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Overall linkage map of the nonstructural proteins of Aichi virus

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ABSTRACT

Aichi virus (AiV), which is associated with acute gastroenteritis in humans, is a member of the genus *Kobuvirus* of the family *Picornaviridae*. Picornavirus genome replication occurs in replication complexes that include viral nonstructural proteins, host proteins and viral RNA. In poliovirus, all nonstructural proteins are found in the replication complexes, suggesting the ability of the viral nonstructural proteins to interact with each other. In this study, we examined the interactions between the AiV nonstructural proteins using a mammalian two-hybrid system. The results showed that all of the tested proteins could interact with more than one protein. We observed homodimerization of five proteins, bidirectional heterodimerization of six protein pairs, and unidirectional heterodimerization of eighteen protein pairs. Among the interactions detected in this study, the 2A–2B, 2A–2BC, 2A–2C, 2BC–3CD, 2BC–3C, 2C–3C, 2C–3CD and 3AB–3C interactions have not been observed in the previous two-hybrid studies with other picornaviruses. The strongest interaction was observed between 2A and 3CD. AiV 2A has already been shown to be involved in genome replication. Domain mapping of the 2A and 3CD interaction in mammalian two-hybrid analysis revealed that the C-terminal quarter of 2A is not required for the interaction with 3CD.

1. Introduction

Picornaviruses are small, nonenveloped, positive-sense, single-stranded RNA viruses. The family *Picornaviridae* is currently classified into eight genera and includes many important human and animal pathogens such as poliovirus (PV), hepatitis A virus, rhinovirus, and foot-and-mouth disease virus. The genome of picornaviruses is 7200–8500 nucleotides in length, and contains a 5′-terminal covalently linked protein, VPg, and a 3′-terminal poly(A) tail. The genome has a single large open reading frame (ORF), which consists of a capsid-coding region, P1, and nonstructural protein-coding regions, P2 and P3. The P1 region encodes VP4, VP2, VP3 and VP1, while the P2 and P3 regions encode 2A, 2B, 2C, 3A, 3B, 3C and 3D. Some viruses encode a leader (L) protein upstream of the P1 region. After virus proteins have been translated as a single large polyprotein from the ORF, the polyprotein is processed by virus-encoded proteases into various functional proteins. The cleavage intermediates, 2BC, 3AB and 3CD, have functions distinct from those of the mature cleavage products. Of the nonstructural proteins, 3B, 3C and 3D are functionally conserved among different genera of picornaviruses, i.e. VPg, protease and RNA-dependent RNA polymerase, respectively (Racaniello, 2006). For 2C, motifs characteristic of nucleoside triphosphate-binding proteins are highly conserved among picornaviruses (Gorbalenya et al., 1990; Racaniello, 2006). 2B, 2C, 2BC, 3A and 3AB have been identified as membrane-associated proteins in several genera (Knox et al., 2005; Krogerus et al., 2007; Teterina et al., 1997; Towner et al., 1996). In contrast, L and 2A vary in amino acid sequence and function among genera.

Aichi virus (AiV) is a member of the genus *Kobuvirus* of the family *Picornaviridae*. AiV was first isolated in 1989 from a stool specimen from a patient with oyster-associated gastroenteritis in Aichi, Japan (Yamashita et al., 1991). The virus has been detected not only in Japan, but also in other Asian countries, Europe, Brazil and Africa (Ambert-Balay et al., 2008; Goyer et al., 2008; Oh et al., 2006; Pham et al., 2007; Yamashita et al., 1995). The proteins encoded by AiV have some unique features. The capsid protein VP0 is present in mature viral particles devoid of cleavage into VP2 and VP4 (Yamashita et al., 1998). The L protein of AiV exhibits no homology to those of other picornaviruses (Yamashita et al., 1998), and has been indicated to be involved in viral RNA replication and encapsidation (Sasaki et al., 2003). AiV 2A, as well as 2A of human parechoviruses, is related to a cellular protein, H-rev107 (Hughes and Stanway, 2000). Recently, it was indicated that AiV 2A is not a protease and that it is involved in viral RNA replication (Sasaki and Taniguchi, 2008).

Picornavirus infection induces rearrangement of intracellular membrane structures, and replication of the genome occurs in replication complexes associated with the rearranged membrane structure (Bienz et al., 1987, 1990, 1992). Replication complexes

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Table 1
Nucleotide sequences of the primers used in this study. The MluI sites and parts of the EcoRV sites are underlined; stop codons are indicated in bold type.

Name	Sense	Sequence	Position
L-Fw	+	5′-CGACGCGTGGATGGCTGCAACACGGGTTTCAC-3′	744–765
L-Rv	–	5′-ATC**TCA**TTGCCGTTGGAGGTTAGTGG-3′	1234–1253
2A-Fw	+	5′-CGACGCGTGGGGCGCCGCCTCAGCGACTC-3′	3792–3810
2A-Rv	–	5′-ATC**TCA**CTGTCGCCTGATGCCTGGG-3′	4181–4199
2B-Fw	+	5′-CGACGCGTGGGGTCTCCTCACCCTCTCTGC-3′	4200–4219
2B-Rv	–	5′-ATC**TCA**ATCTCATTGAGGTTCAAGGGTTGCCC-3′	4675–4694
2C-Fw	+	5′-CGACGCGTGGGGGCTCAAAGACTTCAACG-3′	4695–4713
2C-Rv	–	5′-ATC**TCA**CTGGCGTCTGATGAGGGAGG-3′	5680–5699
3A-Fw	+	5′-CGACGCGTGGGGCAACCGGGTCATCGACGC-3′	5700–5719
3A-Rv	–	5′-ATC**TCA**TTGAGGTTCCTGTGGCGTG-3′	5966–5984
3B-Rv	–	5′-ATC**TCA**TGGCGCTGGATGTGACGAG-3′	6046–6065
3C-Fw	+	5′-CGACGCGTGGGGAATCTCCCCTGCTGTCCC-3′	6066–6085
3C-Rv	–	5′-ATC**TCA**TTGTTGGGTAGTGGCAAATTG-3′	6615–6635
3D-Fw	+	5′-CGACGCGTCTCTCATTGTTCCCACTGC-3′	6636–6655
3D-Rv	–	5′-ATC**TCA**GGCAGCCAGCAGATTTAG-3′	8022–8042
2AΔ1-Fw	+	5′-CGACGCGTGGGCCCCCGACGGCAGTGC-3′	3891–3907
2AΔ2-Fw	+	5′-CCCTCCCATTGGACCCTTGC-3′	3993–4012
2AΔ2-Rv	–	5′-CACCTTGCGGATGGCCCAG-3′	3872–3890
2AΔ3-Fw	+	5′-TCCCTCCCCAACACCGGC-3′	4092–4109
2AΔ3-Rv	–	5′-GAGAATTTCGAGGTAAGG-3′	3975–3992
4170P	+	5′-GTGAAAGCGCTCCCAGGCATCAGG-3′	4170–4193
4103M	–	5′-GTTGGGGAGGGATTCGCCAGTG-3′	4082–4103

contain viral proteins, host proteins and viral RNA (Bienz et al., 1987, 1990, 1992; Belov et al., 2005; Rust et al., 2001; Schlegel et al., 1996). In PV, all nonstructural proteins are found in replication complexes, suggesting that the viral nonstructural proteins interact with each other (Egger et al., 1996). Studies to reach a more holistic understanding of interactions between viral nonstructural proteins have been carried out in PV (Cuconati et al., 1998; Teterina et al., 2006b; Xiang et al., 1998; Yin et al., 2007), coxsackievirus B3 (CV-B3) (de Jong et al., 2002), and porcine teschovirus (PTV) (Zell et al., 2005) by using a yeast two-hybrid system or a mammalian two-hybrid system. These studies have demonstrated different protein–protein interactions between genera.

In this study, we analyzed protein–protein interactions between AiV nonstructural proteins utilizing a mammalian two-hybrid system. The results showed that all of the AiV nonstructural proteins tested have the ability to interact with at least one protein. We observed homodimerization of five proteins, bidirectional heterodimerization of six protein pairs, and unidirectional heterodimerization of eighteen protein pairs. These interactions included ones that have not been observed in previous two-hybrid studies with other picornaviruses, such as the 2A–2B, 2A–2BC, 2A–2C, 2BC–3CD, 2BC–3C, 2C–3C, 2C–3CD and 3AB–3C interactions. The strongest interaction was observed between 2A and 3CD. To further clarify the molecular properties of 2A, we examined the domain of 2A required for binding to 3CD by mammalian two-hybrid analysis using a series of 2A deletion mutants. The results showed that the C-terminal 23 amino acids were not essential for the interaction with 3CD.

2. Materials and methods

2.1. Cells

Vero cells were maintained in Eagle's minimum essential medium supplemented with 5% fetal bovine serum at 37 °C.

2.2. Plasmids

Mammalian two-hybrid analysis was performed using a Checkmate system (Promega). Plasmids pBIND and pACT from the system contain the yeast GAL4 DNA-binding domain and the herpes simplex virus type 1 VP16 activation domain, respectively. The pBIND plasmid also contains the gene of *Renilla* luciferase, which is used to monitor transfection efficiency. A reporter plasmid, pG5luc, expresses firefly luciferase under the control of a minimal TATA box and five GAL4 binding sites. The coding regions of the nonstructural proteins and cleavage intermediates, L, 2A, 2B, 2BC, 2C, 3A, 3AB, 3C and 3D, were amplified by PCR from a full-length cDNA clone, pAV-FL (Sasaki et al., 2001). The 3C and 3CD sequences carrying a C143A mutation that abolishes the 3C protease activity were amplified by PCR from pMAL-3CDmut (Nagashima et al., 2008). The sequences of the primers used are shown in Table 1. The amplified fragments, which contained an MluI site at the 5′ end and a stop codon followed by a part of the EcoRV site at the 3′ end, were digested with MluI, and cloned into the MluI–EcoRV sites of pACT, and then the nucleotide sequences of the inserts of the derived plasmids were confirmed. The derived plasmids were called pACT-L, -2A, -2B, -2BC, -2C, -3A, -3AB, -3C, -3Cm (which contains the C143A mutation), -3CD and -3D. Each MluI–EcoRV fragment derived from these pACT constructs was inserted into the same sites of pBIND, yielding pBIND-L, -2A, -2B, -2BC, -2C, -3A, -3AB, -3C, -3Cm, -3CD and -3D.

2.3. 2A deletion mutants

The sequence from nucleotide (nt) 3891 to the C-terminus of the 2A-coding region was amplified by PCR with primers 2AΔ1-Fw and 2A-Rv (Table 1) using pAV-FL as the template. The derived fragment was cloned into pACT and pBIND as described above, yielding pACT-2AΔ1 and pBIND-2AΔ1. Deletions of nt 3891–3992 (2AΔ2) and nt 3993–4091 (2AΔ3) were introduced by inverse PCR with the primers shown in Table 1 using pACT-2A as a template. The PCR products were self-ligated, and the MluI–EcoRV fragments of the derived plasmids were cloned into these sites of pACT and pBIND, yielding pACT-2AΔ2 and -2AΔ3 and pBIND-2AΔ2 and -2AΔ3. A deletion of nt 4104–4169 (2AΔ4) was introduced by inverse PCR with the primers shown in Table 1 using a plasmid containing nt 3642–5328 as a template. The resultant fragment was self-ligated, and then PCR was performed using the derived plasmid as a template with primers 2A-Fw and 2A-Rv. The PCR product was digested with MluI, and cloned into pACT and pBIND. The resultant plasmids were named pACT-2AΔ4 and pBIND-2AΔ4. The nucleotide sequences of the 2A regions of all of the 2A deletion mutants were verified.