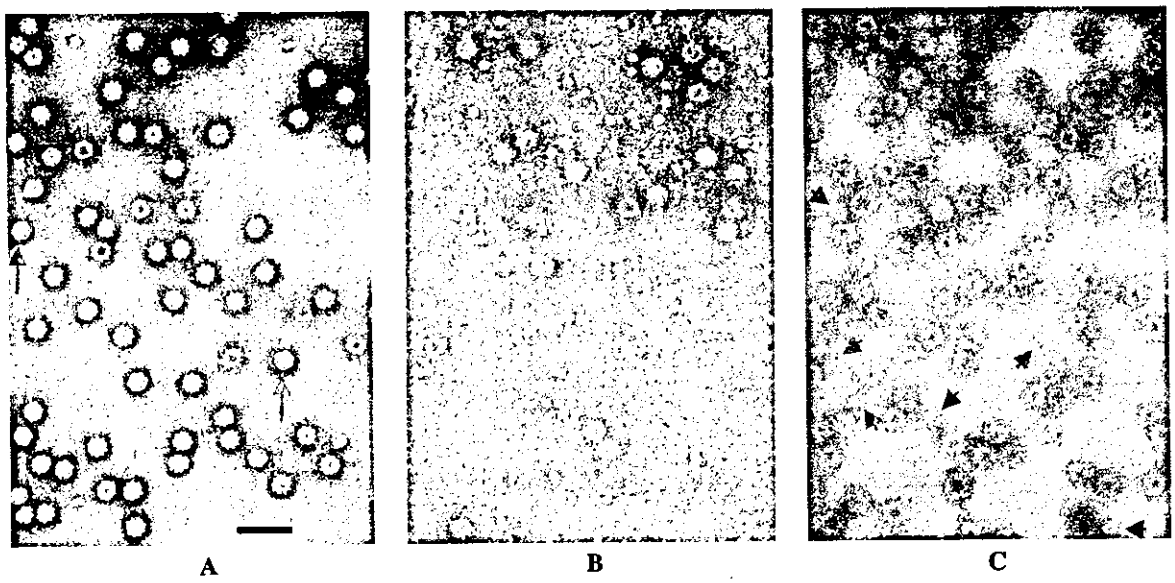

Fig. 3. Electron-microscopic images of CsCl purified (A) Mc114, (B) C12, and (C) NK24 VLPs negative-stained with 4% uranyl acetate (pH 4). The long arrows show the Star of David structure and the short arrows show the small VLPs. The bar indicates 100 nm

shown). Our results indicated that the optimal time to harvest Mc114 VLPs in culture medium was 6 dpi (Fig. 4A).

We also analyzed the time-course expression of Mc114 rVP2 in insect cells by Western blotting with antiserum raised against *E. coli*-expressed Mc114 VP2. However, no bands were detected in either the culture medium or the cell lysate (data not shown).

Western blot cross-reactivity analysis

Western blotting was used to investigate the cross-reactivities among SaV Mc114, C12, and NK24 rVP1 expressed in insect cells. Hyperimmune rabbit antiserum was raised against *E. coli*-expressed Mc114, C12, and NK24 N- and C-terminal VP1. In addition, hyperimmune rabbit antiserum raised against Mc114 VLPs was used. As shown in Fig. 5 and summarized in Table 3, Mc114 (VLP, N- and C-terminal) antisera were reactive with Mc114 and NK24 rVP1 (Mc114 VLP antiserum weakly with NK24 rVP1) but not with C12 rVP1. On the other hand, C12 (N- and C-terminal) antisera were reactive with C12 rVP1 and weakly reactive with Mc114 rVP1, but were not reactive with NK24 rVP1, whereas NK24 (N- and C-terminal) antisera reacted with NK24 rVP1 only.

The three kinds of Mc114 antisera reacted in manner similar to that of Mc114 rVP1, revealing bands at 60K and 55K (Fig. 5), as observed in the time-course analysis (Fig. 4B). These two bands were also detected by C12 N-terminal antiserum with Mc114 rVP1 but not with the C12 C-terminal antiserum. One band was detected by the C12 N-terminal antiserum with C12 rVP1, whereas two bands of

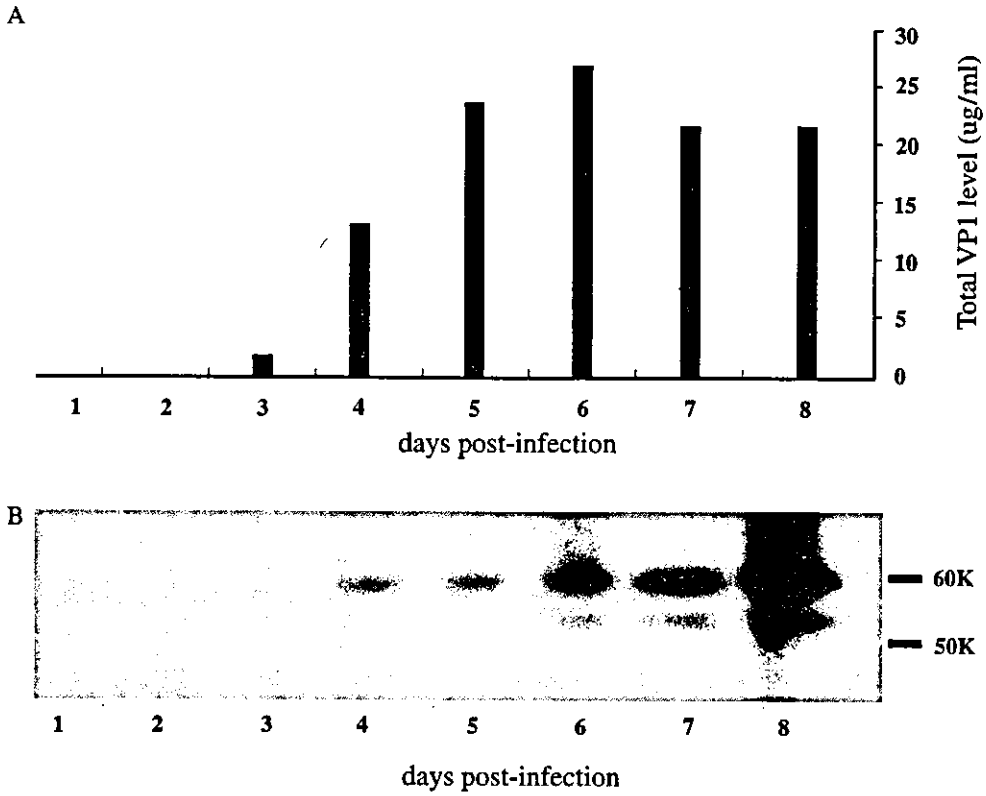

Fig. 4. Time-course expression of the Mc114 rVP1. Confluent Tn5 cells were infected with Mc114 recombinant baculoviruses at MOI of 14.5 and incubated at 26 °C. The culture medium was harvested at the indicated day (days post-infection) and concentrated as described in the Materials and methods. Using the same samples, the expressed proteins were analyzed by (**A**) antigen ELISA with hyperimmune rabbit (capture) and guinea pig (detector) antisera raised against Mc114 VLPs and (**B**) Western blotting with hyperimmune rabbit antiserum raised against Mc114 VLPs. For the antigen ELISA, purified Mc114 VLPs were used as the positive control at concentrations ranging from 500 ng to 0.24 ng

approximately 60K and 58K were detected by the C12 C-terminal antiserum. Two bands of approximately 60K and 55K were detected by NK24 (N- and C-terminal) antisera with NK24 rVP1, and an additional weak band of approximately 45K was detected by the NK24 C-terminal antiserum. Two bands of approximately 60K and 55K were detected by Mc114 VLP and N-terminal antisera with NK24 rVP1, but only a single band of 60K was detected by Mc114 C-terminal antiserum. The significance of these lower bands has not yet been determined.

ELISA cross-reactivity analysis

An antigen ELISA was used to examine the cross-reactivities among Mc114, C12, and NK24 VLPs expressed in insect cells. The ELISA incorporated hyperimmune rabbit (capture) antiserum and guinea pig (detector) antiserum raised against purified Mc114 VLPs. Samples were added to duplicate wells and averaged.

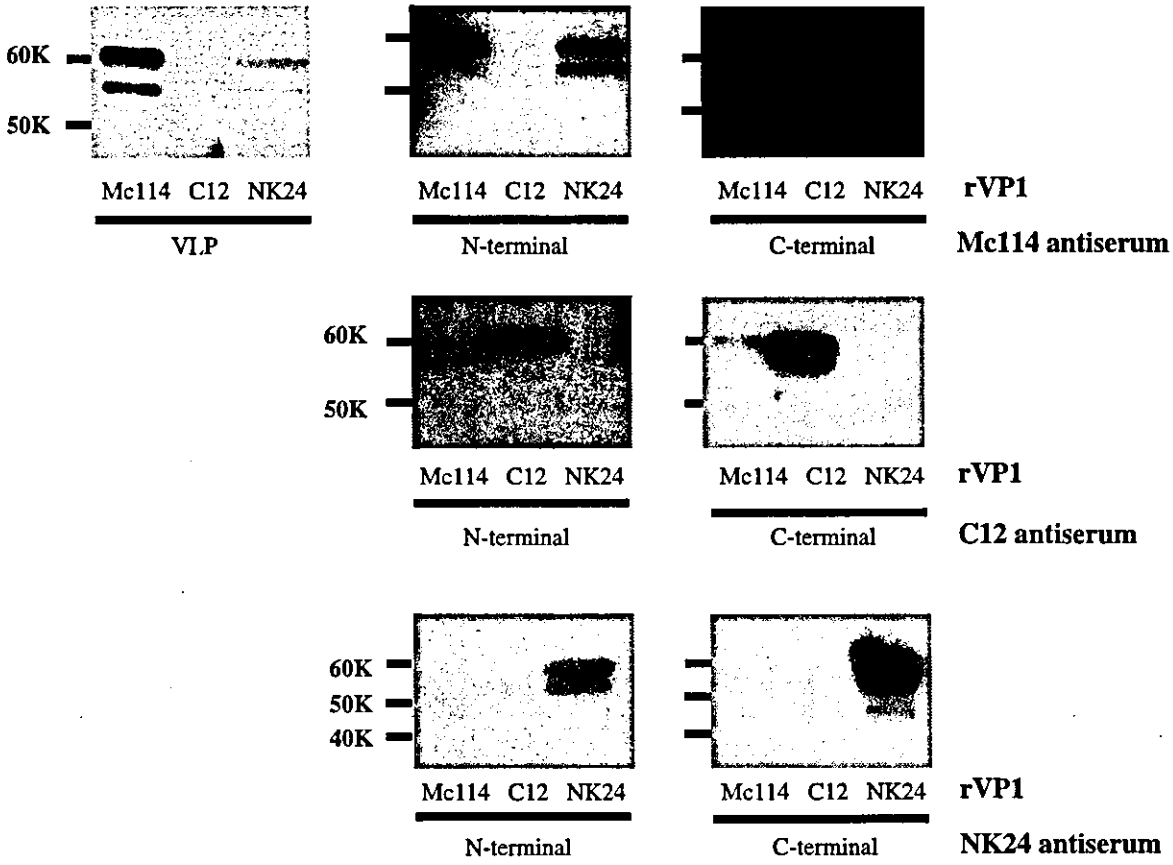

Fig. 5. Western blots of Mc114, C12, and NK24 rVP1 expression in insect cells. Culture medium was harvested at 6 dpi. Seven different hyperimmune rabbit antisera were used, including antisera raised against *E. coli*-expressed Mc114, C12, and NK24 N- and C-terminal VP1, and antisera raised against Mc114 VLPs. The band intensities are relative to each antiserum

Table 3. Reactivities of antiserum with SaV GI, GII, and GV rVP1

rVP1	ELISA	Western blot						
	Mc114 antisera[a]	Mc114 antiserum			C12 antiserum		NK24 antiserum	
	VLP	VLP	N-terminal	C-terminal	N-terminal	C-terminal	N-terminal	C-terminal
Mc114 (GI)	0.46 (9.23)[b]	+	+	+	+[c]	+[c]	−	−
C12 (GII)	0.00	−	−	−	+	+	−	−
NK24 (GV)	0.00	+[c]	+	+	−	−	+	+

[a]The ELISA uses Mc114 VLP (rabbit) antiserum as capture and Mc114 VLP (guinea pig) antiserum as detector
[b]P–N and (P/N ratio) values measured at 492 nm
[c]Weakly reactive

As shown in Table 3, the ELISA detected Mc114 VLPs (A_{492} P–N = 0.46 and P/N ratio = 9.23) but neither C12 VLPs nor NK24 VLPs (A_{492} P–N = 0.00).

Discussion

Human SaV VP1 start contains an amino acid motif, MEG, which is conserved in all human SaV strains and as such is considered to be the putative VP1 start. In this study, we re-examined an earlier suggestion that an upstream sequence from the VP1 start AUG codon is crucial to the formation of human SaV VLP [14]. In addition, we examined the cross-reactivities among SaV GI, GII, and GV rVP1 by Western blotting and the cross-reactivities among SaV GI, GII, and GV VLPs by antigen ELISA. Our results show the following: (i) SaV GI, GII, and GV rVP1 constructs beginning exactly from the predicted VP1 start AUG codon self-assembled VLPs; (ii) an upstream sequence of the predicted VP1 start AUG codon was not an essential element for SaV GI, GII, or GV VLP formation; (iii) the morphological features of the predominant SaV GI, GII, and GV VLPs were similar to those of native SaV, except that GV VP1 also expressed small VLPs; (iv) by Western blot, GI antisera cross-reacted with GV rVP1 but not with GII rVP1, whereas GII antisera cross-reacted weakly with GI rVP1 but did not cross-react with GV rVP1, while GV antisera reacted with GV rVP1 only; and (v) by antigen ELISA, GI VLPs were antigenically distinct from GII and GV VLPs.

Only three other expression studies of human SaV VLP formation have been reported [4, 14, 22]. In one of those studies, an upstream sequence of 73 nucleotides from the predicted VP1 start AUG codon (construct pHou/90-3, Houston/27/90/US strain) was a crucial element for VLP formation [14], whereas one of the other two reports included 39 nucleotides upstream (Sapporo/82/Japan strain) [22]. Recently, the Parkville strain (U73124) was reported to form empty VLPs with a construct that began exactly from the predicted VP1 start. Surprisingly, the Parkville strain had 97.5% and 100% nucleotide identity to pHou/90-3 VP1 and the 73-nucleotide-upstream sequences, respectively. On the other hand, Mc114 had 94.4% and 94.9% nucleotide identity to the Sapporo/82/Japan VP1 and the 39-nucleotide-upstream sequences, respectively. These results suggest that an upstream sequence may not be an essential element for SaV VLP formation, but that some other factor(s) are necessary.

Porcine enteric calicivirus (PEC) of SaV GIII was also expressed using a baculovirus expression system that resulted in the formation of PEC VLPs [9]. The PEC rVP1 construct contained nine nucleotides upstream of the VP1 start AUG codon [9]. That study also included a bacteriophage T7 RNA polymerase promoter of 19 nucleotides (in front of these 9 nucleotides) and a downstream sequence of 98 nucleotides at the 3′ of the VP1 gene. Although the T7 promoter is independent of the baculovirus expression system, Jiang suggested that an element (i.e., an upstream sequence) could be responsible for initiating transcription or translation; alternatively, this element could serve as a spacer between the polyhedrin promoter and the VP1 gene, or as a stabilizer of the RNA after transcription [26]. Interestingly, Jiang also tried to express two other recombinant constructs (pHou/90-1

and pHou/90-2) that included 9 and 29 nucleotides upstream, respectively, but those constructs failed to express rVP1 or form VLPs [14].

Human norovirus (NoV) also belongs to the family *Caliciviridae* and is typically associated with food- and waterborne infections [1]. Human SaV and NoV strains are morphologically, genetically, and antigenically distinct [1, 16]. The NoV genome is organized in a slightly different way than the SaV, since ORF1 encodes all the nonstructural proteins, ORF2 encodes the capsid protein (VP1), and ORF3 encodes a small protein (VP2). In a recent NoV expression study, inclusion of VP2 and poly(A) sequences in NoV rVP1 constructs were found to stabilize VLP formation [3]. In the pHou/90 constructs, both VP2 and poly(A) sequences were absent [14]. Consequently, if SaV VP2 has functions similar to those of NoV VP2, as has been suggested [1], then some crucial factors for SaV VLP formation could be SaV VP2 and poly(A) sequences. Although in a recent report the rVP1 was expressed for a SaV GII strain as determined by SDS-PAGE [24], VLPs were not formed even when an upstream sequence from VP1 start AUG codon, VP2, and poly(A) sequences were included in the construct.

All of our rVP1 constructs formed the typical native SaV Star of David structure and had diameters of 41–48 nm, while NK24 rVP1 also formed smaller VLPs with diameters of 26–31 nm (Fig. 3). The recombinant Sapporo/82/Japan construct had the Star of David form [22], whereas the pHou/90-3 construct did not [14]. Besides the different genogroups and genotypes, the only obvious differences in the constructs that formed this structure were the inclusions of VP2 and poly(A) sequences. These results suggest that VP2 and/or poly(A) sequences are needed to form the Star of David structure, although direct evidence is lacking.

The Mc114 rVP2 was not detected by our Western blotting during the time-course analysis (data not shown), although NoV studies have found that the expression level of NoV rVP2 was low [3]. On the other hand, we did not detect any SaV rVP1 cleavage products either (data not shown), and a similar result was observed in other SaV expression studies [9, 14, 22], whereas for NoV the rVP1 is cleaved at amino acid residue 227, yielding a 32K C-terminal rVP1 product [12, 16]. These data suggest that SaV rVP1 expression could be quite different from that of NoV. Our studies have also found that the yields of purified SaV VLPs were low in comparison with those of NoV VLPs (data not shown), which was also discussed by Guo et al. [9].

Hyperimmune rabbit and guinea pig antisera raised against GI VLPs were used to examine the cross-reactivities among SaV GI, GII, and GV VLPs by an antigen ELISA. As summarized in Table 3, the antigen ELISA was specific only for the homologous GI VLPs. This result was not so unusual, since the amino acid homologies among the VP1 sequences were low (Fig. 2). In the recent structural analysis of SaV VLPs, the outermost domain of VP1, i.e., the P2 domain, was shown to be the most variable region and is likely to confer strain diversity [4]. The predicted P2 domain was the most variable region among these three SaV VP1 sequences, whereas the S and P1 domains were more conserved (Table 3). These data suggest that there were no shared epitopes among GI, GII, and GV VLPs as determined by antigen ELISA, though further studies are needed to confirm this suggestion.

Also, hyperimmune rabbit antisera were raised against *E. coli*-expressed GI, GII, and GV N- and C-terminal VP1 in order to examine the cross-reactivities among SaV GI, GII, and GV rVP1 by Western blotting (Fig. 5 and Table 2). We found that the GI antisera cross-reacted with GV rVP1, but that GV antisera did not cross-react with GI rVP1, i.e., there was only a one-way cross-reactivity. Also, GII antisera cross-reacted weakly with GI rVP1, but GI antisera did not cross-react with GII rVP1, i.e., this too was a one-way cross-reactivity. Figure 2 shows that short continuous residues (8–9 amino acids) had 100% homology between VP1 sequences. These short residues may have represented particular target residues for the polyclonal antibodies that were raised against *E. coli*-expressed VP1. A similar result was observed by Yoda et al. [28] with *E. coli*-expressed NoV VP1. Yoda suggested specific conformational epitope(s) or limited continuous epitope(s) in the NoV VP1 residues that allowed for broad cross-reactivity between NoV GI and GII VP1, which generally have a low amino acid homology. The reason(s) for these SaV one-way cross-reactivities have not yet been determined, nor has the significance of the double (and triple) bands by Western blotting (Fig. 5). Comparable double bands are usually detected for NoV rVP1 expression and were found to include N-terminal cleavage products [2, 16]. However, these SaV extra bands have not yet been determined.

Interestingly, GI VLP rabbit antiserum cross-reacted weakly with GV rVP1 but did not cross-react with GII rVP1 by Western blotting (Fig. 5). Farkas et al. [6] showed that the SaV GV VP1 sequence was related more to GI than GII, based on phylogenetic distance analysis. Also, GI and GV strains possess a predicted ORF3 (VP3?), whereas the ORF3 is absent in GII strains. Further investigations are clearly needed to determine the significance of these novel findings.

Acknowledgments

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We sequenced a 1,012-bp fragment encompassing the first two thirds of the 16S rDNA of ROG140 (accession no. AY692224). The sequence was compared with those of type strains of all members of the former genus *Micrococcus*, and a phylogenetic tree was deduced by the neighbor–joining method (Figure). The sequences of ROG140 and the *K. schroeteri* type strain only differ by an A-to-G substitution at position 747 (*E. coli* numbering). Among the 21 nucleotide differences between the sequences of *K. schroeteri* and the closely related species *K. sedentarius*, 10 are located on a 30-base stretch and constitute a convenient *K. schroeteri* signature (Figure)

Antimicrobial susceptibility testing performed with the disk diffusion method and Etests (AB Biodisk, Solna, Sweden) indicated that the isolate was resistant to penicillins, cephalosporins, kanamycin, tobramycin, erythromycin, clindamycin, sulfonamides, and fusidic acid, but susceptible to imipenem (MIC, 0.25 µg/mL), gentamicin (MIC, 1 µg/mL), trimethoprim (MIC, 0.25 µg/mL), tetracycline (MIC, 0.12 µg/mL), linezolid (MIC, 0.25 µg/mL), vancomycin (MIC, 0.125 µg/mL), teicoplanin (MIC, 0.06 µg/mL), and rifampicin (MIC, <0.002 µg/mL). Unlike the original isolate reported by Becker et al. (1), isolate ROG140 was resistant to ofloxacin and ciprofloxacin (MICs, 8 µg/mL). Conversely, moxifloxacin displayed excellent in vitro activity (MIC, 0.05 µg/mL). As moxifloxacin was more rapidly microbicidal than vancomycin in an animal model of *Staphylococcus aureus* prosthetic valve endocarditis (4), it might present a potential advantage against infections caused by *K. schroeteri*, especially when the oral route is favored.

The natural habitat of *K. schroeteri* remains unknown. The only isolates of *K. schroeteri* identified so far originated from blood or cardiac material, although *Kytococcus* literally means "a coccus from the skin." Our attempts to recover *K. schroeteri* from the mouth, nose, or skin of our patient were unsuccessful. In a recent study, Szczerba et al. were able to isolate most micrococcal species, including *K. sedentarius* but not *K. schroeteri*, from human skin and mucosa (5). However, at that time the authors may not have been aware of this newly described species. The mode of contamination also remains unclear. In the original description (1), *K. schroeteri* endocarditis had developed in the patient <3 months after she underwent cardiac surgery, which suggested perioperative contamination. Here, we describe a late onset, subacute infection 3 years after surgery, which is more likely to have been caused by hematogenous spread.

Although *Micrococcus*-like organisms cause endocarditis infrequently (6), the description of 2 independent infections due to a new species in a short period is intriguing and suggests a specific pathogenicity, at least on prosthetic heart devices. By demonstrating the presence of the bacteria in the infected site, this report establishes *K. schroeteri* as a genuine pathogen in this clinical setting and should prompt further investigations to identify its natural habitat and virulence determinants. At present, commercial systems are not able to identify *K. schroeteri*. However, gram-positive cocci that are strictly aerobic, oxacillin-resistant, and arginine dihydrolase-positive should be recognized as potential *Kytococcus* species and taken into account when endocarditis is suspected.

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Viral Gastroenteritis in Mongolian Infants

To the Editor: Viral agents of gastroenteritis affect millions of persons of all ages worldwide (1). The major agents include rotavirus, norovirus, sapovirus, astrovirus, and enteric adenovirus. Rotavirus is the most frequent cause of acute sporadic childhood gastroenteritis (1), whereas norovirus infects both adults and children and is mainly associated with

outbreaks of acute gastroenteritis (1). These viruses are commonly transmitted by foodborne, person-to-person, fecal-oral, and environmental routes.

In 1999, the infant death rate was 37.3 per 1,000 live births in Mongolia (2). Bacterial pathogens, such as *Shigella flexneri* and *Salmonella*, are commonly detected in hospitalized patients with gastroenteritis, but no data exist concerning viral agents of gastroenteritis in hospitalized patients or in the general community (2).

This preliminary community-based molecular epidemiologic study was the first to report viral agents of gastroenteritis in Mongolian infants. Stool specimens collected from July to August 2003 from 36 infants belonging to 25 different households from 2 areas in Mongolia were screened for rotavirus, norovirus, sapovirus, astrovirus, and adenovirus. The 2 areas were Tov Province, which included Zuun Mod (provincial center) and Bayanchandmani (provincial district center), and Ulaanbaatar area (capital city), which included Chingeltei, Bayangol, Songinok-harikhan, and Bayanzurkh. A total of 48 stool specimens, which were randomly selected from negative-enterovirus specimens (poliovirus and nonpolio enterovirus (Minako Kuramitsu, unpub. data), were screened. Of the 36 infants in the study, 2 specimens were collected 3 weeks apart from each of 12 infants, and 1 specimen was collected from each of 24 infants. In 10 households, specimens were collected from 2 or 3 siblings. Clinical symptoms were recorded when available.

RNA extraction, cDNA synthesis, and polymerase chain reaction (PCR) were performed as described elsewhere (3); for norovirus genogroup (G) I (GI), PCR, G1SKF, and G1SKR primers were used, and for norovirus GII PCR, G2SKF, and G2SKR primers were used (4). For sapovirus, a nested PCR approach was used for all human genogroups (5). For the first sapovirus PCR, SV-F11 and SV-R1 primers were used, while for the nested PCR, SV-F21, and SV-R2 primers were used. For astrovirus PCR, Mon244, and 82b primers were used (6). All PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining. For rotavirus and adenovirus screening, a rapid dry-spot latex agglutination test, Diarlex Rota-Adeno (Orion Diagnostica, Espo, Finland) was used.

Reverse transcription (RT)–PCR products were excised from the gel and purified by the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Nucleotide sequences were prepared with the terminator cycle sequence kit (version 3.1) and determined with the ABI 3100 avant sequencer (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were aligned with Clustal X and the distances were calculated by Kimura's 2-parameter method (3). The nucleotide sequence data determined in this study have been deposited in GenBank under accession no. AY590250–AY590262.

Specimens from 12 (33%) of 36 infants were positive for viral agents of gastroenteritis. Specimens from 9 infants were positive for noroviruses, specimens from 2 infants were positive for astroviruses, and a specimen from 1 infant was positive for sapovirus. All specimens were negative for rotavirus and adenovirus. Ten isolated norovirus sequences (9 persons) were classified according to the recent capsid-based sequence scheme of Kageyama et al. (7). Two norovirus sequences belonged to genogroup I/genotype 11 (G1/11), 4 sequences belonged to GII/3, 1 sequence belonged to GII/7, and 3 sequences belonged to GII/6 (Table).

In 1 household, 2 female infants (isolates 213-3 and 214-3, respectively) were infected with a norovirus GI/11 strain that shared 100% nucleotide identity. This strain was

Table. Mongolian infants positive for viral agents of gastroenteritis

Virus	Genogroup/genotype	Specimen*	Symptom†	Age (mo.)	Sex
Norovirus	GI/11	213-3‡	NA	4	F
Norovirus	GI/11	214-3‡	NA	24	F
Norovirus	GII/6	101-1	None	5	F
Norovirus	GII/3	109-1	Diarrhea	6	F
Norovirus	GII/6	205-3	NA	5	F
Norovirus	GII/3	209-1	Diarrhea	3	M
Norovirus	GII/3	317-1§	NA	24	M
Norovirus	GII/6	613-1	None	5	M
Norovirus	GII/7	613-3	NA	5	M
Norovirus	GII/3	609-3§	NA	5	M
Astrovirus	GI	121-3	NA	4	M
Astrovirus	GI	201-3	NA	5	M
Sapovirus	GI	217-1	Diarrhea	1	F

*First 3 numbers before the hyphen refer to the infant; number after the hyphen refers to the week the specimen was collected.
†NA, not available.
‡Two siblings from the same household.
§Only 1 of the siblings from this household was infected.
¶Astrovirus GI = serotype 1.

likely the same and suggests a common source of contamination or person-to-person transmission. Strains belonging to this new genotype have only been detected in Japan and Switzerland (7).

In a different household, 2 different norovirus strains were detected 3 weeks apart in a 5-month-old male infant (isolates 613-1 and 613-3, respectively). These 2 isolated norovirus sequences shared 77.5% nucleotide identity and clustered into two different genotypes, GII/6 (isolate 613-1) and GII/7 (isolate 613-3). In spite of this infection, the infant had no symptoms of gastroenteritis during excretion of the first norovirus strain.

In 4 other households, 4 infants (isolates 109-1, 609-3, 317-1, and 209-1) were infected with norovirus strains belonging to GII/3. These 4 isolated sequences shared >98% nucleotide identity to Arg320 sequence (AF190817), which was previously found to be a recombinant norovirus (8). This result suggests these 4 strains are also recombinant noroviruses, though further sequence analyses of other genetic regions are needed to confirm this result.

Astrovirus was detected in 2 male infants from different households. One infant was 4 months of age (isolate 121-3), and the other infant was 5 months of age (isolate 201-3). These 2 isolated astrovirus sequences had 100% nucleotide identity, which suggests a common source of contamination. These isolated astrovirus sequences shared 98% nucleotide identity to astrovirus Oxford virus sequence (genogroup I). Sapovirus was detected in 1 stool specimen (isolate 217-1) from a 1-year-old female with diarrhea. The isolated sapovirus sequence shared 98% nucleotide identity to sapovirus Manchester virus sequence (genogroup I). Rotavirus and adenovirus were not detected in any of these specimens; further studies, including those of hospitalized infants, may be useful since infants with rotavirus infections are commonly admitted to hospitals (9).

Our preliminary findings have shown that norovirus was a common agent of gastroenteritis (9 of 36 persons) in Mongolian infants. In a recent report on norovirus gastroenteritis, the risk of contracting gastroenteritis was high when another household member was infected and slightly higher when that member was a child (10). In our study, we found 2 siblings infected with an identical norovirus strain during the same period. In Mongolia, diarrhea has become a major health-care problem (2), therefore, general education in sanitation and hygiene practices may help reduce the transmission of these viruses and lessen the frequency of this disease.

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Mutational study of sapovirus expression in insect cells

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Abstract

Human sapovirus (SaV), an agent of human gastroenteritis, cannot be grown in cell culture, but expression of the recombinant capsid protein (rVP1) in a baculovirus expression system results in the formation of virus-like particles (VLPs). In this study we compared the time-course expression of two different SaV rVP1 constructs. One construct had the native sequence (Wt construct), whereas the other had two nucleotide point mutations in which one mutation caused an amino acid substitution and one was silent (MEG-1076 construct). While both constructs formed VLPs morphologically similar to native SaV, Northern blot analysis indicated that the MEG-1076 rVP1 mRNA had increased steady-state levels. Furthermore, Western blot analysis and an antigen enzyme-linked immunosorbent assay showed that the MEG-1076 construct had increased expression levels of rVP1 and yields of VLPs. Interestingly, the position of the mutated residue was strictly conserved residue among other human SaV strains, suggesting an important role for rVP1 expression.

Introduction

The family *Caliciviridae* is made up of four genera, *Sapovirus, Norovirus, Lagovirus,* and *Vesivirus,* which contain sapovirus (SaV), norovirus (NoV), rabbit hemorrhagic disease virus, and feline calicivirus strains, respectively. Human SaV and NoV strains are agents of gastroenteritis. The prototype strain of human SaV, the Sapporo virus, was originally discovered from an outbreak of gastroenteritis in an orphanage in Sapporo, Japan, in 1977 [1]. Chiba et al. identified viruses with the typical animal calicivirus morphology, called the "Star of David" structure, by electron microscopy (EM). SaV strains were recently divided into five genogroups (GI to GV), of which GI, GII, GIV, and GV strains infect humans, while GIII strains infect porcine species [2]. The SaV GI, GIV, and GV genomes are each predicted to contain three main open reading frames (ORFs), whereas SaV GII and GIII have two ORFs. SaV ORF1 encodes for non-structural proteins and the major capsid protein (VP1). SaV ORF2 (VP2) and ORF3 (VP3) encoded proteins of yet unknown functions. The NoV genome is organized in a slightly different way than the SaV, since ORF1 encodes all the nonstructural proteins, ORF2 encodes the capsid protein (VP1), and ORF3 encodes a small protein (VP2).

Human SaV and NoV strains are noncultivable, but expression of the recombinant VP1 (rVP1) in a baculovirus expression system results in the self-assembly of virus-like particles (VLPs) that are morphologically similar to native SaV [3,4] In a recent NoV expression study, a single amino acid substitution in the rVP1 gene affected VLP formation but not rVP1 expression [5]. In a different study,

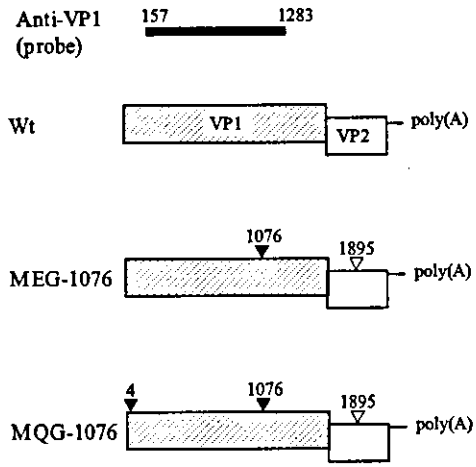

Figure 1
Schematics of the SaV constructs, Wt, MEG-1076, and MQG-1076, containing the rVP1, rVP2, and poly(A) sequences. Each construct began at the predicted AUG start. The triangles show the positions of the nucleotide point mutations. The black triangle had an amino acid substitution in the VP1, whereas the open triangle in the VP2 gene did not change amino acid sequence. An RNA probe (anti-VP1) was used to monitor the transcription of rVP1 mRNA in which contained the native sequence, i.e., lacking the mutation at 1076.

inclusions of NoV ORF3 and poly(A) sequences in a construct increased the expression levels of NoV rVP1 and the stability of VLPs when compared to constructs without these sequences [6]. Recently, cryo-EM analysis of SaV VLPs and X-ray crystallography analysis of NoV VLPs predicted the SaV shell (S) and protruding domains (subdomains P1 and P2) that were based the NoV domains [7,8]. Chen et al. also described strictly and moderately conserved amino acid residues in the capsid protein among the four genera in family *Caliciviridae*.

The purpose of this study was to compare the time-course expression of two different SaV rVP1 constructs in a baculovirus expression system by Northern blotting, Western blotting, enzyme-linked immunosorbent assay (ELISA), and EM. Our novel results have indicated that nucleotide point mutations increased the yields of SaV VLPs in insect cells, offering an alternative explanation for the increased expression levels of rVP1 and yield of VLPs.

Results

Wt, MQG-1076, and MEG-1076 constructs

Expression of SaV rVP1 in a baculovirus expression system results in the self-assembly of VLPs [4]. However, during PCR amplification nucleotide point mutations occurred in our initial MQG-1076 construct, at nucleotide positions 4 and 1076 in VP1, which resulted in two amino acid substitutions at residues 2 and 358, respectively, and a silent nucleotide mutation at position 1895 in VP2 (Fig. 1). Despite these two substitutions the MQG-1076 construct formed VLPs morphological similar to native SaV (data not shown). In order to further investigate these substitutions we expressed another construct (MEG-1076 construct) having only one substitution, at residue 358 in VP1 (Fig. 1). This construct also formed VLPs. Finally we expressed a construct (Wt construct) without these nucleotide point mutations, i.e., having the native sequence. The Wt construct also formed VLPs, however the expression level of rVP1 was noticeably lower than those of the MQG-1076 and MEG-1076 constructs in which had similar levels (data not shown). In order to compare expression levels, we infected Wt and MEG-1076 recombinant baculoviruses each at a multiplicity of infection (MOI) of 14.5 in 2.7 × 10^6 confluent Tn5 cells in 1.5 ml of Ex-Cell 405 medium followed by incubation at 26°C. RNA transcription and rVP1 expression experiments were run in parallel for the Wt and MEG-1076 constructs.

Northern blot analysis

Total RNA was extracted from the cells at 1, 2, 3, 4, 5, 6, 7, and 8 days postinfection (dpi) for Wt and MEG-1076 constructs. Equal amounts (500 ng) of total RNA were added to a 2% agarose gel containing formaldehyde and stained with SYBR Gold (Fig. 2A). The rVP1 mRNA was then analysed by Northern blot with a probe specific for the VP1 gene (native sequence) corresponding to the VP1 position 157 to 1283 (Fig. 1). The rVP1 mRNA transcript was predicted to be approximately 2300 nucleotides long. As shown in Figure 2B, rVP1 mRNA was detected for each construct. This result showed that the insert sequence and some part of the baculovirus vector, approximately 300 nt, was transcribed, although the exact location(s) on the vector has yet to be determined. Nevertheless, the MEG-1076 construct had increased band intensities, indicating an increased steady-state level, when compared to those of the Wt construct (Fig. 2B). For the Wt construct, rVP1 mRNA was detected at 1 dpi, peaked at 2 dpi, decreased at 3 and 4 dpi, and then decreased to undetectable levels at 5, 6, 7, and 8 dpi. For the MEG-1076 construct, rVP1 mRNA was detected at 1 dpi, peaked at 2 dpi, had steady-state levels at 3 and 4 dpi, and then decreased at 5 dpi but could still be detected at 6, 7, and 8 dpi. These results indicated that the MEG-1076 rVP1 mRNA also had greater stability when compared to those of the Wt rVP1 mRNA.

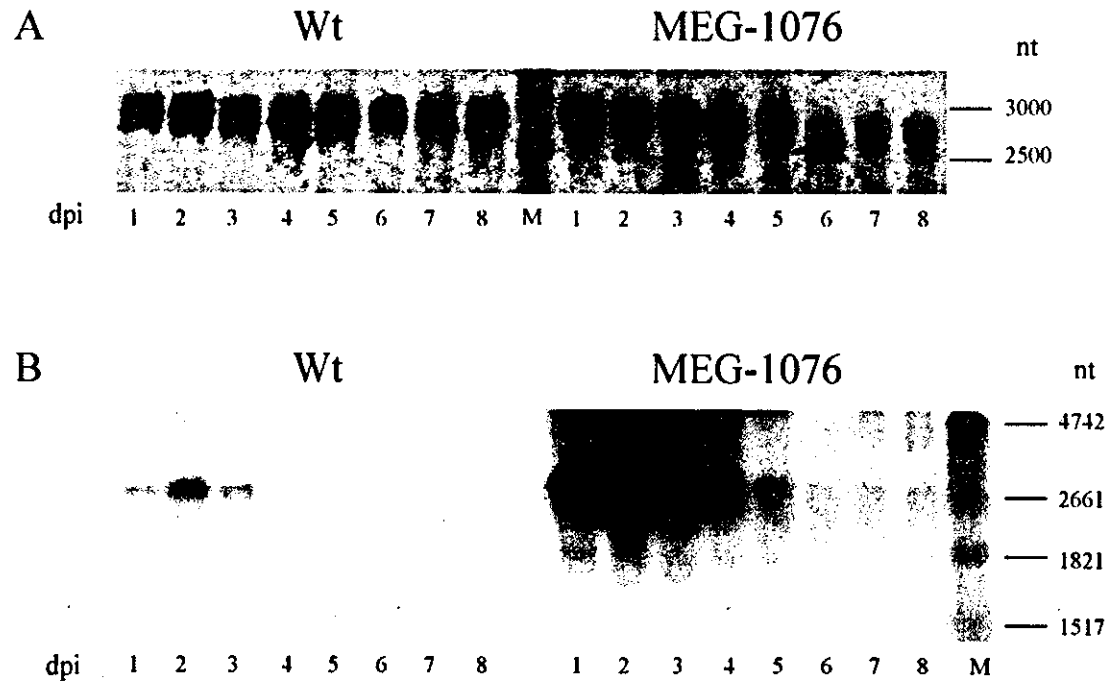

Figure 2
Northern Blot analysis of Wt and MEG-1076 rVP1 mRNA. The total RNA was purified from the cells at 1, 2, 3, 4, 5, 6, 7, and 8 dpi. (A) The relative amounts of total RNA for each construct. (B) The steady-state levels of rVP1 mRNA with an anti-VP1 probe specific for the VP1 gene, corresponding to the VP1 nucleotide position 157 to 1283.

Western Blot analysis

Western blot analysis was used to compare the expression levels of Wt and MEG-1076 rVP1. The culture medium was separated from the cell lysate 1, 2, 3, 4, 5, 6, 7, and 8 dpi as described in the Materials and Methods. Equal volumes of culture medium and cell lysate at each dpi were used for both constructs. Proteins were separated by SDS-PAGE, electrotransferred to PVDF, and detected with a 1:3000 dilution of hyperimmune rabbit Mc114 VLP antiserum. A band at the predicted rVP1 size (60 K) was first detected in the culture medium at 2 and 4 dpi for MEG-1076 and Wt constructs, respectively, which increased each day thereafter as evidenced by an increase in band intensity (Fig. 3A). As indicated by increased band intensities, the MEG-1076 construct expressed increased levels of rVP1 (60 K) than those of the Wt construct. Similarly, these results were reproduced using different MOIs in order to address the variability in virus stock quality (data not shown).

A thin band of approximately 55 K was also detected in the culture medium that appeared at 4 and 5 dpi for Wt and MEG-1076 constructs, respectively, and increased each day thereafter. In a different experiment, we determined the amino acid sequence of the MQG-1076 upper and lower bands by an Edman's degradation method. We discovered that the first three amino acid residues were MQG for both the upper and lower bands. This result indicated that the 55 K bands for these constructs were likely truncated or C-terminal deleted forms of rVP1. A

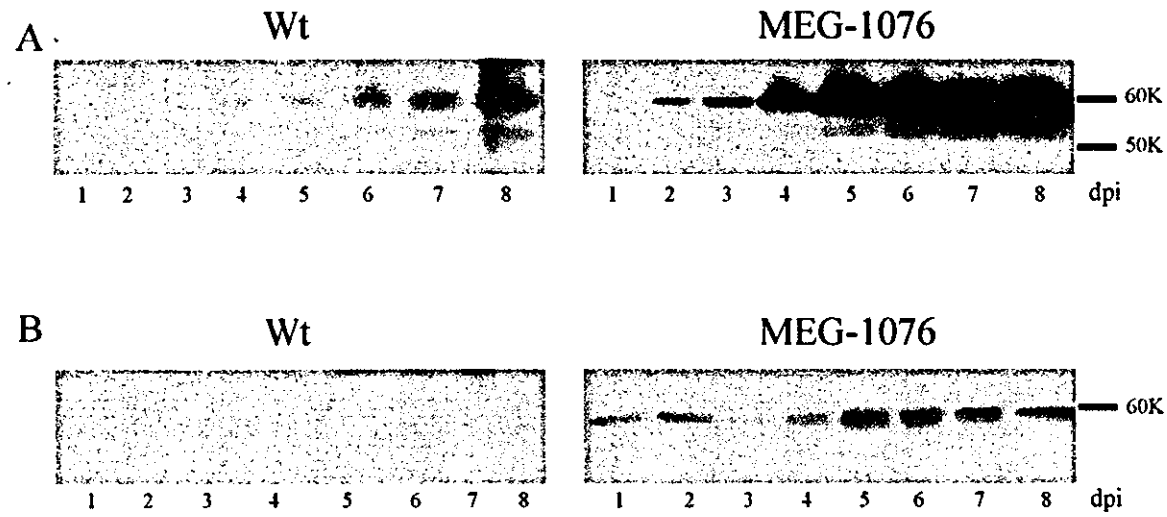

Figure 3
Western blot analysis of Wt and MEG-1076 rVP1. Confluent Tn5 cells were infected with Mc114 recombinant baculoviruses at MOI of 14.5 and incubated at 26°C. The culture medium, including the cells, were harvested 1, 2, 3, 4, 5, 6, 7, and 8 dpi as described in the materials and methods. (A) The cell culture medium was concentrated by ultracentrifugation, resuspended in 20 μl of Grace's medium, and 5 μl was mixed with loading dye and loaded into each well. (B) The cell lysate was separated from the culture medium, resuspended in 200 μl of Grace's medium, and 5 μl was mixed with loading dye and loaded into each well.

thin band of 60 K was detected at every dpi in the cell lysate for the MEG-1076 construct (Fig. 3B), however the intensity of this band did not increase to the same extent as the MEG-1076 60 K band in the culture medium (Fig. 3A). This suggested that immediately after translation the majority of rVP1 was rapidly exported from the cells to the culture medium, though a fraction accumulated within the cells. This may also explain why no 60 K bands were detected in the cell lysate for Wt construct.

The VP2 amino acid sequence was the same in all constructs. We did not detect rVP2 during the time-course expression of the MQG-1076 construct using the antiserum raised against *E. coli* expressed VP2 (data not shown).

Antigen ELISA and EM analysis of Wt and MEG-1076 VLPs
An antigen ELISA system was used to compare the yields of Wt and MEG-1076 VLPs at 1, 2, 3, 4, 5, 6, 7, and 8 dpi. The ELISA incorporated hyperimmune rabbit (capture) and guinea pig (detector) antisera raised against purified Mc114 VLPs [4]. The ELISA first detected VLPs at 2 and 3 dpi for MEG-1076 and Wt constructs, respectively (Fig. 4). For both constructs, the yields of VLPs increased each day thereafter, however the MEG-1076 construct had increased yields of VLPs than those of the Wt construct at 4, 5, 6, 7, and 8 dpi, approximately 6-fold increase. EM was used to verify the VLP formation of each of these constructs. We first detected VLPs at 4 dpi in the culture medium for both constructs and the numbers of VLPs increased each day thereafter (data not shown).

Amino acid analysis
The MEG-1076 construct contained a nucleotide point mutation in which resulted in an amino acid substitution at position 358 in VP1. We aligned 21 different VP1 amino acid sequences of SaV GI, GII, and GV strains and found this residue was strictly conserved, but more importantly, there was a strictly conserved amino acid motif at this site, NGDV (data not shown). However, when we included a porcine SaV GIII strain and a recently identified SaV GIV strain (PEC and Hou-7, respectively), only the GD site was strictly conserved, though several other

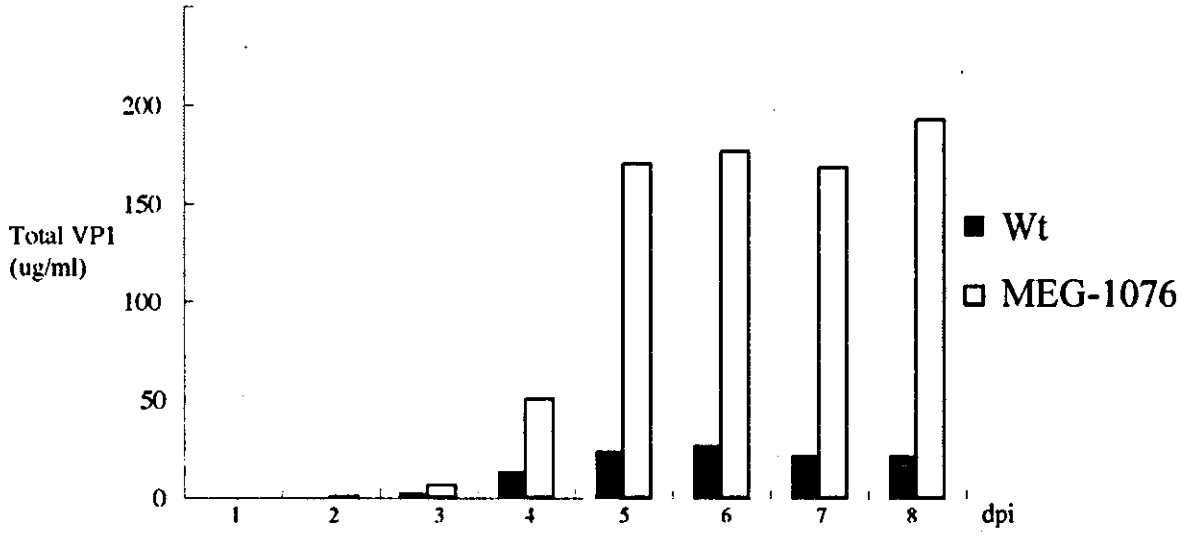

Figure 4
Antigen ELISA analysis of Wt and MEG-1076 VLPs. The ELISA used hyperimmune rabbit (capture) and guinea pig (detector) antiserum raised against Mc114 VLPs. For the antigen ELISA, purified Mc114 VLPs were used as the positive control at concentrations ranging from 500 ng to 0.24 ng.

amino acids nearby were also strictly conserved (Fig. 5). Further analysis of other SaV GIV strains are clearly needed in order to examine the possibility that the NGDV motif was moderately conserved in other human SaV strains. Figure 5 also showed that the predicted SaV P2 domain had very few conserved amino acid residues. Apart from the strictly conserved GD motif, the only other strictly conserved motif in the P2 domain was at the 5' end.

Discussion

Expression of the human SaV rVP1 in a baculovirus expression system was first reported in 1997 [9]. In that study, the full-length VP1 gene, ORF2, and poly(A) sequences were included in a construct (Sapporo strain, GI). The second human SaV reported to form VLPs was with a construct (Houston/90 strain, GI) using only the VP1 sequence, i.e., lacking ORF2 and poly(A) sequences [10], while the third human SaV reported to form VLPs used a construct (Parkville strain, GI) with only VP1 and ORF2 sequences, i.e., lacking poly(A) sequence [7]. We recently expressed human SaV GI, GII, and GV rVP1 with constructs (Mc14, C12, and NK24 strains, respectively) that included ORF2 and poly(A) sequences [4]. Additional information on human SaV rVP1 expression is lacking, although it appeared that the yields of human SaV VLPs were typically low for these three genogroups.

In this study, we compared the time-course expression of two different Mc114 SaV rVP1 constructs in a baculovirus expression system (Fig. 1). The MEG-1076 construct had two nucleotide point mutations, one in the VP1 gene in which resulted in an amino acid substitution, and one in the VP2 gene in which was silent. Although both constructs formed VLPs morphological similar to native SaV, the levels of transcription, translation, and VLP formation were clearly different. As shown in Figure 2B, the MEG-1076 rVP1 mRNA had increased steady-state levels and greater stability when compared to those of the Wt rVP1 mRNA. This difference was understood to be due to the nucleotide mutations in the MEG-1076 construct, since a similar result was observed in a NoV expression study [6]. Bertolotti-Ciarlet et al. found that a nucleotide point mutation in a NoV rVP1 construct (ORF2-*AUG* → *ACG*-ORF3+3' UTR construct, represented in bold) had decreased levels of rVP1 mRNA at 36 hours post-infection,

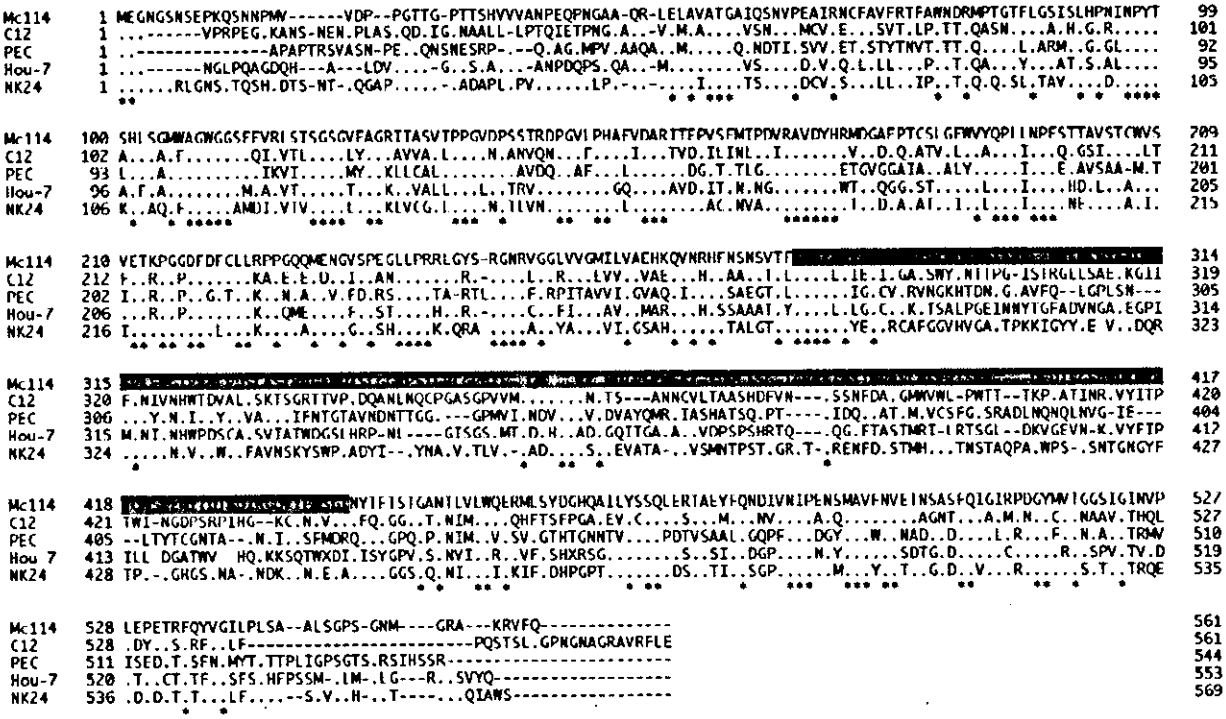

Figure 5
VP1 amino acid alignment of SaV GI, GII, GIII, GV, and GV strains. We originally aligned 21 SaV GI, GII, and GV sequences but to simplify the figure we used one representative strain from each genogroup. The green bar shows the SaV P2 domain predicted by Chen et al. [7]. The asterisks indicate conserved amino acids. We originally aligned 21 different VP1 amino acid sequences of SaV GI, GII, and GV strains and found the residue (N) at position 358 (yellow) was strictly conserved (data not shown), but SaV GIII and GIV strains (PEC and Hou-7, respectively) had other residues at this position. The alignment of the five SaV genogroups showed the amino acid motif, GD, was strictly conserved (red) and several other amino acids surrounding the residue at position 358 were also strictly conserved (red).

by approximately 50%, when compared to a construct without the mutation (ORF2+ORF3+3' UTR construct). Bertolotti-Ciarlet suggested that the RNA secondary structure or changes in the mRNA stability could be responsible for the different steady-state levels, but this was not proven.

Also, the MEG-1076 construct had increased levels of rVP1 expression and yields of VLPs in the culture medium when compared to those of the Wt construct (Fig. 3A). On the other hand, the concentration of rVP1 in the cell lysate remained more or less the same during the time-course expression for the MEG-1076 construct. And for the Wt construct, rVP1 was not detected in the cell lysate, although this may have been related to the low expression levels (Fig. 3B). Our results showed that the MEG-1076 construct had a 6-fold increase in yields of VLPs in the culture medium (Fig. 4), which corresponded to approximately 80 μg of CsCl purified VLPs from 200 ml of culture medium (at 6 dpi), but less than 5 μg of CsCl purified VLPs in the cell lysate (data not shown). These results suggested that either (i) immediately after translation the majority of rVP1 was exported from the cells to the culture medium where the majority of VLPs were folded but a fraction were simultaneously folded within the cells or (ii) VLPs were folded within the cells and then the majority of VLPs were immediately exported from the cells to the culture medium, though a fraction remained within the cells.

In a recent NoV expression study, a single amino acid substitution in the rVP1 gene affected VLP formation but not rVP1 expression [5]. In that study, a (native) histidine residue at position 91 (relative to NoV Snow Mountain Virus strain amino acid VP1 sequence) was found to be essential for VLP formation and a construct with a substituted (mutant) arginine residue at this position failed to form

VLPs despite expressing rVP1. Interestingly, that study found a single amino substitution was critical for the formation of VLPs, whereas our results showed that a single amino acid substitution was beneficial, i.e., increased the yields of VLPs. Bertolotti-Ciarlet found that inclusions of NoV ORF3 and poly(A) sequences in a construct increased the expression levels of NoV rVP1 and the stability of VLPs when compared to constructs without these sequences; and suggested that expression of other caliciviruses (NoV and SaV) rVP1 that resulted in low yields or unstable VLPs may be due to constructs that lacked the VP2 gene [6]. An alternative explanation was that point mutations influenced steady-state levels of mRNA and stability, which in turn influenced VLP formation. In our case, one or two nucleotide point mutations caused an enhancement of transcription, leading to increased yields of SaV VLPs in insect cells. Furthermore, many of these studies that expressed calicivirus rVP1 in insect cells only examined rVP1 expression and yields of VLPs but not rVP1 mRNA transcription [11-14]. However, another reason for the increased yields of VLPs may be associated with adaptation of SaV rVP1 to the baculovirus expression system and insect cells, since a similar result was observed with porcine enteric calicivirus in primary kidney cells [15].

Although the growth rate and replication efficiency of the recombinant baculoviruses themselves and differences in the levels of virus replication might account for such variation, we observed similar results using other MOIs, that is, the MEG-1076 construct continued to express greater yields of VLPs than the Wt construct (data not shown). Another explanation may have been differences in the extents to which these baculoviruses induce apoptosis and all these may result from features in the baculovirus skeleton rather than from the inserted SaV sequence. Such effects might for instance affect the number of adherent cells harvested or the degradation rates of both proteins and RNAs. However, we found that the MQG-1076 construct, developed from a separate experiment, had similar expression levels to that of the MEG-1076 construct (data not shown), which may eliminate the possibility that the baculovirus skeleton played a role in the increased yields of VLPs. On the other hand, we could not demonstrate whether the nucleotide mutations in VP1 and/or in ORF2 affected the transcription, a construct with only one of these mutations would be needed. Nevertheless, our results indicate that translation was exclusively affected by the single amino acid substitution in VP1. Therefore, the final increase in yields of VLPs may have been coupled at multiple levels, involving one or both of the nucleotide mutations in VP1 and VP2.

We did not detect rVP2 during the time-course expression of the MQG-1076 construct (data not shown). The Wt and MEG-1076 constructs had an identical amino acid sequence, which would suggest a similar negative-result. NoV studies have found that inclusion of VP2 increases the stability of VLPs, though the expression level of NoV rVP2 was low [6]. These results may suggest that (i) SaV rVP2 was expressed at undetectable levels, (ii) SaV rVP2 was not expressed in the insect cells, or (iii) SaV rVP2 was degraded in the insect cells. The SaV GI, GIV, and GV genomes are each predicted to encode a third ORF (ORF3) overlapping the VP1 gene, whereas SaV GII and GIII have only two ORFs. The functions of SaV ORF2 and ORF3 still remain unknown.

The amino acid substitution (N → S) for the MEG-1076 construct occurred in the VP1 gene at residue 358. This asparagine residue was recently identified as a moderately conserved residue among the caliciviruses capsid proteins [7], but more importantly, the residue was strictly conserved among 21 different SaV GI, GII, and GV strains and belonged to a strictly conserved amino acid motif, NGDV (Fig. 5). However, when we included SaV GIII and GIV strains (PEC and Hou-7, respectively) we found that only the GD amino acids were strictly conserved though several other amino acids nearby were also strictly conserved (Fig. 5). These data further suggested that this site played an important role in the regulation of SaV VLP formation.

Recently, the cryo-EM analysis of SaV was determined and compared to NoV X-ray crystallography structure [7]. Chen et al. analysed 30 different VP1 amino acid sequences of calicivirus strains belonging to the four genera in the family *Caliciviridae* and identified strictly and moderately conserved residues, and predicted the P1 and P2 domains of SaV VP1 based on NoV X-ray crystallography structure. Based on these predictions, the residue at position 358 (amino acid sequence) was found as a moderately conserved residue among the caliciviruses. This arginine residue was predicated to be in the P2 domain, which is defined as the outer most protruding domain for NoV and thought to provide strain diversity [16]. Further high-resolution structural analysis of SaV VLPs is clearly needed in order to determine the precise domains and regions of SaV. However, our expression results have indicated that only approximately 80 μg of purified VLPs from 200 ml of culture medium was possible (data not shown), thus in order to determine the X-ray crystallography structure of SaV, a minimum increase in expression level of about 20-fold would be required: a challenging feat.

Materials and methods

Virus strain, RNA extraction, cDNA synthesis

SaV GI Mc114 strain (GenBank accession number, AY237422) was isolated from a male infant seven months of age from the McCormic Hospital, Chiang Mai, Thailand on the 7th May 2001 [17]. RNA extraction and cDNA synthesis were performed as previously described [18].

PCR and sequencing

Our initial SaV rVP1 construct (MQG-1076 construct) was amplified with ExTaq DNA polymerase. However, this construct was later found to have two nucleotide point mutations in ORF1 at positions 4 (GAG → CAG) and 1076 (AAT → AGT) and one nucleotide point mutation in ORF2 at position 1895 (GTG → GTA) (relative to the VP1 start and represented in bold). Primer and PCR errors likely introduced these mutations. These three nucleotide point mutations resulted in two amino acid substitutions in the VP1 gene, one at the second residue, where glutamic acid (E) → glutamine (Q), and one at residue 358, where asparagine (N) → serine (S). The nucleotide point mutation in ORF2 did not result in an amino substitution. Despite the two amino acid substitutions, the MQG-1076 construct formed VLPs. We designed another construct (MEG-1076) using the pDEST8-MQG-1076 as template but with a new sense primer and used KOD-plus DNA polymerase according to the manufacture's instructions (Toyobo, Japan). The MEG-1076 construct had the same nucleotide point mutations at positions 1076 in VP1 and 1895 in VP2 as the MQG-1076 construct but not at nucleotide 4 in VP1 (Fig. 1). Lastly, we designed a third construct with the native sequence (Wt construct) using KOD-plus DNA polymerase and the original cDNA [4]. PCR-amplified fragments were cloned into the Gateway Expression System (Invitrogen, Carlsbad, Calif.) as previously described [4]. The insert sequences of the pDONR8 plasmids were confirmed, including the partial upstream and downstream sequences on the plasmids in which were found to be identical for the Wt and MEG-1076 constructs. Sequencing was performed as previously described [18].

Expression of rVP1 in insect cells

Recombinant bacmids were transfected into Sf9 cells (Riken Cell Bank, Japan) and the recombinant baculoviruses was collected as previously described [4]. The expression of the rVP1 constructs were analyzed by infecting recombinant baculoviruses at a MOI of 14.5 in 2.7 × 10^6 confluent Tn5 cells in 1.5 ml of Ex-Cell 405 medium followed by incubation at 26°C. The total culture medium was harvested 1, 2, 3, 4, 5, 6, 7, and 8 dpi. The culture medium was centrifuged for 10 min at 3,000 × *g*, and further centrifuged for 30 min at 10,000 × *g*. The VLPs in the culture medium were further concentrated by ultracentrifugation for 2 h at 45,000 rpm at 4°C (Beckman TLA-55 rotor), and then resuspended in 30 μl of Grace's medium. The cell lysate from the first centrifuge was resuspended in 200 μl of Grace's medium and stored at 4°C.

Northern blotting

Total RNA was prepared from the attached cells at 1, 2, 3, 4, 5, and 6 dpi with 1 ml of Isogen (Nippon Gene, Japan). For 7 and 8 dpi, the cell culture medium (containing unattached cells) was collected and centrifuged for 5 min at 3,000 × *g*, the supernatant removed, and then the cells were dissolved with 1 ml of Isogen. The cells were stored at -80°C. RNA was purified by a chloroform/ ethanol method (Nippon Gene, Japan). Briefly, RNA was mixed with chloroform, centrifuged at 12,000 × *g* for 15 min at 4°C, and the aqueous layer collected. This was repeated once, and then the aqueous layer collected and mixed with isopropanol and stored overnight at -20°C. The solution was mixed, centrifuged at 12,000 × *g* for 15 min at 4°C, and the supernatant discarded. The pellet was resuspended in 80% ethanol, centrifuged at 12,000 × *g* for 15 min at 4°C. This was repeated once, and then the pellet air-dried and resuspended in 25 μl of TE, and stored at -80°C. The amounts of purified RNA were determined spectrophotometrically (Bio-Rad, USA). The same amounts (500 ng) of total RNA were loaded for each construct and each dpi onto a 2% denaturing agarose gel containing formaldehyde. The amounts of total RNA were compared using SYBR Gold staining (Invitrogen, USA). RNA was transferred to a positively charged nylon transfer membrane (Hybond-N+; Amersham Biosciences, Ireland) under vacuum (VacuGene XL; Pharamacia LKB, Sweden) and analyzed by Northern blotting according to the DIG Northern Starter Kit (Roche, USA), except for a minor modification. Briefly, a RNA probe corresponding to Mc114 VP1 position 157 to 1283 (anti-VP1) was generated from a PCR fragment (native sequence) according to the manufacture's instructions (Roche, USA). Hybridization was performed overnight at 68°C with anti-VP1 in 10 ml of ultrasensitive hybridization buffer (Ambion, Canada). After hybridization, immunological detection was performed according to the manufacture's instructions (Roche, USA).

Western blotting, ELISA, EM, and protein sequencing

Western blotting, ELISA, and EM were used to examine rVP1 expression as previously described [4]. However, it should be acknowledged that the hyperimmune rabbit and guinea pig antisera were raised against the MQG-1076 VLPs. Protein sequences were determined by an Edman's degradation method.

Amino acid alignment

VP1 nucleotide sequences were translated using Genetyx software (software development Co. Version 11.2.2) and submitted to online ClustalW at DDBJ http://spiral.genes.nig.ac.jp/homology/welcome-e.shtml. In total, we aligned different 21 SaV GI, GII, GIII, GIV, and GV sequences, and included: Arg39, AY289803; Bristol, AJ249939; C12, AY603425; Cruise ship/00, AY289804; PEC, AF182760; Dresden, AY694184; Hou-7, AF435814; Houston/86/US, U95643; Houston/27/90/US, U95644; London/29845/92/UK, U95645; Lyon/598/97/F, AJ271056; Manchester, X86560; Mc2, AY237419; Mc10,

AY237420; Mex340/1990, AF435812; Mex14917/00, AF435813; NK24, AY646856; Parkville, U73124; Potsdam, AAG01042; Plymouth, X86559; Sapporo/82/Japan, U65427; and Sakaeo-15, AY646855.

Competing interests

The author(s) declare that they have no competing interests.

Authors' contributions

GH carried out the study and wrote the manuscript. KK, TO, KN, and NT participated in the design of the study and helped to draft the manuscript.

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