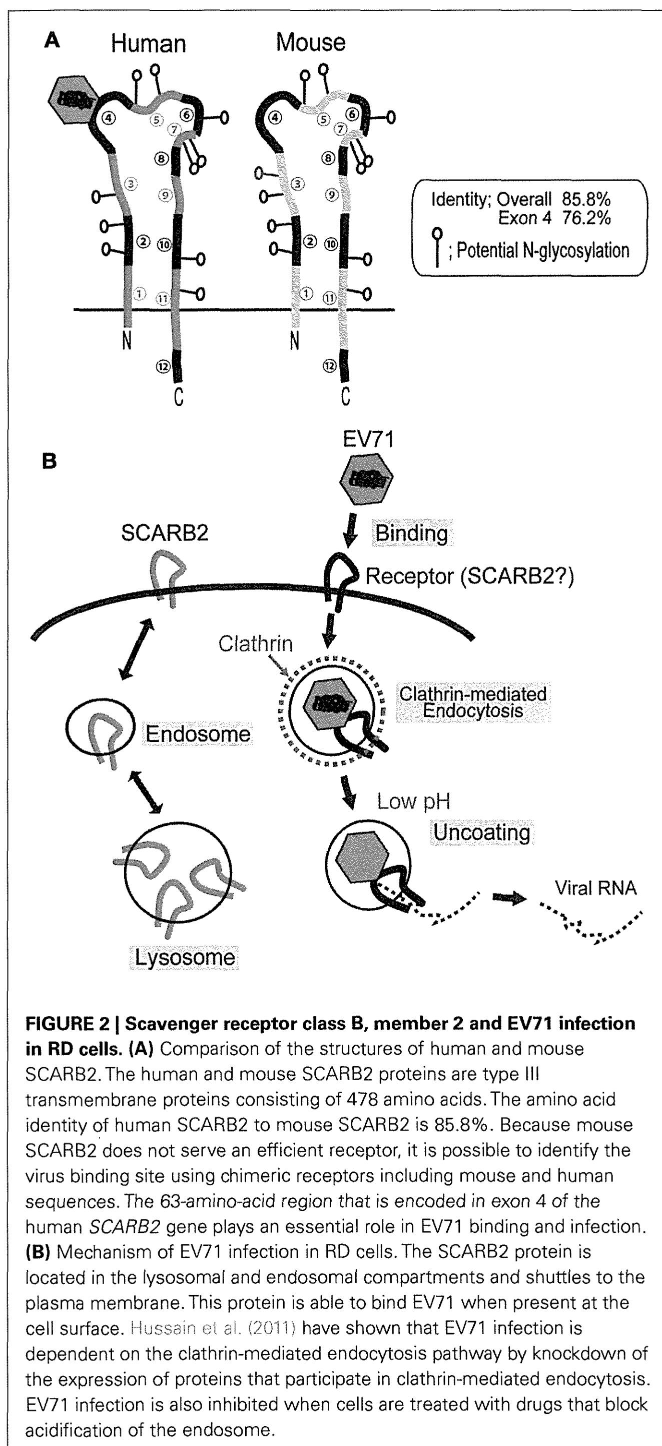

FIGURE 2 | Scavenger receptor class B, member 2 and EV71 infection in RD cells. (A) Comparison of the structures of human and mouse SCARB2. The human and mouse SCARB2 proteins are type III transmembrane proteins consisting of 478 amino acids. The amino acid identity of human SCARB2 to mouse SCARB2 is 85.8%. Because mouse SCARB2 does not serve an efficient receptor, it is possible to identify the virus binding site using chimeric receptors including mouse and human sequences. The 63-amino-acid region that is encoded in exon 4 of the human *SCARB2* gene plays an essential role in EV71 binding and infection. **(B)** Mechanism of EV71 infection in RD cells. The SCARB2 protein is located in the lysosomal and endosomal compartments and shuttles to the plasma membrane. This protein is able to bind EV71 when present at the cell surface. Hussain et al. (2011) have shown that EV71 infection is dependent on the clathrin-mediated endocytosis pathway by knockdown of the expression of proteins that participate in clathrin-mediated endocytosis. EV71 infection is also inhibited when cells are treated with drugs that block acidification of the endosome.

EV71 infection has been shown to be sensitive to the disruption of clathrin-mediated endocytosis and the acidic endosomal pH. Infection of RD cells with EV71 is largely blocked by anti-SCARB2 antibody or soluble SCARB2 (Yamayoshi et al., 2009), suggesting that the SCARB2-dependent pathway is the main pathway of EV71 infection in RD cells. SCARB2 is also known to be internalized by clathrin-dependent endocytosis (Le Borgne et al., 2001; Rodionov et al., 2002). The evidence has not directly shown that SCARB2 is involved in this pathway. The involvement of SCARB2 in this pathway should be confirmed experimentally.

It is known that the poliovirus receptor (CD155) and the major group rhinovirus receptor (intercellular adhesion molecule-1) are able to bind poliovirus and rhinovirus, respectively, and induce a conformational change that leads to the uncoating of the viral genome. Chen et al. (2012) reported that SCARB2, but not PSGL-1, induced a conformational change from native 160S virions to 135S particles. The change was enhanced under an acidic environment (pH 5.6; Chen et al., 2012). It should be further determined whether SCARB2 induces a conformational change to empty capsids and whether VP4 is released during SCARB2-mediated conformational change.

ANIMAL MODEL OF EV71 INFECTION

To study the neuropathogenicity of EV71, experiments using live animals are essential. The most reliable animal model is the monkey model (Hashimoto et al., 1978; Chumakov et al., 1979b; Hashimoto and Hagiwara, 1982; Hagiwara et al., 1983, 1984; Nagata et al., 2002, 2004; Arita et al., 2005, 2007; Zhang et al., 2011) because the species barrier caused by receptor differences is not a critical problem. The localization patterns of EV71-induced lesions in monkeys after intraspinal and intravenous inoculation were highly consistent with those observed in humans with severe EV71 encephalitis at autopsy (Lum et al., 1998; Wang et al., 1999; Chan et al., 2000; Shieh et al., 2001). Infected monkeys showed acute flaccid paralysis, which is a sign of involvement of the pyramidal tract, and tremor and ataxia, which are signs of involvement of the extrapyramidal tract. Histopathological changes were observed in the cerebellar and pontine vestibular nuclei, and in the spinal cord. Although the monkey is an excellent model for study of EV71 neuropathogenicity, monkey models have disadvantages with respect to handling, ethics, and cost. Experiments to identify the virulence determinants in the EV71 genome are limited to one report (Arita et al., 2005).

EV71, like other CVAs, is able to infect suckling mice. Some investigators use the suckling mouse model (Chumakov et al., 1979b; Chen et al., 2004; Ong et al., 2008). A problem with this system is that mice lose susceptibility to EV71 as they age (Yu et al., 2000; Chua et al., 2008). To circumvent this problem, some research groups have isolated mouse-adapted EV71 (Wang et al., 2004, 2011; Chua et al., 2008). Khong et al. (2012) found that 2-week-old mice deficient in type I and type II interferon receptors are susceptible to EV71 strains that were not artificially adapted to mice. Because mouse SCARB2 and PSGL-1 do not function as EV71 receptors in mice (Nishimura et al., 2009; Yamayoshi et al., 2009; Yamayoshi and Koike, 2011), EV71 infection in suckling mice is mediated by an unknown mechanism distinct from the SCARB2- and PSGL-1-mediated mechanisms. Indeed, EV71 exhibits different tissue tropism in suckling mice than in humans. In addition to infecting the CNS, EV71 replicates efficiently in the muscle of mice, unlike in humans (Chen et al., 2004; Wang et al., 2004; Chua et al., 2008; Ong et al., 2008).

In poliovirus, critical nucleotides or amino acids that influence the neurovirulence have been reported (Evans et al., 1985; Omata et al., 1986; Kawamura et al., 1989; Westrop et al., 1989). To identify such neurovirulence determinants in EV71, attempts were made

to determine nucleotide changes that influence the virulence level in suckling mice (Arita et al., 2008; Chua et al., 2008; Li et al., 2011; Huang et al., 2012). Some virulence determinants were mapped to the capsid region. Because the viral capsid is involved in the binding to the receptor and in other steps of infection, such as uncoating and stabilization of the virion, it is difficult to claim that amino acid changes in the capsid region are responsible for adaptation or virulence.

One of the strategies that can be used to overcome the problems discussed above is to generate transgenic mice that express the human EV71 receptor(s). Transgenic mice expressing human PVR, CD155, are susceptible to poliovirus and are used for the study of poliovirus pathogenicity (Ren et al., 1990; Koike et al., 1991; Horie et al., 1994; Abe et al., 1995). To this end, transgenic mice that express human PSGL-1 driven by the CMV promoter were generated (Liu et al., 2011). The expression of human PSGL-1 by this method was not sufficient to cause disease. It is obvious that the CMV promoter does not mimic the native PSGL-1 promoter in humans. Transgenic mice that express PSGL-1 with a distribution identical to that in human tissues are desirable. It seems that human SCARB2 expression in mice is necessary for the development of disease. A transgenic mouse model that develops disease that resembles the severe neurological diseases observed in humans will greatly contribute to the study of EV71 pathogenicity.

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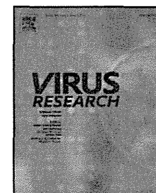

3CD, but not 3C, cleaves the VP1/2A site efficiently during Aichi virus polyprotein processing through interaction with 2A

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Polyprotein processing

ABSTRACT

Picornavirus genomes are translated into a single large polyprotein, which is processed by virus-encoded proteases into individual functional proteins. 3C of all picornaviruses is a protease, and the leader (L) and 2A proteins of some picornaviruses are also involved in polyprotein processing. Aichi virus (AiV), which is associated with acute gastroenteritis in humans, is a member of the genus *Kobuvirus* of the family *Picornaviridae*. The AiV L and 2A proteins have already been shown to exhibit no protease activity. In this study, we investigated AiV polyprotein processing by 3C and 3CD using a cell-free translation system. 3C and 3CD were capable of processing the polyprotein in *trans*; 3C, however, cleaved the VP1/2A site inefficiently, while 3CD cleaved this site almost completely. Mammalian two-hybrid and coimmunoprecipitation assays showed an interaction between 2A and 3CD. Using a 3CD mutant and various 2A mutants of substrate proteins, we showed a clear correlation between the 2A–3CD interaction and the VP1/2A cleavage by 3CD. Thus, this study suggests that tight interaction of 3CD with the 2A region of a precursor protein is required for efficient cleavage at the VP1/2A site.
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1. Introduction

Members of the family *Picornaviridae* infect mammals and birds. This family consists of 12 genera, and contains important human and animal pathogens, such as poliovirus, enteroviruses, human rhinoviruses, hepatitis A virus, and foot-and-mouth disease virus (FMDV). Picornaviruses are single-stranded, positive-sense RNA viruses. Their genomes are 7100–8500 nucleotides (nt) in length, and contain a single, long open reading frame encoding a polyprotein. The coding region is divided into a capsid-coding P1 region, and nonstructural protein-coding regions P2 and P3. After being translated, the polyprotein is cleaved by virus-encoded proteases into individual proteins. Some processing intermediates, such as 2BC, 3AB and 3CD, are known to have different functions from their final processing products.

Picornavirus genomes encode one or more proteases and a specific amino acid sequence around the cleavage sites involved in polyprotein processing (reviewed by Martínez-Salas and Ryan, 2010). 3C is a protease (Palmenberg et al., 1979; Hanecak et al., 1982; Klump et al., 1984), which is common to all picornaviruses, and cleaves most cleavage sites of the polyprotein. The leader (L) protein of aphtho- and erboviruses cleaves at its own C-terminus to release itself from the polyprotein (Strebel and Beck, 1986; Hinton et al., 2002). 2A of enteroviruses undergoes cleavage at its own N-terminus cotranslationally (Toyoda et al., 1986; Sommergruber et al., 1989). The NPG/P motif, which is present at the 2A/2B junction in many picornaviruses including aphtho-, cardio-, and erboviruses, and at the 2A1/2A2 junction of Ljungan virus (Johansson et al., 2002) and duck hepatitis virus type 1 (Ding and Zhang, 2007; Kim et al., 2006; Tseng et al., 2007), is required for "cleavage" through a mechanism different from the proteolytic reaction called ribosomal skip (Donnelly et al., 2001). In addition, 3CD, a precursor to the 3C protease and the 3D RNA-dependent RNA polymerase, functions as a protease, and is known to cleave certain cleavage sites more efficiently than 3C in poliovirus and FMDV (Jore et al., 1988; Ryan et al., 1989; Ypma-Wong et al., 1988).

Aichi virus (AiV) is a member of the genus *Kobuvirus* of the family *Picornaviridae* (Yamashita et al., 1998). This virus was first isolated from patients with gastroenteritis in Japan (Yamashita et al., 1991). AiV has been detected in other Asian countries, Brazil, Europe and Africa so far, and is thought to be a causative agent of gastroenteritis (Ambert-Balay et al., 2008; Goyer et al., 2008; Oh et al., 2006; Pham et al., 2007; Sdiri-Loulizi et al., 2008; Yamashita et al., 1995; Yang et al., 2009). L and 2A of AiV contain no protease motifs, and no NPG/P motif is present at the 2A/2B junction. In addition, when a mutation was introduced into the active motif (GXCG) of the 3C protease within the AiV polyprotein, the polyprotein was not processed (Sasaki and Taniguchi, 2008). These findings indicate that the proteases involved in AiV polyprotein processing are only 3C and the 3C-containing precursor protein. In this study, we examined AiV polyprotein processing by 3C and 3CD supplied in *trans* using a cell-free translation system. The results showed that 3C

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and 3CD could cleave all cleavage sites in the polyprotein, but 3CD cleaved the VP1/2A site much more efficiently than 3C. Mammalian two-hybrid and coimmunoprecipitation assays showed a strong interaction between 2A and 3CD. Furthermore, on analyses using a 3CD mutant and various 2A mutants of substrate proteins, we found a good correlation between the 2A–3CD interaction and the VP1/2A cleavage by 3CD. Thus, this study suggests that efficient cleavage at the VP1/2A site by 3CD requires tight binding of 3CD to the 2A region of the substrate protein.

2. Materials and methods

2.1. Plasmids

2.1.1. Full-length cDNA clones of AiV

A full-length cDNA clone of AiV (pAV-FL) and a replicon, in which the P1 region is replaced by the firefly luciferase (Luc) gene (pAV-FL-Luc-5′rzm), were described previously (Sasaki et al., 2001; Nagashima et al., 2003; Sasaki and Taniguchi, 2008). pAV-FL-Luc-5′rzm-3Cm, in which TG at nt 6492–6493 was mutated to GC to inactivate the 3C protease, was described previously (Sasaki and Taniguchi, 2008). A *Csp*45I–*Hin*dIII fragment containing nt 6480 to the poly(A) tail of pAV-FL was replaced by the corresponding fragment of pAV-FL-Luc-5′rzm-3Cm, generating pAV-FL-3Cm.

2.1.2. Plasmids for a mammalian two-hybrid assay

pACT-2A, pACT-3CDm, pBIND-2A and pBIND-3CDm were described previously (Ishikawa et al., 2010). The 2A-coding region containing the NC-AA mutation was amplified by PCR from pAV-FL-5′rzm-NC-AA (Sasaki and Taniguchi, 2008), and ligated to the pACT and pBIND plasmids (Promega) as described previously (Ishikawa et al., 2010), generating pACT-2A(NC-AA) and pBIND-2A(NC-AA). A mutation to change of HWAI (aa 27–30) to AWAA was introduced into pACT-2A by inverse PCR-mediated mutagenesis using primers 2A-2-Fw-normal (5′GCCCCCGACGGCAGTGCC) and 2A-AWAA-Rv (5′CACCTTGCGTGCGGCCCATGCGAC), then pACT-2A(HI-AA) and pBIND-2A(HI-AA) were constructed as described previously (Ishikawa et al., 2010).

2.1.3. pIRES plasmids

Various DNA fragments were amplified by PCR, digested with appropriate restriction enzymes, as summarized in Table 1, and then ligated to pAV-FLΔ741-8043, a cassette plasmid for AiV IRES-dependent protein expression in a cell-free translation system (Nagashima et al., 2008), generating pIRES-3C, pIRES-3Cm, pIRES-3CD, pIRES-3CDm, pIRES-VP1–2A, pIRES-Luc-2A, and pIRES-3CDΔ1-1-3Cm. To construct pIRES-3CD(QQ-AA), in which the C-terminal two amino acids of 3C, Gln-Gln, were changed to Ala-Ala, a BamHI–PstI fragment of pIRES-3CD was cloned into pUC118, and then the mutation was introduced into this generated plasmid by inverse PCR-mediated mutagenesis using primers 3D-P (5′TCTCTCATTGTTCCCACTGCTG) and 3C3′QQ-AA-M (5′TGCTGCGGTAGTGGCAAATTGAGTG). Then, the BamHI–PstI fragment containing the mutation was introduced into the corresponding sites of pIRES-3CD to yield pIRES-3CD(QQ-AA). pIRES-3CDΔ1-1(QQ-AA) was constructed as summarized in Table 1.

2.1.4. 2A mutants of pIRES-Luc and pAV-FL-Luc-5′rzm

A SacI–*Hin*dIII fragment of pIRES-Luc-2A was subcloned, and inverse PCR-mediated mutagenesis was performed using this subclone and the primers described above or previously (Ishikawa et al., 2010) to introduce the Δ2, Δ3, Δ4, and HI-AA mutations. Then, the SacI–MluI fragment containing the mutation was substituted for the corresponding fragment of pIRES-Luc-2A, generating pIRES-Luc-2AΔ2, -2AΔ3, -2AΔ4, and -2A(HI-AA). To construct pIRES-Luc-2A(NC-AA), a DNA fragment was amplified from pAV-FL-5′rzm-NC-AA using primers Sac-3772P (Nagashima et al., 2003) and Mlu-2A stop-M (Table 1), digested with SacI and MluI, and then ligated to the corresponding sites of pIRES-Luc-2A.

A SacI–XhoI fragment of pAV-FL-Luc-5′rzm was subcloned, and PCR-based mutagenesis was performed using the derived clone to introduce the Δ2, Δ3, Δ4 and 2A(HI-AA) mutations as described above. Then the SacI–MluI fragment containing the mutation was substituted for the corresponding fragment of pAV-FL-Luc-5′rzm, yielding pAV-FL-Luc-5′rzm-2AΔ2, -2AΔ3, -2AΔ4, and -2A(HI-AA). pAV-FL-Luc-5′rzm-2A(NC-AA) was described previously (Sasaki and Taniguchi, 2008).

2.2. Cell-free translation reaction

Plasmids were linearized by digestion with *Hin*dIII, and then subjected to *in vitro* transcription using a T7 RiboMAX Express Large Scale RNA Production System (Promega). The cell-free translation reaction was performed using Vero cell S10 extracts in the presence of L-[^{35}S]Met/Cys (EasyTag EXPRE^{35}S^{35}S Protein Labeling Mix; PerkinElmer) as described previously (Nagashima et al., 2005). Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), and radiolabeled proteins were detected using a BAS2000 imaging analyzer (Fujifilm).

Table 1
Construction of pIRES plasmids.

Clone	Primer	Sequence of primer	Template for PCR	Restriction enzyme used for cloning
pIRES-3C	Sal-Met-6066P	AAGTCGACGTCATGGGAATCTCCCCTGCTGTCCCC	pAV-FL-Luc-5′rzm	SalI–MluI
	Mlu-Stop-6635M	Described in Nagashima et al. (2008)		
pIRES-3Cm	Sal-Met-6066P	Described above	pAV-FL-Luc-5′rzm-3Cm	SalI–MluI
	Mlu-Stop-6635M	Described in Nagashima et al. (2008)		
pIRES-3CD	Sal-Met-6066P	AAGTCGACGTCATGGGAATCTCCCCTGCTGTCCCC	pAV-FL-Luc-5′rzm	SalI–*Hin*dIII
	M-13-20			
pIRES-3CDm	Sal-Met-6066P	Described above	pAV-FL-Luc-5′rzm-3Cm	SalI–*Hin*dIII
	M-13-20			
pIRES-VP1–2A	Sac-VP1-P	AAGAGCTCACCATGGCCCTCACCGAAGACCTCGAT	pAV-FL	SacI(blunt end)–MluI
	Mlu-2A Stop-M	AACGCGTTACTGTCGCCTGATGCCTGGGA		
pIRES-Luc-2A	Sal-ATG-Luc-P	AAAGTCGACGTCATGGAAGAGGCCAAAAACATAAAGAAAGGC	pAV-FL-Luc-5′rzm	SalI–MluI
	Mlu-2A Stop-M	AACGCGTTACTGTCGCCTGATGCCTGGGA		
pIRES-3CDΔ1-1-3Cm	Sal-Met-6066P	Described above	pAV-FL-Luc-5′rzm-3Cm	SalI-blunt end
	3D-5 RV	ATCTCAGCCTTTGTTTGCGGGGGTGAC		
pIRES-3CDΔ1-1(QQ-AA)	Sal-Met-6066P	Described above	pIRES-3CD(QQ-AA)	SalI-blunt end
	3D-5 RV	Described above		

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2.3. Mammalian two-hybrid analysis

The mammalian two-hybrid assay was carried out using a Checkmate kit (Promega) as described previously (Ishikawa et al., 2010). In this system, an interaction between a transcription activation domain-fused protein expressed from a pACT construct and a DNA-binding domain-fused protein expressed from a pBIND construct results in the transcription of a reporter firefly luciferase gene. pACT or one of its derivatives and pBIND or one of its derivatives were transfected into Vero cells together with pG5luc, a reporter plasmid. At 48 h after transfection, cell lysates were prepared and subjected to the luciferase assay. Transfection efficiency was normalized by the activity of *Renilla* luciferase, which was also expressed from pBIND. The higher of the values obtained with the combination of the pBIND construct and the empty pACT, and the combination of the pACT construct and the empty pBIND was used as a negative control.

2.4. Antiserum and antibody

Rabbit anti-3C antiserum was prepared by immunization with GST-tagged 3C expressed in *Escherichia coli*. Rabbit anti-luciferase antibody was purchased from Santa Cruz.

2.5. Immunoprecipitation

A cell-free translation reaction mixture (2 μl) was diluted with 200 μl of NP-40 buffer (50 mM Tris [pH 7.5], 200 mM NaCl, 1% NP-40), and then mixed with antibodies and Protein G Sepharose (Amersham). After incubation at 4 °C overnight, the Protein G Sepharose was washed five times with NP-40 buffer, and then radiolabeled immunoprecipitates were analyzed by SDS-PAGE, and then detected with a BAS2000.

3. Results

3.1. Polyprotein processing by 3C or 3CD

We previously demonstrated that an AiV polyprotein with a 3C protease-inactivating mutation is not processed (Sasaki and Taniguchi, 2008). This means that any viral protein other than 3C and its precursors is not involved in polyprotein processing. We examined polyprotein processing by 3C or 3CD supplied in *trans* using a cell-free translation system (Fig. 1A and B). The translation reaction was programmed with RNA to express a polyprotein (AV-FL) (Fig. 1B, lane 1), RNA to express the polyprotein with a protease-inactivating mutation (AV-FL-3Cm; lane 2), AV-FL-3Cm RNA together with RNA to express 3C (IRES-3C; lane 3), or AV-FL-3Cm RNA together with RNA to express 3CD (IRES-3CD; lane 4). The profile of polyprotein processing by 3CD supplied in *trans* was similar to that of the natural polyprotein (lanes 1 and 4). On the other hand, when the polyprotein was cleaved by 3C in *trans*, the amount of VP1 was reduced, and an approximately 40-kDa polypeptide was accumulated (lane 3), which was hardly observed on processing of the natural polyprotein and polyprotein processing by 3CD supplied in *trans*. The deduced molecular mass of VP1–2A is 41.4 kDa, and the electrophoretic mobility of this polypeptide corresponded to that of solely synthesized VP1–2A polypeptide (Fig. 1C, lanes 1 and 2). These results indicate that this protein is VP1–2A.

We examined whether inefficient cleavage at the N-terminus of 2A occurs also when the polyprotein in which the P1 region is replaced by the firefly luciferase gene is synthesized together with 3C (Fig. 2). Since the amino acid sequence of 2A contains no methionine and only one cysteine, 2A is not labeled with L-[^{35}S]Met/Cys efficiently and cannot be detected with this system (Sasaki and Taniguchi, 2008). Therefore, inhibition of cleavage at the N-terminus of 2A was monitored as the accumulation of Luc-2A and the decrease in the amount of Luc. On processing by 3C, the amount of Luc decreased and Luc-2A accumulated (Fig. 2B and C, lane 3), compared with processing by 3CD or 3CD(QQ-AA), a mutant that is not cleaved into 3C and 3D (lanes 4 and 5). These results indicate that 3C and 3CD can cleave all cleavage sites, but the VP1/2A site requires 3CD rather than 3C for efficient cleavage.

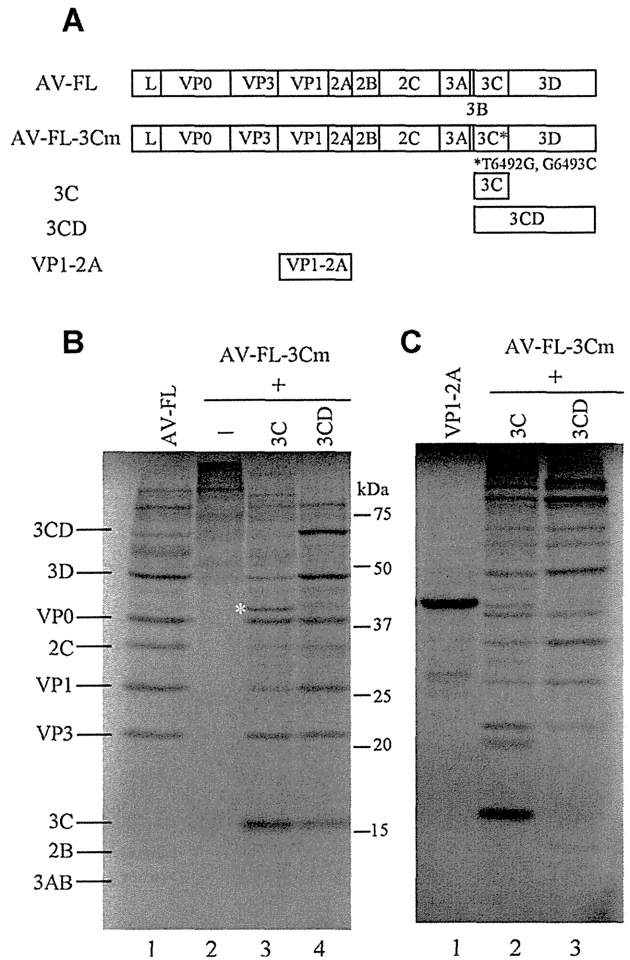

Fig. 1. Processing of the AiV polyprotein by 3C or 3CD supplied in *trans*. (A) Schematic diagram of polypeptides synthesized by cell-free translation. (B) and (C) Cell-free translation. Radiolabeled proteins were analyzed by SDS-PAGE. The asterisk indicates VP1–2A.

3.2. Effects of mutations in the 2A region on polyprotein processing by 3CD, and on the interaction between 2A and 3CD

We previously observed that some mutations introduced into the 2A region of a polyprotein affected cleavage at the VP1/2A junction during polyprotein processing (Sasaki and Taniguchi, 2008). In addition, in another study, we analyzed the interactions among the AiV nonstructural proteins by a mammalian two-hybrid assay, and showed that the 2A–3CD interaction was strongest among all protein pairs tested. On the other hand, 3C showed only weak binding ability to 2A (Ishikawa et al., 2010). Then, we attempted to investigate whether the binding ability of 3CD to 2A is related to cleavage by 3CD at the VP1/2A site by mutational analysis of 2A. First, five kinds of 2A mutations (Fig. 3A) were introduced into pAV-FL-Luc-5′rzm to examine the effects of the mutations on polyprotein processing. In addition, the binding ability of 2A mutants to 3CD was analyzed by the mammalian two-hybrid assay.

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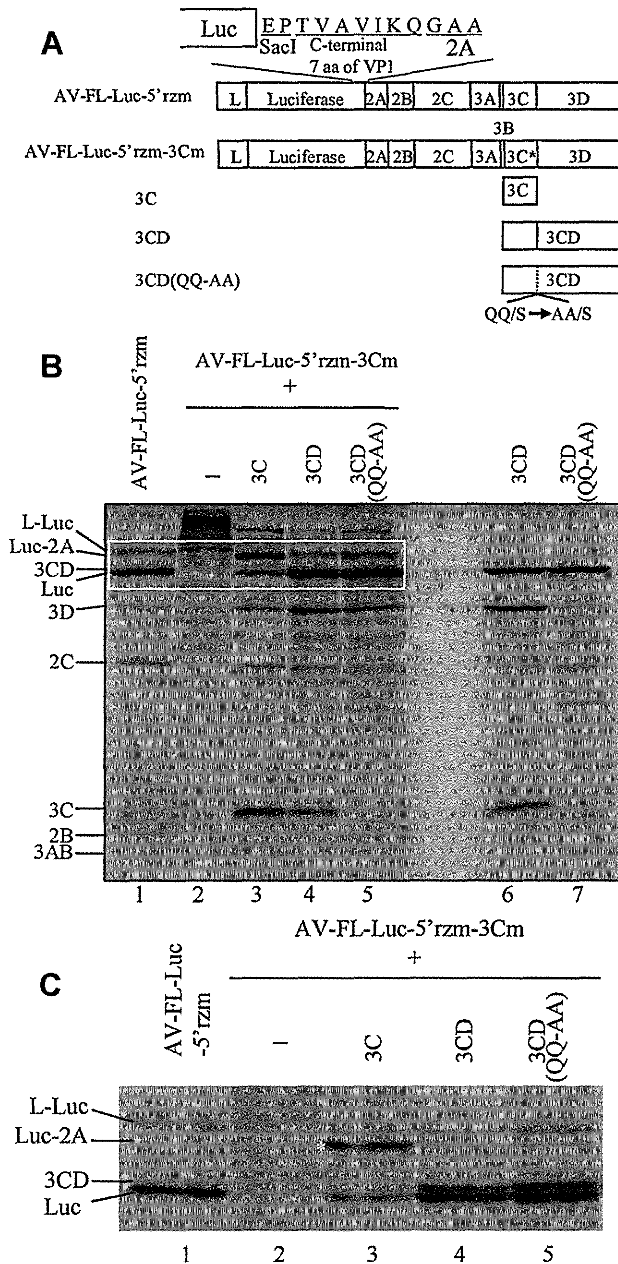

Fig. 2. Processing of the AiV polyprotein in which the P1 region was replaced by the luciferase gene by 3C or 3CD supplied in *trans*. (A) Schematic diagram of polypeptides synthesized by cell-free translation. The amino acid sequence between the luciferase gene (Luc) and 2A is shown. (B) and (C) Cell-free translation. Radiolabeled proteins were analyzed by (B) 12% or (C) 7.5% SDS-PAGE. Proteins boxed in (B) are shown in (C). The asterisk indicates Luc-2A.

The processing pattern for the mutant polyproteins is shown in Fig. 3B. Of the five mutants, the amount of Luc was decreased in the 2AΔ2, 2AΔ3 and 2A(HI-AA) mutants, and the accumulation of Luc-2AΔ2, Luc-2AΔ3 and Luc-2A(HI-AA) was observed (lanes 2, 3 and 5). (The identity of Luc-2AΔ2 and Luc-2AΔ3 was predicted by comparison with the electrophoretic mobilities of Luc-2AΔ2 and Luc-2AΔ3 synthesized alone [Fig. 4A].) These observations suggest that cleavage at the N-terminus of 2A is inhibited in the 2AΔ2, 2AΔ3 and 2A(HI-AA) mutants.

Next, the effects of the NC-AA and HI-AA mutations on the interaction with 3CD were examined by the mammalian two-hybrid assay. The ability to interact with 3CD was maintained in the NC-AA mutant, but lost in the HI-AA mutant (Fig. 3C). The previous study showed that 2AΔ2 and 2AΔ3 lost the ability to interact with 3CD, whereas 2AΔ4 maintained it (Ishikawa et al., 2010). Thus, the 2A mutations inhibiting cleavage at the N-terminus of 2A impaired the ability to interact with 3CD.

3.3. *Cleavage of Luc-2A or its mutants by 3CD, and the interaction between these proteins*

As a simpler substrate for examining cleavage at the VP1/2A site by 3CD, we constructed pIRES-Luc-2A. RNA transcribed from this plasmid expresses a Luc-2A polypeptide, which is composed of Luc followed by the two amino acids encoded by a SacI-recognition site, the C-terminal 7 amino acids of VP1, and 2A (Fig. 4A). When Luc-2A was synthesized together with 3CD(QQ-AA), the protein corresponding to Luc-2A disappeared and Luc appeared (lane 3), indicating efficient cleavage of Luc-2A at the N-terminus of 2A. The five kinds of 2A mutations shown in Fig. 3A were introduced into Luc-2A, and then cleavage by 3CD(QQ-AA) was examined (Fig. 4A). Consistent with the results of processing of the full-length polyprotein containing these mutations (Fig. 3B), cleavage at the N-terminus of 2A was inhibited in the 2AΔ2, 2AΔ3, and 2A(HI-AA) mutants (lanes 4, 5, and 7), but not in the 2AΔ4 and 2A(NC-AA) mutants (lanes 6 and 8).

To investigate the interaction of 3CD with these substrates, coimmunoprecipition analysis was performed (Fig. 4B). 3CD(3Cm), the protease-inactivated form of 3CD, was synthesized together with Luc-2A or its mutants by a cell-free translation reaction, and proteins were immunoprecipitated with anti-3C or anti-Luc antibody. Luc-2A, Luc-2AΔ4, and Luc-2A(NC-AA) (lanes 1, 4, and 6) were coimmunoprecipitated with 3CD(3Cm) more efficiently than Luc-2AΔ2, Luc-2AΔ3, and Luc-2A(HI-AA) (lanes 2, 3, and 5). This is consistent with the results of the mammalian two-hybrid assay. Thus, a good correlation was observed between cleavage at the N-terminus of 2A and the 2A–3CD interaction.

3.4. *Abilities of a 3CD mutant to process Luc-2A and the polyprotein and to interact with Luc-2A*

3CDΔ1-1, a 3CD mutant with deletion of the C-terminal 53 aa, was constructed (Fig. 5). First, the ability of 3CDΔ1-1 to cleave the N-terminus of 2A was investigated (Fig. 5A). 3CD(QQ-AA), 3CDΔ1-1(QQ-AA), or 3C was synthesized together with Luc-2A (lanes 4–6) or the polyprotein (lanes 7–9). During processing of the polyprotein by 3CDΔ1-1(QQ-AA) as well as 3C, Luc-2A was accumulated (lanes 8 and 9). In addition, 3CDΔ1-1(QQ-AA) and 3C cleaved Luc-2A less efficiently than 3CD(QQ-AA), as indicated on comparison of the amounts of Luc-2A (lanes 4–6).

Next, interaction with 2A was examined by coimmunoprecipitation analysis (Fig. 5B). The protease-inactivated form of 3CD, 3CDΔ1-1 or 3C was synthesized together with Luc-2A, and the proteins were immunoprecipitated with anti-3C or anti-Luc antibody. 3CD was coimmunoprecipitated efficiently with Luc-2A (lanes 4 and 7), but 3CDΔ1-1 and 3C were not (lanes 5, 6, 8, and 9). Thus, 3CD could interact and form a stable complex with the 2A-containing substrate, whereas 3CDΔ1-1, as well as 3C, lost the binding ability to 2A. Loss of the ability of 3CDΔ1-1 to interact with 2A was confirmed by the mammalian two-hybrid assay (data not shown). Thus, efficient cleavage at the N-terminus of 2A by 3CD was correlated with the interaction between 2A and 3CD, also in analyses using a 3CD mutant.

4. Discussion

During picornavirus polyprotein processing, the structural protein precursor is separated from the nonstructural protein

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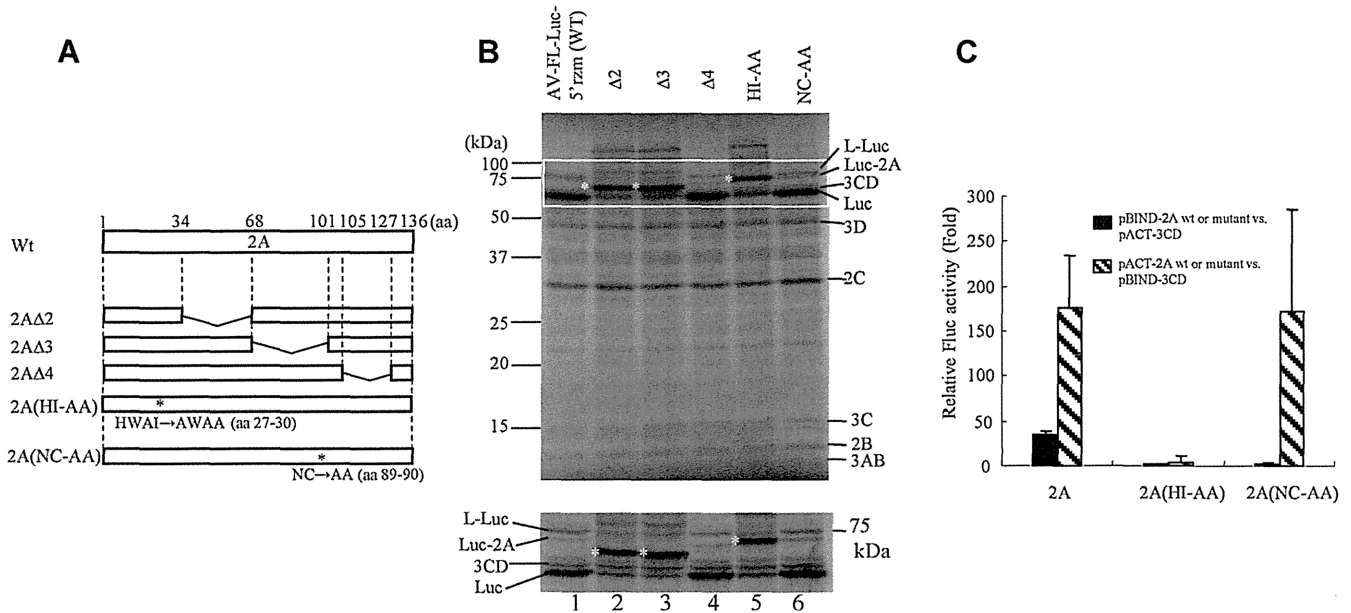

Fig. 3. Effects of mutations introduced into the 2A region on polyprotein processing and on the interaction with 3CD. (A) Schematic diagram of mutations introduced into the 2A-coding region. (B) Cell-free translation of AV-FL-Luc-5′rzm RNA and its 2A mutants. Radiolabeled proteins were analyzed by 12% (upper panel) or 7.5% (bottom panel) SDS-PAGE. Proteins boxed in the upper panel are shown in the bottom panel. The asterisks indicate Luc-2A with the 2A mutation. (C) Mammalian two-hybrid analysis of the interaction between 3CD and 2A or 2A mutants. The normalized firefly luciferase activity is represented as fold activation compared to a negative control. The experiment was repeated at least three times. Standard deviation bars are shown.

precursor with or without 2A cotranslationally or rapidly after translation. In spite of the fact that 3C is a protease that cleaves most sites of the polyprotein, cleavage at the N- or C-terminus of 2A is carried out through mechanisms other than 3C-mediated cleavage in many picornaviruses. In viruses belonging to many genera, a NPGP motif located at the C-terminus of 2A leads to cotranslational “cleavage” through a mechanism different from a proteolytic reaction (Donnelly et al., 2001). 2A of enteroviruses is a protease, and the N-terminus of 2A is cleaved by 2A itself cotranslationally (Toyoda et al., 1986; Sommergruber et al., 1989).

AiV 2A contains neither the protease motif nor the NPGP motif. It has been indicated that only 3C and a 3C-containing precursor protein are involved in polyprotein processing (Sasaki and Taniguchi, 2008). This study showed that 3C and 3CD could process the polyprotein, but only the VP1/2A cleavage site requires 3CD rather than 3C for efficient cleavage. Analyses using a 3CD mutant and substrates containing various 2A mutations showed a correlation between efficient VP1/2A cleavage by 3CD and binding of 3CD to 2A, suggesting that efficient VP1/2A cleavage is accomplished by stable interaction between 3CD and the 2A region of the substrate.

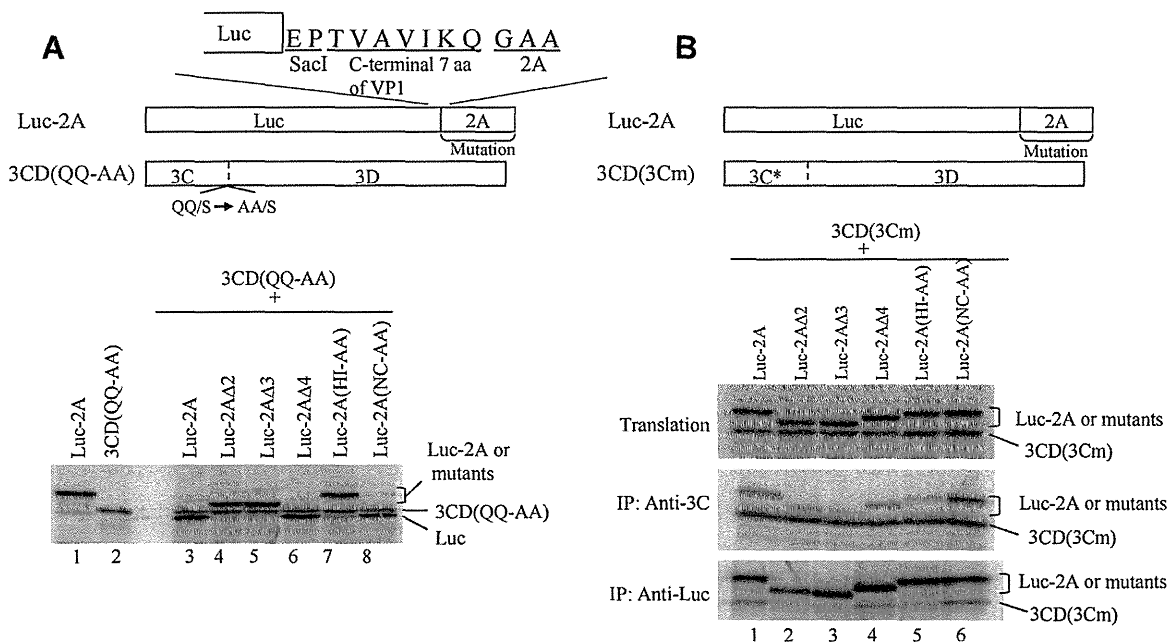

Fig. 4. Effects of mutations introduced into the 2A region of Luc-2A on cleavage at the N-terminus of 2A and on the interaction with 3CD. (A) Cell-free translation and cleavage of Luc-2A or its mutants with 3CD(QQ-AA). (B) Luc-2A or its mutants were cotranslated with 3CD(3Cm) in the cell-free translation system (upper panel), and then immunoprecipitation analysis was performed using anti-3C (middle panel) or anti-Luc (bottom panel) antibody.

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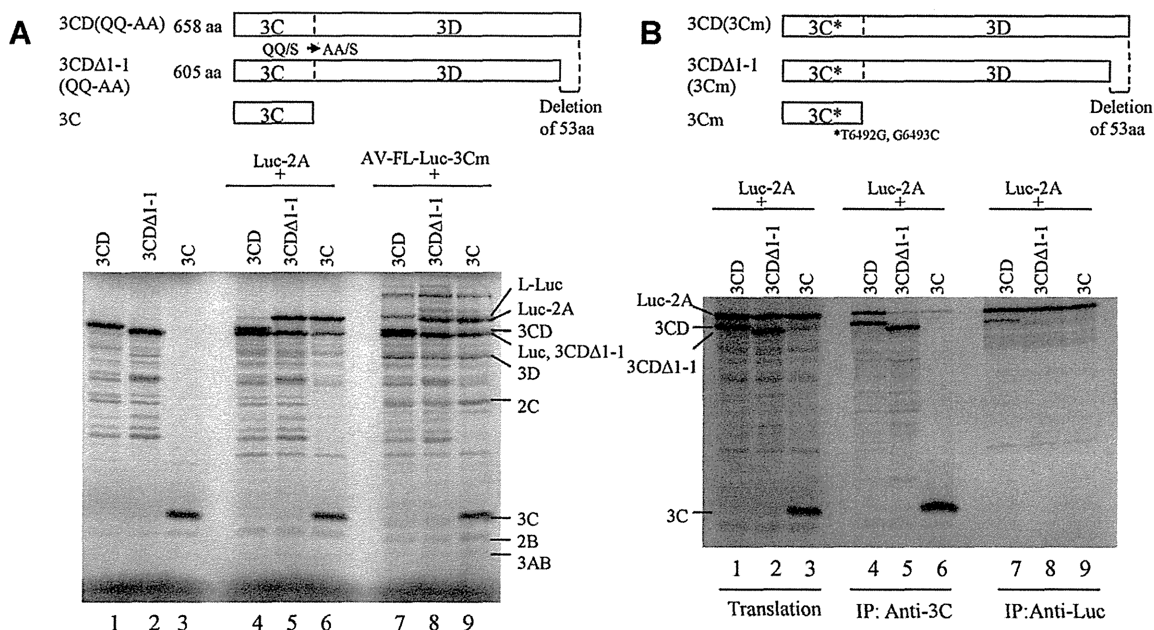

Fig. 5. Effects of a C-terminal 53-aa deletion in 3CD on cleavage at the N-terminus of 2A and on the interaction with 2A. (A) Cell-free translation and cleavage of Luc-2A or the polyprotein by 3CD(QQ-AA), 3CDΔ1-1(QQ-AA), or 3C. (B) Luc-2A was cotranslated with 3CD(3Cm), 3CDΔ1-1(3Cm), or 3Cm in the cell-free translation system, and then immunoprecipitation analysis was performed using anti-3C or anti-Luc antibody.

For 2A, two-amino acid mutation of aa 27 and 30 and deletion of aa 34–67 (2AΔ2) or aa 68–100 (2AΔ3) eliminated the interaction with 3CD. For 3CD, which is 658-aa long, the C-terminal 53-amino acid deletion abolished the 2A–3CD interaction. The mammalian two-hybrid analysis has already shown that 3C and 3D have only a weak binding ability to 2A (Ishikawa et al., 2010). The whole structures of 2A and 3CD, rather than the specific region of each protein, may be important for stable interaction between the two proteins.

It has been reported for other picornaviruses that certain cleavage sites are processed more efficiently by 3CD than 3C. For poliovirus, the P1 precursor protein is processed efficiently by 3CD but not by 3C (Jore et al., 1988; Ypma-Wong et al., 1988). For FMDV, 3CD processes the P1–2A precursor, particularly the VP1/2A cleavage site, more efficiently than 3C (Ryan et al., 1989). In both cases, the possibility that the 3D sequence is required for interaction with or for enhancing the affinity with the substrate is discussed (Ypma-Wong et al., 1988; Ryan et al., 1989); however, this possibility has not been confirmed experimentally.

Human parechovirus 2A, as well as AiV 2A, belongs to the H-rev107 family of proteins (Hughes and Stanway, 2000), and contains neither the proteolytic activity motif nor the NPG/P motif. It has been reported that the VP1–2A protein of parechovirus can be cleaved by 3C in *trans* very efficiently in a bacteria expression system. However, cleavage of P1–2ABC synthesized in a rabbit reticulocyte lysate by bacteria-expressed 3C results in the accumulation of a protein that migrates more slowly than the predicted molecular mass of P1, although it has not been determined whether this protein is P1–2A (Schultheiss et al., 1995). It is of interest to determine whether 3CD is required for efficient VP1/2A cleavage and processing of P1 in parechovirus, and, if so, whether interaction of 3CD with 2A or the P1 precursor protein is involved in cleavage at these sites.

The amino acid pair at the VP1/2A junction is Gln/Gly (Fig. 4A), the typical dipeptide recognized by picornavirus 3C. Indeed, 3C could cleave this site, albeit inefficiently. We assume that the active site of 3C may be inaccessible to the VP1/2A cleavage site in the polyprotein, for example, this cleavage site may not be located on the surface of the molecule. Binding of 3CD to the 2A region within the precursor protein will lead to a conformational change around the VP1/2A junction of the precursor. As a result, the VP1/2A cleavage site will be properly positioned and recognized by the 3C protease active site of 3CD. In our previous study, 3C and 3CD did not necessarily interact strongly with all nonstructural proteins (Ishikawa et al., 2010). This suggests that only the VP1/2A site requires a stable interaction with 3CD for efficient cleavage, and that other cleavage sites can be cleaved by 3C or 3CD without such an interaction.

It is possible that cleavage of the VP1/2A site by 3CD, not by 3C, is involved in temporal regulation to separate the structural protein precursor from the nonstructural protein precursor. In AiV, the L-P1 polypeptide was found during polyprotein processing, but P1–2A was an abnormal product (Sasaki and Taniguchi, 2008), suggesting that cleavage at the P1/2A site is a comparatively rapid reaction after polyprotein translation. The 3CD region of the intact polyprotein may interact with the 2A region, leading to primary and intramolecular cleavage of the polyprotein; however, it remains to be determined whether this site is indeed the primary cleavage site. Additionally, it is possible that the 2A–3CD interaction is maintained after processing of the polyprotein. The resultant 2A/3CD complex may play a role in viral RNA replication. AiV 2A has been shown to be required for viral RNA replication (Sasaki and Taniguchi, 2008). VP1/2A cleavage through the 2A–3CD interaction may facilitate efficient formation of the 2A/3CD complex. Further research is needed to clarify the significance of cleavage at the VP1/2A site by 3CD on AiV replication.

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MINIREVIEW

Saffold Virus, a Novel Human Cardiovirus with Unknown Pathogenicity

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Although cardioviruses have been thought to mainly infect rodents, a novel human cardiovirus, designated Saffold virus (SAFV), was identified in 2007. SAFV is grouped with Theiler-like rat virus and Theiler's murine encephalomyelitis virus (TMEV) in the species *Theilovirus* of the genus *Cardiovirus* of the family *Picornaviridae*. Eight genotypes of SAFV have now been identified. SAFV has been isolated from nasal and stool specimens from infants presenting with respiratory and gastrointestinal symptoms as well as from children with nonpolio acute flaccid paralysis; however, the relationship of SAFV to this symptomatology remains unclear. Of note, the virus has also been isolated from the cerebrospinal fluid specimens of patients with aseptic meningitis. This finding is of interest since TMEV is known to cause a multiple sclerosis-like syndrome in mice. The involvement of SAFV in various diseases (e.g., respiratory illness, gastrointestinal illness, neurological diseases, and type I diabetes) is presently under investigation. In order to clarify the pathogenicity of SAFV, additional epidemiological studies are required. Furthermore, identification of the SAFV cellular receptor will help establish an animal model for SAFV infection and help clarify the pathogenesis of SAFV-related diseases. In addition, investigation of the tissue-specific expression of the receptor may facilitate development of a novel picornavirus vector, which could be a useful tool in gene therapy for humans. The study of viral factors involved in viral pathogenicity using a reverse genetics technique will also be important.

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Members of the genus *Cardiovirus* are positive, single-stranded RNA viruses that belong to the family *Picornaviridae*. Cardioviruses have been thought to mainly infect rodents; however, the possible existence of authentic human cardioviruses has been debated over the years. In 2007, a novel human cardiovirus, designated Saffold virus (SAFV), was isolated from the stool sample of a girl presenting with a fever of unknown origin (31).

The nucleotide sequence of the SAFV isolate showed a strong similarity to that of Theiler-like rat virus (TRV), which had been previously isolated from rats in Japan (41). Phylogenetic analysis grouped SAFV with TRV, Theiler's murine encephalomyelitis virus (TMEV), and Vilyuisk human encephalomyelitis virus (VHEV) in the species *Theilovirus* (34). SAFV was subsequently isolated from nasal and stool specimens of infants presenting with respiratory or gastrointestinal symptoms and from children with nonpolio acute flaccid paralysis. The virus has also been identified in specimens from asymptomatic patients (1, 2, 13). Seroepidemiologic studies have shown that SAFV is a common and widespread virus that causes infection in early childhood (9, 66). In this review, we will discuss findings related to this novel human cardiovirus and focus on its potential pathogenicity for humans.

DISCOVERY OF A NOVEL HUMAN CARDIOVIRUS

In the1990s, virology textbooks noted that the *Cardiovirus* genus included two species: encephalomyocarditis virus (EMCV) and TMEV (55). The natural hosts for both species are mainly mice, although EMCV has been isolated from over 30 host species, including various mammals, birds, and invertebrates (65).

In 2003, a virus was isolated from sentinel rats exposed to cage bedding previously used by TMEV-seropositive adult rats in Japan (41). The nucleotide sequence showed a strong similarity to that of TMEV. Therefore, the virus was designated TRV.

For over 100 years, a form of human encephalomyelitis called Vilyuisk encephalitis (VE) has been known to affect the Yakut people who inhabit the Vilyuy Valley in Siberia (22, 35). Between 1954 and 1957, viral isolates thought to be linked to the disease were recovered from human clinical specimens. The virus isolates, VHEV, cross-reacted fully with TMEV and weakly with EMCV (6). It was unclear whether the virus was the human pathogen causing VE, a TMEV inadvertently recovered during isolation and passage in mice, or a recombinant between these two (49). A similar scenario occurred in the case of the isolation of hemagglutinating virus of Japan (HVJ), Sendai virus. HVJ was recovered from mice inoculated with an autopsy specimen from an infant with pneumonia, which was epidemic in Sendai in the early 1950s (7). The agent was later shown to be indigenous to mice, and therefore it remains unclear whether it is the pathogen that caused the pneumonia.

In 1981, an 8-month-old girl presented with a fever of unknown origin. A virus from a stool sample grew well in human fetal diploid kidney cells but failed to grow in primary monkey kidney, A-549, BSC, and RD cells. The virus also grew in suckling mice. Further characterization was carried out at the State of California Viral and Rickettsial Disease Laboratory. As determined by electron microscopy, the agent had a diameter of 28 to 30 nm and appeared to be a typical picornavirus. It was acid stable and temperature sensitive (with no growth at 33°C) (31). The virus was designated SAFV after the middle name of Morris Saffold Jones, the senior author of the corresponding report in 2007. A Saffold-like virus was subsequently isolated from a nasopharyngeal sample collected from a 23-month-old child in 2008 (1). These viruses were subsequently designated SAFV-1 and SAFV-2, respectively (34). SAFV-3 was later isolated from a stool sample obtained from

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a 13-month-old boy who presented with vomiting (66). Eight SAFV genotypes have now been identified (2, 3).

SAFV is a member of the *Cardiovirus* genus, which consists of two species: *Encephalomyelitis virus* and *Theilovirus.* The species *Theilovirus* includes SAFV as well as TMEV, TRV, and VHEV (34).

PROPERTIES AND GENOME STRUCTURE OF SAFV

Since SAFV belongs to the genus *Cardiovirus* of the family *Picornaviridae*, its properties and genome structure are similar to those of other cardioviruses. The genome is approximately 8,050-nucleotides (nt) long. The 5′ untranslated region (UTR) is approximately 1,040-nt long, with a type II internal ribosome entry site (IRES) similar to that of the murine cardioviruses, TMEV and EMCV (14, 38, 46, 47). The 3′ UTR is approximately 120-nt long with a poly(A) tract. The poly(A) tail of cardiovirus is reported to be short (35 nt) (44); however, a 3′-RACE experiment demonstrated that one of the clones derived from the JPN08-404 strain (HQ902242) had a 124-nt-long poly(A) tract (data not shown).

The genome of picornaviruses contains a higher-order RNA structure, the *cis*-acting replication element (CRE), used as a template for 3D polymerase-mediated VPg uridylation. In the case of the murine cardioviruses (TMEV and EMCV), CRE is located in the VP2-coding region and consists of a stem-loop structure (38). SAFV also contains a conserved sequence of CRE in the VP2-coding region (66).

The 2A protein of cardioviruses lacks protease activity. Instead, cardioviruses have an asparagine-proline-glycine-proline (NPG/P) amino acid sequence at the 2A-2B junction. This sequence is known to trigger polyprotein cleavage at the 2A-2B boundary via a nonproteolytic mechanism, called a ribosomal skip (36). The NPG/P amino acid sequence is conserved in the 2A-2B junction of SAFV (36).

L PROTEIN

Viruses in the *Aphthovirus*, *Cardiovirus*, *Erbovirus*, *Kobuvirus*, *Teschovirus*, and *Sapelovirus* genera of the *Picornaviridae* family contain an L-coding region at the most 5′ terminus of the open reading frame (ORF) (43). Only the L proteins of the aphthoviruses and cardioviruses have been studied in any detail.

In foot-and-mouth disease virus (FMDV), an aphthovirus, L protein is a papain-related thiol protease (23). FMDV L autocatalytically cleaves the viral polyprotein between its C terminus and the N terminus of VP4 (45, 58). It also cleaves the translation initiation factor eIF4G; the latter cleavage inhibits cellular translation, resulting in a shutoff of host protein synthesis (11).

In contrast to the L protein of aphthoviruses, the L protein of cardioviruses has a very different sequence that has an N-terminal zinc finger and a C-terminal acidic domain (38). Cardiovirus L is not a protease but plays a key role in the regulation of virus translation (15), the phosphorylation of nucleoporins (48), and the inhibition of transcription of alpha/beta interferon (IFN-α/β) (24). L protein of EMCV has been reported to have an antiapoptotic activity related to a mitochondrial-dependent pathway (52).

Special attention has been directed to the L protein of TMEV, a member of the *Theilovirus* species. TMEV L is a multifunctional protein that is important in neurovirulence, viral RNA encapsidation, anti-IFN activity, and viral persistence (29). In contrast to EMCV L protein, L protein of TMEV has a proapoptotic activity (16, 42) or an antiapoptotic activity, depending on the TMEV strain and the particular cell types (57). TMEV L is thought to be an important factor in inducing the pathology found after infection of the mouse. The special properties of TMEV L may be a result of the presence of an S/T-rich domain present in the C terminus, in addition to the zinc finger and acidic domains found in the case of EMCV (21, 29).

Of note, SAFV has a hybrid L protein with features of both the *Theilovirus* and EMCV species; SAFV has a partially deleted S/T-rich domain along with a zinc finger and an acidic domain (34). Studies of the function of SAFV L are likely to be important in order to clarify the virus's pathogenicity.

L* PROTEIN

Attenuated Theiler's original (TO) subgroup strains of TMEV have an alternative translation initiation site at nt 1079, just downstream from the authentic initiation site for the polyprotein at nt 1066. From this alternative translation initiation site, a small 17- to 18-kDa protein, referred to as L*, is synthesized out-of-frame with the polyprotein. The synthesis of L* is specific to the TO subgroup strains of TMEV because its initiating AUG is present only in these strains and not in neurovirulent GDVII subgroup strains (where the L* AUG is replaced by an ACG) (33, 39). Because L* protein is synthesized only in TO subgroup strains, it is thought to play a role as a determinant for viral persistence and demyelization, which are seen after infection of mice with TO subgroup strains. L* protein is also important for virus growth in macrophages and exhibits antiapoptotic activity in certain cell types (26, 27). Of note, SAFV lacks an AUG initiating codon at the position used by TMEV to translate L* protein; however, there is an ACG in this region. If a non-AUG-initiated start codon were used to synthesize SAFV L*, as has been reported in the case of TMEV (61), the presence of stop codons predict that the L* protein would be only 57 amino acids (SAFV-1, California/81) or 34 amino acids (SAFV-2, Can112051-06, and SAFV-3, JPN08-404) (28, 34) in length. At this time, it remains unclear whether an L* protein of SAFV is expressed and, if so, whether it has functional activity (2).

SAFV-RELATED VIRUSES

The *Theilovirus* species of cardioviruses includes TMEV, TRV, and VHEV. Among them, TMEV is the most extensively studied because of its unusual phenotype. TMEV is divided into two subgroups of strains. GDVII subgroup strains are highly virulent. Intracerebral and peripheral routes of inoculation cause an acute fatal polioencephalomyelitis in mice. Infected mice show progressive flaccid paralysis, and almost all infected mice die within 2 weeks. Neither virus persistence nor demyelination is observed in the few surviving mice (4, 29, 54). On the other hand, TO subgroup strains cause a milder encephalomyelitis 1 to 2 weeks postinoculation (p.i.). Mice recover and then develop a chronic, progressive demyelinating disease with spastic paralysis 1 to 2 months p.i. Since its pathological findings are reminiscent of multiple sclerosis (MS), TO subgroup strain-induced demyelinating disease serves as an excellent animal model for this disease.

An understanding of the mechanisms of TMEV persistence and demyelination remains incomplete. Infectious cDNAs constructed in the late 1980s to the early 1990s (5, 18, 37, 53, 59) have been used to prepare recombinant viruses from the GDVII and DA (or BeAn) strains in order to clarify the region(s) responsible for TMEV biological activities. Although the precise region(s) responsible for virus persistence and demyelination is unclear, several regions have been highlighted. The capsid proteins, especially

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VP1 and VP2, were found important for some of the biological activities (29). In addition to these structural proteins, the two nonstructural viral proteins, L and L*, play an important role in TMEV disease pathogenesis (4, 29, 54).

Investigation of TMEV indicates that the role(s) of the capsid proteins (VP1 to VP4) as well as the nonstructural proteins (L and L*) will need to be clarified to better understand the pathogenesis of SAFV-induced disease. An infectious SAFV cDNA which was recently constructed by Himeda et al. (28) will be a most useful tool for a reverse genetics study.

THE PATHOGENICITY OF SAFV IN HUMANS

The data that follow indicate that SAFV-1 is a worldwide infection that occurs early in life and involves the respiratory and gastrointestinal systems. SAFV-2 and SAFV-3 have been isolated in North and South America, Europe, and Asia (1, 2, 8, 9, 13, 31, 50, 51, 66), while SAFV genotypes 4 to 8 have been isolated in South Asia (2). Zoll et al. showed by virus neutralization studies that SAFV-3 infection occurs early in life (>75% seropositivity at 24 months) and that the seroprevalence reaches >90% in older children and adults in several countries in Europe, Africa, and Asia (66). Chiu et al. reported that 91% of U.S. adults carry antibodies to SAFV-2, of which 80% generate neutralizing antibodies (9).

In Japan between 2009 and 2010, 1,525 nasopharyngeal swab specimens were obtained from patients younger than 18 years with acute respiratory illness. SAFV-2 sequences were detected by nested reverse transcription (RT)-PCR in 3.5% of patients (30). In addition, SAFV-3 sequences were detected by nested RT-PCR in 1.4% of 423 nasopharyngeal swab specimens from patients with acute respiratory illness (60). Of note, however, Chiu et al. examined 719 respiratory specimens (89% from patients with acute respiratory illness) for SAFV by real-time quantitative RT-PCR (qRT-PCR) and found all were negative (8).

Screening of 751 stool specimens from 498 individuals in a gastrointestinal cohort found 1.2% of 498 individuals were positive for SAFV (genotypes 2 and 3). All positive stool specimens were from children (<2 years old) (8). Of note, SAFV was implicated as a cause of enteric disease in Minnesota in 2008 (19).

Attention has been directed to whether SAFV is a possible cause of central nervous system (CNS) disease, since the closely related TMEV causes a demyelinating CNS disease that resembles MS (29). The isolation of SAFV-3 (JPN08-404) from the cerebrospinal fluid (CSF) of a 9-year old boy with aseptic meningitis (28) is noteworthy and suggests that SAFV may have CNS tropism and pathogenicity. However, Chiu et al. examined 400 CSF specimens from patients with neurological diseases (aseptic meningitis, encephalitis, and 40 cases of MS) and found that all were negative for SAFV by qRT-PCR (8).

In summary, epidemiological studies have failed to provide a clear picture of the relationship between SAFV infection and actual disease in humans. Further studies that include a control group of healthy persons will help clarify this issue.

THE PATHOGENICITY OF SAFV IN EXPERIMENTAL ANIMALS

Animal experiments have been carried out in order to study the tropism and pathogenicity of SAFV. Hertzler et al. found that high doses of SAFV-2 intracerebrally inoculated into FVB/n mice produced paralysis with neuropathological changes consistent with acute encephalomyelitis, particularly in the limbic system (25). Of special note was the presence of inflammation in the spinal cord white matter. Sorgeloos et al. presented data describing the results of experiments involving intraperitoneal inoculation of SAFV-2 and SAFV-3 into 129/Sv mice. Both of these SAFVs infect the heart and the CNS; however, the major viral load was in the pancreas. SAFV-3 is more neurotropic than SAFV-2, and intracerebral inoculation of SAFV-3 into FVB/n mice caused acute encephalitis (56). These data suggest that SAFV is neurotropic in mice.

The finding that the major viral load following SAFV-2 and SAFV-3 intraperitoneal inoculation was in the pancreas is noteworthy. Several viruses in the *Picornaviridae* family, particularly Coxsackie B viruses (members of the *Human enterovirus B* species), have been implicated in the etiology of type I diabetes (17, 20, 32, 63, 64), a disease characterized by the destruction of insulin-producing β cells in the pancreas. In addition, EMCV is known to induce pancreatitis and type I diabetes in rodents. The fact that EMCV infection can also lead to encephalomyelitis, myocarditis, orchitis, and sialodacryoadenitis in rodents (12) and that it has been isolated from two febrile patients (10, 40) will help guide further investigations of the relationship of SAFV to disease. Furthermore, epidemiological studies will be important to investigate the relationship between SAFV and type I diabetes.

FUTURE PROSPECTS

The relationship of SAFV to disease in humans remains unclear. Data suggest that this virus may have diverse potential pathogenicity (e.g., respiratory illness, gastrointestinal illness, neurological diseases, and/or type I diabetes). In order to clarify the pathogenicity of SAFV, further epidemiological studies are needed. In addition, the establishment of an animal model of SAFV infection will be useful in investigating the pathogenesis(es) of SAFV-induced diseases. An infectious full-length cDNA clone of SAFV (28) will be a powerful tool in reverse genetic studies in order to identify viral factors involved in pathogenicity. The preparation of a chimeric virus expressing green fluorescent protein may make it possible to identify the receptor for SAFV infection, as has been carried out in the case of enterovirus 71 infection (62). The identification of the receptor for virus infection would enable the preparation of transgenic mice as a novel animal model for SAFV infection. Furthermore, a clarification of the tissue-specific expression pattern of the receptor in human tissues will facilitate studies on SAFV tropism and pathogenicity. If SAFV is not found to be pathogenic in humans, it may still be a useful tool in gene therapy as a novel picornavirus vector. Therefore, further studies to clarify the pathogenicity of SAFV are needed.

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ACBD3-mediated recruitment of PI4KB to picornavirus RNA replication sites

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Phosphatidylinositol 4-kinase IIIβ (PI4KB) is a host factor required for genome RNA replication of enteroviruses, small non-enveloped viruses belonging to the family *Picornaviridae*. Here, we demonstrated that PI4KB is also essential for genome replication of another picornavirus, Aichi virus (AiV), but is recruited to the genome replication sites by a different strategy from that utilized by enteroviruses. AiV non-structural proteins, 2B, 2BC, 2C, 3A, and 3AB, interacted with a Golgi protein, acyl-coenzyme A binding domain containing 3 (ACBD3). Furthermore, we identified previously unknown interaction between ACBD3 and PI4KB, which provides a novel manner of Golgi recruitment of PI4KB. Knockdown of ACBD3 or PI4KB suppressed AiV RNA replication. The viral proteins, ACBD3, PI4KB, and phophatidylinositol-4-phosphate (PI4P) localized to the viral RNA replication sites. AiV replication and recruitment of PI4KB to the RNA replication sites were not affected by brefeldin A, in contrast to those in enterovirus infection. These results indicate that a viral protein/ACBD3/PI4KB complex is formed to synthesize PI4P at the AiV RNA replication sites and plays an essential role in viral RNA replication.
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Introduction

All known positive-strand RNA viruses utilize intracellular membranes, such as the endoplasmic reticulum (ER), the ER-Golgi intermediate compartment, the Golgi, endosomes, or lysosomes, for genome replication. Virus infection results in remodelling of intracellular membranes, and replication complexes, where viral RNA is replicated, are formed associated with membranes. Virus-induced membrane structures are thought to increase the local concentrations of components required for replication, to provide a scaffold for anchoring the replication complexes, to prevent the activation of certain host defense mechanisms that can be triggered by dsRNA produced during virus RNA replication, and to provide certain lipids required for genome synthesis (reviewed in Miller and Krijnse-Locker, 2008).

The family *Picornaviridae* is a group of non-enveloped, single-stranded positive-sense RNA viruses. Picornaviruses include many important pathogens for humans and animals, such as poliovirus, enterovirus 71, rhinoviruses, hepatitis A virus (HAV), and foot-and-mouth disease virus (FMDV). Each genome is 7200–8500 nucleotides in length, and has a single large open reading frame (ORF) consisting of a capsid-coding P1 region, and non-structural protein-coding P2 and P3 regions. Some viruses encode a non-structural protein, leader (L) protein, upstream of the P1 region. After a large polyprotein has been translated from a single ORF, the polyprotein is processed by virus-encoded proteases into 11–12 final cleavage products. Of the non-structural proteins, 2B, 2C, and 3A, and the cleavage intermediates, 2BC and 3AB, are membrane-associated proteins (Towner *et al*, 1996; Teterina *et al*, 1997; Knox *et al*, 2005; Moffat *et al*, 2005; Krogerus *et al*, 2007), and have been reported to be involved in membrane reorganization (Cho *et al*, 1994; Aldabe *et al*, 1996; Egger *et al*, 2000; Suhy *et al*, 2000). In addition, cellular factors are thought to be required for membrane reorganization for picornavirus replication. Many studies have shown the involvement of Golgi-specific Brefeldin A (BFA) resistance factor 1 (GBF1) and ADP-rybosilation factor 1 (Arf1), which participate in the cellular secretory pathway, in the replication of enteroviruses such as poliovirus and coxsackievirus B3 (CVB3) (Belov *et al*, 2005, 2007, 2008; Lanke *et al*, 2009; Wessels *et al*, 2006a,b). Furthermore, the formation of membranous vesicles by enterovirus infection is also proposed to occur through COPII-mediated vesicle budding from the ER (Rust *et al*, 2001), or an autophagy-mediated process (Suhy *et al*, 2000; Jackson *et al*, 2005).

Recently, a model by which enteroviruses remodel membranes for viral RNA replication was proposed (Hsu *et al*, 2010). According to the model, enterovirus RNA replication begins at the Golgi/*trans*-Golgi network (TGN). Viral protein 3A anchored to membranes binds and modulates GBF1/Arf1 to enhance recruitment of phosphatidylinositol 4-kinase IIIβ (PI4KB) to the sites for viral RNA replication on the membranes, over COPI. PI4KB catalyses the production of phophatidylinositol-4-phosphate (PI4P). The produced PI4P binds to soluble viral 3D RNA polymerase and recruits it to the membranes to facilitate viral RNA synthesis. Enhanced recruitment of PI4KB on the membranes decreases anterograde transport and leads to the emergence of PI4P-enriched organelles for enteroviral RNA replication adjacent to the ER exit sites.

Aichi virus (AiV) is a member of the family *Picornaviridae* (Yamashita *et al*, 1998), and belongs to the genus *Kobuvirus*, a different genus from the genus *Enterovirus*, to which poliovirus and CVB3 belong. AiV was first isolated from patients with oyster-associated acute gastroenteritis in 1989 in Japan

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(Yamashita *et al*, 1991). The virus has been detected in gastroenteritis outbreaks or sporadic cases of diarrhoea not only in Japan, but also in other Asian countries, Brazil, Europe, and Africa, and is suggested to be a causative agent of gastroenteritis (Yamashita *et al*, 1995, 2000; Oh *et al*, 2006; Pham *et al*, 2007; Ambert-Balay *et al*, 2008; Goyer *et al*, 2008; Sdiri-Loulizi *et al*, 2008; Reuter *et al*, 2009; Yang *et al*, 2009). We have performed several studies to obtain an understanding of the mechanism of this virus replication, such as the characterization of some non-structural proteins and the *cis*-acting replication element at the 5′-terminus of the genome (Sasaki *et al*, 2003; Nagashima *et al*, 2005; Sasaki and Taniguchi, 2003, 2008; Ishikawa *et al*, 2010); however, the host factors involved in AiV replication remain to be elucidated.

In this study, we demonstrated that PI4KB is an essential host factor for RNA replication of AiV as in the case of enteroviruses. However, we found that AiV utilizes a different strategy to recruit PI4KB to the site of genome replication from that used by enteroviruses. We showed that AiV non-structural proteins, 2B, 2BC, 2C, 3A, and 3AB, interact with a Golgi resident protein, acyl-coenzyme A binding domain containing 3 (ACBD3). In addition, we found that ACBD3 interacts with PI4KB. No direct interaction between PI4KB and the viral proteins was detected. Knockdown of ACBD3 or PI4KB reduced virus RNA replication. Immunofluorescence microscopy revealed colocalization among the viral non-structural proteins, ACBD3, PI4KB, PI4P lipids, and dsRNA in viral RNA-replicating cells. Thus, these results indicate that a viral protein/ACBD3/PI4KB complex is formed to synthesize PI4P at the AiV RNA replication sites and plays an essential role in viral RNA replication.

Results

2B, 2BC, 2C, 3A, and 3AB interact with ACBD3

To identify host proteins involved in AiV genome replication, we searched for host proteins that interact with the AiV 2B, 2C, and 3A proteins by screening a HeLa cell cDNA library in the yeast two-hybrid system. As a result, ACBD3 was identified as a binding partner of 3A.

Using a mammalian two-hybrid system, all of the non-structural proteins including 3A were tested for binding to ACBD3. In this system, interaction between a transcription activation domain-fused protein expressed from a pACT construct and a DNA-binding domain-fused protein expressed from a pBIND construct results in transcription of the reporter firefly luciferase gene. Interaction between 3A and ACBD3 induced a 270-fold increase in luciferase activity compared with the negative control (Figure 1A). Interestingly, strong activation of luciferase expression (40- to 158-fold increase in luciferase activity) was observed also for 2B, 2BC, 2C, and 3AB.

To further confirm the interaction of these viral proteins with ACBD3, FLAG-tagged ACBD3 was co-expressed with HA-tagged L, 2B, 2BC, 2C, 3A, or 3AB in 293T cells, and then a co-immunoprecipitation assay was performed. Consistent with the results of the mammalian two-hybrid assay, 2B, 2BC, 2C, 3A, and 3AB were co-immunoprecipitated with ACBD3, but the L protein was not (Figure 1B).

Furthermore, to examine whether these viral proteins interact with ACBD3 directly, a maltose binding protein (MBP) pull-down assay was performed using glutathione S-transferase (GST)-fused ACBD3 (GST–ACBD3) and MBP-fused viral proteins (MBP–2B, MBP–2C, MBP–3A, and MBP–3AB) expressed in *Escherichia coli*. Expression of MBP-fused 2BC was not enough to use for this experiment. GST–ACBD3 was pulled down with the MBP-fused viral proteins, but not with MBP (Figure 1C).

These results indicate that 2B, 2BC, 2C, 3A, and 3AB have the ability to interact with ACBD3.

2B, 2BC, 2C, 3A, and 3AB interact with the C-terminal region of ACBD3

To determine the region in ACBD3 required for binding to the viral proteins, the ability of various deletion mutants of ACBD3 to interact with 2B, 2BC, 2C, 3A, or 3AB was investigated in the mammalian two-hybrid system (Figure 2A and B). For 2B, 2C, 3A, or 3AB, mut3 and mut5, both of which contain a C-terminal region (aa 327–528), maintained the ability to induce a greater than $\sim$15-fold increase in luciferase activity compared with negative controls. Also for 2BC, 7- and 4-fold increases in luciferase activity were detected with mut3 and mut5, respectively. On the other hand, mutants without the C-terminal region lacked the ability to enhance luciferase expression. These results suggest that the C-terminal region of ACBD3 is essential for binding to 2B, 2BC, 2C, 3A, and 3AB. Further detailed experiments to identify the binding domain to these viral proteins were carried out using a series of deletion mutants of mut3 (Figure 2C and D). For the five viral proteins, mut3Δ1 with the deletion of aa 328–373 enhanced luciferase expression to a similar level to mut3, but the other mutants did not. This suggests that aa 374–528 of ACBD3 are important for binding to the viral proteins.

ACBD3 colocalizes with 2B, 2C, 3A, and dsRNA in AiV RNA-transfected cells

To investigate whether ACBD3 colocalizes with viral proteins or replicating viral RNA, an AiV replicon RNA (AV-FL-Luc-5′rzm) containing a firefly luciferase gene was transfected into Vero cells by electroporation, and then an immunofluorescence assay was carried out. The replicating viral RNA was detected by staining double-stranded replicative intermediates and replicative forms using an antibody recognizing dsRNA in which the helix length is >40 bp, as carried out for other positive-stranded RNA viruses including picornavirus (Miller *et al*, 2006; Harwood *et al*, 2008; Knoops *et al*, 2008; Berger *et al*, 2009; DeWitte-Orr *et al*, 2009, Hyde *et al*, 2009). 2B, 2C, and 3A were detected as patchy clusters in the cytoplasm at 4 h after transfection (Figure 3A), and accumulated in the perinuclear region at 6 h (only the data for 3A are shown in Figure 3A). At 4 h, when viral RNA replicates actively (see Figure 5A or C), 2B, 2C, and 3A colocalized with dsRNA (Figure 3B). ACBD3, which also formed patchy clusters in the cytoplasm at 4 h after transfection, colocalized with 2B, 2C, 3A (Figure 3A), and dsRNA (Figure 3B). These results indicate that ACBD3, 2B, 2C, and 3A (and possibly also the precursor proteins 2BC and 3AB) are present in the viral RNA replication sites.

Knockdown of ACBD3 inhibits AiV RNA replication

To investigate whether ACBD3 is involved in AiV RNA replication, replication of AV-FL-Luc-5′rzm RNA was examined in Vero cells treated with small interfering RNA (siRNA) targeting ACBD3 or control siRNA. At 72 h after treatment

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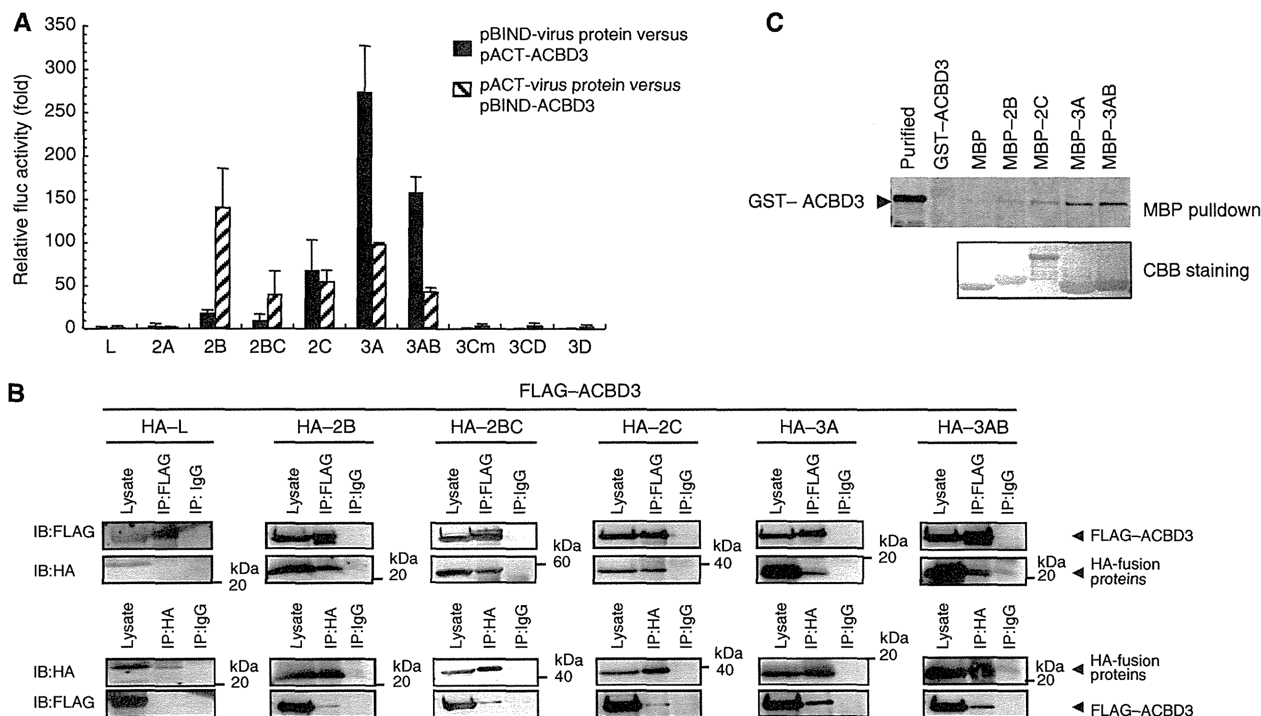

Figure 1 2B, 2BC, 2C, 3A, and 3AB interact with ACBD3. (**A**) The mammalian two-hybrid assay. The indicated combination of a pACT construct and a pBIND construct was transfected into Vero cells together with pG5luc encoding a firefly luciferase. Cell lysates were prepared at 48 h after transfection and assayed for firefly luciferase activity. Transfection efficiency was normalized by the activity of *Renilla* luciferase, which was simultaneously expressed from pBIND. The higher value of normalized luciferase activities obtained in cells transfected with the combination of the pBIND construct and empty pACT and with the combination of the pACT construct and empty pBIND was used as a negative control. The normalized firefly luciferase activity was represented as fold activation compared with a negative control. The experiment was repeated at least three times. Standard deviation bars are shown. (**B**) Co-immunoprecipitation of ACBD3 with 2B, 2BC, 2C, 3A, or 3AB. FLAG-tagged ACBD3 was co-expressed with HA-tagged L, 2B, 2BC, 2C, 3A, or 3AB. Proteins were immunoprecipitated with anti-FLAG (upper panel), anti-HA (lower panel) antibodies, or control IgG, and the resulting immunoprecipitates and whole cell lysates were analysed by immunoblotting with anti-FLAG and anti-HA antibodies. IB, immunoblotting; IP, immunoprecipitation. (**C**) MBP pull-down assay. MBP-fused viral proteins or MBP immobilized on amylose resin were mixed with purified GST–ACBD3, and proteins binding to the resin were analysed by SDS–PAGE, followed by immunoblotting with anti-GST antibody (upper panel). After immunoblotting, proteins on a PVDF membrane were stained with Coomassie brilliant blue to detect MBP-fused viral proteins or MBP (lower panel). Figure source data can be found in Supplementary data.

with siRNA targeting ACBD3, the amount of ACBD3, but not that of α-tubulin, was apparently decreased (Figure 3C). An effect of knockdown of ACBD3 on cell viability was not observed (data not shown). At 72 h after treatment with siRNA targeting ACBD3 or control siRNA, cells were transfected with AV-FL-Luc-5′rzm RNA by lipofection, and then cell lysates were prepared at various times after transfection and subjected to the luciferase assay. Knockdown of ACBD3 resulted in a decrease in luciferase activity. At 10 h after transfection, luciferase activity was decreased by ∼70% (Figure 3C). Here, Vero cells were used to exclude the effect of siRNA-induced interferon response, but a similar result was obtained using HeLa cells (data not shown). These results indicate that ACBD3 plays an important role in AiV RNA replication.

Localization of other Golgi proteins in AiV RNA-transfected cells

ACBD3 localizes to the Golgi through interaction with giantin, a Golgi protein, and the giantin-binding domain of ACBD3 has been mapped to the C-terminal region (aa 373–528) (Sohda *et al*, 2001). This region overlaps with the region important for binding to the AiV proteins (aa 374–528) (Figure 2). We then examined localization of giantin in viral RNA-replicating cells (Figure 4A). To distinguish RNA-transfected cells, a viral non-structural protein, the L protein, was immunostained. In mock-transfected cells, both ACBD3 and giantin were located in the Golgi. At 2 h after electroporation with viral RNA, the dispersion of ACBD3 and giantin was observed in some cells; however, the L protein could not be detected, because of its insufficient accumulation. At this time point, ACBD3 colocalized with giantin. As infection progressed, the redistribution of ACBD3 and giantin from the Golgi to the cytoplasm became more apparent. Importantly, at 4 h, giantin was dispersed throughout the cytoplasm, whereas ACBD3 formed clusters. As shown in Figure 3, ACBD3 colocalized with dsRNA or the viral proteins in such structures. At 4 h after electroporation, the intensity of immunofluorescence for giantin seems to be decreased compared with that in mock-transfected cells; however, no degradation of giantin was observed in an immunoblot analysis using anti-giantin antibody (data not shown).

We also examined the localization of other Golgi proteins, GM130, a *cis*-Golgi marker, and TGN46, a *trans*-Golgi marker. At 2 h after transfection with replicon RNA, dispersion of GM130 and TGN46 appears to begin like giantin, and they colocalized with ACBD3 (Figure 4B and C). GM130 was redistributed to form clusters in the cytoplasm at 4 h after transfection, but did not colocalize with ACBD3 (Figure 4B). TGN46 also became dispersed in the cytoplasm with the

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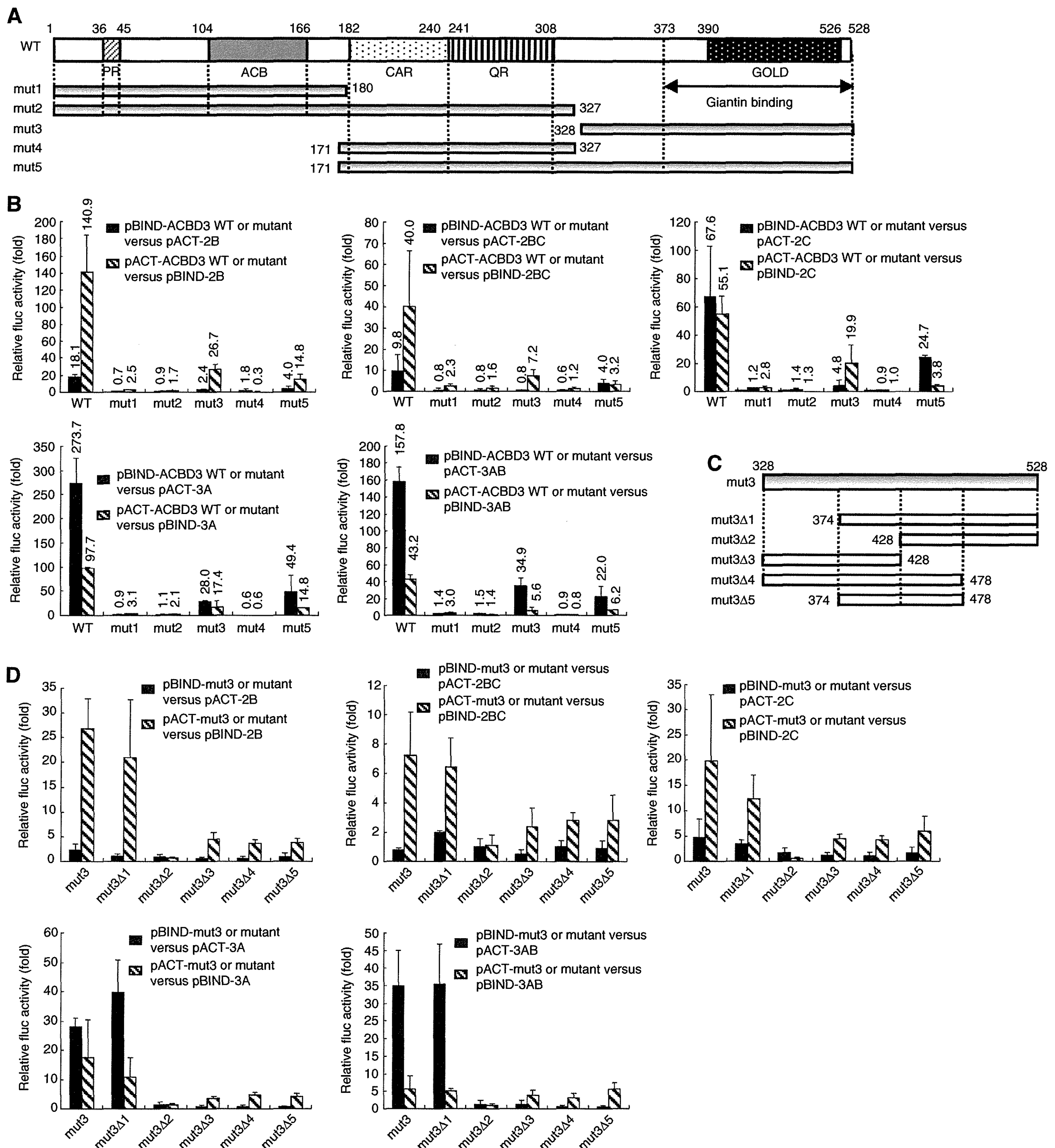

Figure 2 The C-terminal region of ACBD3 interacts with 2B, 2BC, 2C, 3A, and 3AB. (**A**, **C**) Schematic representation of (**A**) full-length ACBD3 (WT) and its mutants (mut1–mut5) and (**C**) mut3 (amino acids 328–528) and its mutants (mut3Δ1–Δ5). ACBD3 contains characteristic domains as follows: PR, proline-rich domain; ACB, ACB region; CAR, charged amino acid-rich domain; QR, glutamine-rich domain; and GOLD, Golgi dynamic domain. Numbers indicate amino-acid positions. The region between amino acids 373 and 528 is required for binding giantin. (**B**, **D**) Mammalian two-hybrid analyses were performed to examine interactions (**B**) between the ACBD3 mutants (mut1-5) and 2B, 2BC, 2C, 3A, or 3AB, and (**D**) between the mut3 mutants (mut3Δ1–Δ5) and 2B, 2BC, 2C, 3A, or 3AB, and the results are represented as described in Figure 1A. All experiments were repeated at least three times. Standard deviation bars are shown.

progression of infection, and did not colocalize with ACBD3 (Figure 4C). Thus, in viral RNA-replicating cells, other Golgi proteins examined did not colocalize with ACBD3.

AiV RNA replication requires PI4KB activity

It was recently reported that PI4KB is an essential host factor for enterovirus RNA replication (Hsu *et al*, 2010; Arita *et al*, 2011). We investigated whether PI4KB is also important for AiV replication. First, we examined the effect of a PI4KB-specific inhibitor, T-00127-HEV1 (Arita *et al*, 2011), on viral RNA replication. Vero cells were electroporated with AV-FL-Luc-5′rzm RNA, and then cultured in medium containing 0, 1, or 5 μM T-00127-HEV1. T-00127-HEV1 inhibited AiV RNA replication in a dose-dependent manner, and the replication

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