Leucine at Position 383 of Fusion Protein Is Responsible for Fusogenicity of Wild-Type Mumps Virus in B95a Cells

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Introduction

Mumps virus is a nonsegmented, single-stranded, negative-sense RNA virus. It is a member of the genus Rubulavirus in the family Paramyxoviridae. The mumps virus genome encodes 7 proteins in the following genome order: nucleocapsid (N), phospho (P), matrix (M), fusion (F), small hydrophobic (SH), hemagglutinin-neuraminidase (HN) and large (L) protein genes [1]. There are 2 envelope glycoproteins, F and HN. The HN protein is involved in virus attachment to a sialic acid receptor on the surface of host cells. Attachment of the HN protein leads to conformational changes which induce the subsequent conformational alteration in the F protein in the cascade reaction of cell fusion [1–4].

Acute infection by mumps virus is characterized by self-limiting parotid gland swelling and several complications, including aseptic meningitis, deafness, orchitis...
and pancreatitis. The mumps virus is still circulating throughout the world, and genotype classification of the wild type is useful for the identification of its transmission pathway. Recently, circulating mumps viruses have been divided into 12 genotypes, from A to L, based upon the sequence diversity of the SH gene [5]. From our previous reports on the molecular epidemiology of the mumps virus in Japan, currently circulating mumps virus strains were divided into 4 different genotypes, B, G, J and L [5, 6].

The conventional virological diagnostic method involves virus isolation from clinical samples. Isolation of the circulating mumps virus strain is an essential method for the diagnosis of the patients and monitoring the antigenicity of wild-type circulating strains. The efficiency of virus isolation depends mainly upon the virus load of infectious virus particles in clinical specimens and upon the sensitivity of cells used in virus isolation. Vero cells have been used for the virus isolation of several pathogenic agents such as measles virus, enterovirus and herpes viruses and also show high sensitivity to the mumps virus. Kobune et al. [7] reported that measles virus was isolated from clinical samples using B95a cells derived from marmoset B lymphocytes at a higher efficiency than using Vero cells. Recently, mumps virus was efficiently isolated using B95a cells, rhesus monkey kidney cells, human colorectal adenocarcinoma epithelial cells and other cell lines [8, 9]. In this study, the fusion inducibility of mumps virus strains was investigated in Vero and B95a cells, using the Hoshino mumps vaccine strain (genotype B) and some other wild strains (genotypes B, G, J and L). Wild circulating strains of genotypes B, G and L induced cytopathic effects (CPE) in B95a cells, but the Hoshino vaccine strain did not. Expression plasmids of the F and HN proteins of mumps virus were constructed from the Hoshino vaccine seed strain KO3 and wild strains, and the fusion inducibility of these plasmids was examined in Vero and B95a cells under the control of T7 RNA polymerase.

**Materials and Methods**

**Mumps Virus Strains**

The mumps vaccine seed strain of Hoshino KO3 and 8 currently circulating wild-type mumps virus strains, genotypes B (MPi/Mie.JPN/98.NK-H and MPi/Himeji.JPN/2001-355), G (MPi/Sapporo.JPN/02.A158, MPi/Mie.JPN/2-2002 and MPi/Tokyo.JPN/00.SV13), J (MPi/Mie.JPN/94.OH-Mie and MPi/Sapporo.JPN/2000.K-4) and L (MPi/Tokyo.JPN/02.SII-10) in Japan, were used [6]. The Hoshino vaccine seed strain KO3 was developed by attenuation through 22 passages in chick embryonic cells from wild-type mumps virus isolated in 1972 [10]. The currently circulating wild strains were isolated in Vero cells and propagated within 5 passages.

**Cell Lines**

Vero cells were propagated in minimum essential medium (MEM) supplemented with 5% fetal bovine serum. B95a cells, a marmoset B cell line transformed by Epstein-Barr virus, were cultured in RPMI 1640 medium supplemented with 8% fetal calf serum. All cells were cultured at 37°C in 5% CO₂.

**Immunofluorescent Staining**

Vero and B95a cells were cultured on a Lab Tek 8-well chamber slide (Nunc, N.Y., USA) and infected with the mumps Hoshino vaccine strain and other wild strains. Cells were fixed with acetone and stained by the indirect immunofluorescent assay using anti-mumps antiserum raised in rabbits immunized with the purified Enders strain of mumps virus, followed by rhodamine-labeled monoclonal antibody against rabbit IgG (Sigma, Steinheim, Germany). Expression of the F protein was confirmed by immunofluorescent staining using monoclonal antibodies against F protein, kindly supplied by Dr. Takeuchi, Department of Infection Biology, Graduate School of Comprehensive Human Sciences and Institute of Basic Medical Sciences, University of Tsukuba.

**Mumps Virus PCR and Sequencing**

Total RNA was extracted from 200 μl of culture supernatants using a magnetic beads RNA purification kit (TOYOBO Co., Ltd., Osaka, Japan), the RNA pellet was suspended in 25 μl of distilled water, and 3 μl was reverse transcribed to cDNA with AMV reverse transcriptase (Life Sciences, St. Petersburg, Fla., USA), using MpF0+ (5'-GTCGATGACTCTACTGAGTAC-3') for the F gene and MpF921+ (5'-TCTATAATTCAATTCGCCAG-3') for the HN gene as positive-sense primers. First PCR was performed with KOD-plus polymerase (TaKaRa Biomedicals, Tokyo, Japan), using MpF0+ and MpHNI- (5'-CCATATTCCGGAAGCGAGTTCGGGAA-3') for the F gene and MpF921+ and MpL1- (5'-AACCGGTTCTCAGACATCAC-3') Nested PCR was performed with MpF-ATG (5'-TCTCGTACTCTGACGAATACGTTGAG-3') and MpF-TAA (5'-GCGGGAGCTCTAGATCTACCTAAGTATG-3') primers, which amplified 1,617 nucleotides of the F gene, and MpHN-ATG (5'-CTCTTTGTTACTGTCGTGGAAG-3') and MpF-HN (5'-TCTAGAGCTCTAAGTGATGAGTCTAATAC-3') primers, which amplified 1,749 nucleotides of the HN gene, as previously reported [6, 11]. Underlined sequences are linker restriction enzyme sites of KpnI and SacI. The nucleotide sequence was analyzed employing the dye terminator method using the ABI 3130 DNA sequencer (Applied Biosystems Japan, Tokyo). Nucleotide alignments and phylogenetic analysis were performed with GENETYX-MAC version 12 (Software Development Co., Ltd., Tokyo, Japan).

**Construction of F and HN Expression Plasmids of Mumps Virus**

The F and HN genes were amplified by nested PCR using linker primers (MpF-ATG, MpF-TAA, MpHN-ATG and MpHN-TAA) under the control of T7 RNA polymerase. Vero cells were infected with the Hoshino vaccine strain followed by rhodamine-labeled monoclonal antibody against rabbit IgG and anti-mumps antiserum raised in rabbits immunized with the purified mumps virus. Underlined sequences are linker restriction enzyme sites of KpnI and SacI.

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KO3 Hoshino vaccine seed strain and from the wild-type genotypes B, G, J and L, by cloning at the multicloning sites located downstream of the T7 promoter.

The construction of recombinant and point-mutated plasmids is shown in figure 1. Recombinant plasmids (pKO3/SV13-F and pSV13/KO3-F) were constructed from the F expression plasmids of the KO3 Hoshino vaccine strain and MPi/Tokyo. JPN/00.SV-13 (genotype G) using the PacI site at nucleotide position 635 from the F ATG. Point mutations were introduced by PCR at amino acid positions 383, 479 and 488, based on pKO3-F and pSV13-F.

**Results**

**Cell Fusion in Vero and B95a Cells**

Currently circulating mumps virus strains in Japan were divided into 4 different genotypes, B, G, J and L [5, 6, 7, 8, 9, 10, 11, 12, 13].
6]. Wild-type strains showed marked cell fusion in Vero cells, compared with the Hoshino vaccine strain seed KO3. B95a cells were infected with Hoshino vaccine strain seed KO3, MPi/Himeji.JPN/2001-355 (Himeji 355; genotype B), MPi/Tokyo.JPN/02.SIII-10 (SIII-10; genotype L), MPi/Sapporo.JPN/2000.K-4 (Sapporo K-4; genotype J), MPi/Sapporo.JPN/02.A158 (A158/2002; genotype G) and MPi/Mie.JPN/2-2002 (Mie.JPN/2.2002; genotype G), and the cells were stained with indirect immunofluorescent staining, using polyclonal antibodies against the mumps Enders strain (genotype A).

**Sequence Analysis of F and HN Genes**

Deduced amino acid differences in the F and HN proteins among the Hoshino vaccine strain and the other 5 wild strains, genotype B (MPi/Mie.JPN/98.NK-H), G (MPi/Sapporo.JPN/00.A158, MPi/Tokyo.JPN/00.SV13), J (MPi/Mie.JPN/94.OH-Mie) and L (MPi/Tokyo.JPN/02.SIII10), are shown in table 1. Among these strains, 13–16 amino acid changes were identified in the F protein region and 6–20 in the HN protein region. Five amino acids at positions 3, 4, 8, 14 and 383 of the F protein and one at position 119 of the HN protein were unique to the KO3 Hoshino vaccine strain.

**Fusion Inducibility in B95a Cells**

Expression plasmids of the F and HN proteins were used to cotransfect Vero and B95a cells, and the fusion inducibility was investigated. When Vero cells were cotransfected with pKO3-F and pKO3-HN, small-cell fusion was observed, and large-cell fusion was demonstrated when pSV13-F and pSV13-HN were the transfectants (data not shown). Different combinations of F and HN expression plasmids were used to transfect in B95a cells,
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Hoshino = KO3 Hoshino vaccine strain; NK-H = MPI/Mie.JPN/98.NK-H (genotype B); A158 = MPI/Sapporo.JPN/02.A158 (genotype G); SV13 = MPI/Tokyo.JPN/00.SV13 (genotype G); SIII-10 = MPI/Tokyo.JPN/02.SIII-10 (genotype L).

Figures in bold show the unique amino acid positions of the KO3 Hoshino vaccine strain.

Table 1. Amino acid differences of F and HN proteins among mumps vaccine and wild-type strains
and the results are shown in figure 3. When B95a cells were cotransfected with pKO3-F and pKO3-HN or pSV13-HN, cell fusion was not observed (fig. 3a and b), but extensive fusion was noted when pSV13-F was used as the F expression partner in B95a cells (fig. 3c and d). Fusion inducibility in B95a cells depends on the plasmid expressing the wild-type F protein.

Fusion Inducibility of Recombinant F Protein Expression Plasmids

Two recombinant plasmids, pSV13/KO3-F and pKO3/SV13-F, were constructed using pKO3-F and pSV13-F with a PacI restriction enzyme site located at nucleotide position 635 from the F ATG. The results of fusion inducibility with these recombinant plasmids are shown in figure 3e and f. When pSV13/KO3-F was used in B95a cells, no obvious cell fusion was noted. However, when pKO3/SV13-F was applied as an F expression partner in B95a cells, extensive fusion was observed. These results suggested that fusion inducibility depended on the 6 amino acids at positions 333, 369, 383, 393, 479 or 488 of the F-coding region. Among 6 amino acid differences, 383 Leu, 479 Iso and 488 Iso were common in genotype G, as shown in table 1. All isolates of genotype G induced large-cell fusion in B95a cells, and thus, common amino acid changes seemed to be crucial amino acids for cell fusion in B95a cells. Six recombinant plasmids with a single amino acid substitution were constructed through intro-
Inducing a mutation by PCR. Glutamine (Q) at position 383 of the KO3 F protein was replaced with leucine (L), designated as pKO3-F Q383L. Similarly, pSV13-F L383Q, pKO3-F V479 I, pSV13-F I 479 V, pKO3-F V488 I and pSV13-F I488V were constructed. The results regarding the fusion inducibility of these point-mutated plasmids as the expression partner in B95a and Vero cells are shown in figure 4. When pSV13-F I479V and pSV13-F I488V were used to transfect B95a cells, extensive cell fusion was observed similarly to pSV13-F. No syncytium occurred in B95a cells transfected with pSV13-F L383Q, but large-cell fusion was demonstrated when transfected with pKO3-F Q383L. The other KO3-F-based plasmids did not induce cell fusion in B95a cells. Amino acid substitution at position 383 influenced the fusion inducibility in B95a cells. However, the substitution of amino acids at the other 2 positions, 479 and 488, led to no significant difference in the fusion inducibility of the original plasmid.

pSV13-F and pKO3-F were cotransfected with pKO3-HN in Vero cells. pKO3-F induced cell fusion in Vero cells, and extensive cell fusion was observed when pSV13-F was used (fig. 4i and j).

pKO3-F Q383L, pSV13-F L383Q, pKO3-F V479I and pKO3-F V488I were used to cotransfect B95a cells and stained with monoclonal antibody against mumps F protein. F protein was expressed in B95a cells after transfection with point-mutated plasmids (fig. 5a, c, e and f). pKO3-F Q383L induced cell fusion when used as a cotransfectant with pKO3-HN (fig. 5b), but SV13-F L383Q did not (fig. 5d).

Discussion

Mumps virus replicates in various cell lines of both human and nonhuman origin. The susceptibility depends on the receptor expression on the surface of cells and HN protein of mumps virus. Sialic acid has been reported as a receptor for mumps virus [1]. Vero cells are most commonly used for the isolation of mumps virus.

Fig. 4. The results of fusion inducibility in different point-mutated F protein expression plasmids in B95a and Vero cells. Point-mutated plasmids based on pKO3-F (d) [pKO3-F Q383L (a), pKO3-F V479I (b) and pKO3-F V488I (c)] were constructed, and they were used to cotransfect B95a cells with pKO3-HN. Similarly, pSV13-F L383Q (e), pSV13-F I 479 V (f) and pSV13-F I488V (g) were constructed, based on pSV13-F (h), and they were used for cotransfection with pKO3-HN. pKO3-F (i) and pSV13-F (j) cotransfected Vero cells with pKO3-HN, with subsequent staining with Giemsa’s solution.
from clinical samples. Knowles and Cohen [9] demonstrated that mumps virus was successfully isolated in rhesus monkey kidney or B95a cells, with an isolation rate of 40% from clinical samples, similar to that in Vero cells, and all positive samples were obtained within 2 days after the onset of illness. They investigated virus isolation only for genotype C strains. Afzal et al. [8] examined mumps virus growth in various cell lines and found that human colorectal adenocarcinoma epithelial and Vero cells were the most susceptible cell lines. However, they did not investigate the susceptibility of B95a cells.

Recently, circulating mumps viruses were divided into 12 genotypes from A to L based upon the sequence diversity of the SH gene [5]. In Japan, current circulating mumps virus strains could be divided into 4 different genotypes, B, G, J and L, from the 1970s to the present [6]. Irrespective of the diversity of the SH sequence, Örvell et al. [14] proposed a genotype classification based upon the HN region, but data on the sequence diversity of the HN region were limited. The SH protein is present in infected cells but not in virus particles. The function of the SH protein is believed to be related to virus replication, but this has not been fully investigated [15]. Virus antigenicity is based on the surface envelope HN and F glycoproteins. The HN protein binds to the sialic acid receptor in the initial process of infection [2–4]. The active form of the HN protein is a tetramer, and F protein is a trimer. Native F0 protein is subsequently cleaved into the 2 fragments of F1 and F2. The well-conserved hydrophobic domain (fusion peptide) at the amino terminus of the F1 subunit is considered to be directly involved in the fusion event [2–4]. Sequences downstream of the fusion peptide and upstream of the transmembrane anchor domain typically show a 4-3 (heptad) pattern of hydrophobic repeats, designated as heptad repeats (HR) 1 and 2, respectively [2–4]. In the prefusion state, HR1 and HR2 of the F protein undergo intermolecular binding, and trimerization of the F protein forms a stable 6-helix bundle. The HN protein plays an important role in virus attachment to sialic-acid-containing host-cell surface receptors. This alters conformational changes in the HN protein to induce further conformational alteration in the F protein. The fusion peptide protrudes to the lipid bilayer of the cell membrane in the fusion process. This structure is unstable and tends to refold to the stable 6-helix bundle structure, inducing cell membrane fusion [4]. Recently, other domains were reported: HR3 between HR1 and HR2, and HR4 in the F2 region. Mutational analyses of HR3 and HR4 domains have revealed that they are related to the folding of the F protein molecular or surface expression [16, 17]. In this study, recombinant point-mutated plasmids exhibited varying fusion inducibility, but there was no significant difference in the expression of the F protein at amino acid positions 383, 479 and 488, as shown in figure 5.

Fig. 5. F protein expression by point-mutated plasmids. pKO3-F Q383L (a), pSV13-F L383Q (c), pKO3-F V479I (e) and pKO3-F V488I (f) were used to transfect B95a cells. pKO3-F Q383L (b) and pSV13-F L383Q (d) cotransfected B95a cells with pKO3-HN. Transfected cells were stained using monoclonal antibody against mumps F protein.
The Hoshino vaccine strain induced small plaques in Vero cells [10], but small plaque inducibility was not conclusively related to the attenuation or low rea
togencity. Information on the molecular analysis of func-
tional differences in each genotype is limited. In this re
cport, the wild-type isolates induced CPE in both Vero and B95a cells, but the Hoshino vaccine strain did not induce CPE in B95a cells. Other wild circulating strains produced CPE with differing extents of cell fusion. It is now generally thought that most paramyxovirus cell fu-
sions usually require the coexpression of both F and HN proteins in the same cell, and a biochemical interaction was reported [2–4, 18]. The F and HN expression plas-
mids were constructed from 4 different genotypes, and we investigated their fusion inducibility. When the pKO3-F plasmid was used as an F expression partner, no extensive cell fusion was demonstrated. These results suggested that F protein of the wild strain was respon-
sible for the fusion inducibility in B95a cells. The find-
ings of chimerical and point-mutation experiments using different recombinant F protein expression plasmids demonstrated that Leu at position 383 of the wild type was responsible for the fusion inducibility in B95a cells and that Glu at position 383 was specific to the Hoshino vaccine strain, inducing small plaque formation in Vero cells and no fusion in B95a cells. It remains unknown whether the poor fusogenicity is related to the attenu-
ation or lower pathogenicity. A highly neurovirulent strain (88-1961 wild-type) showed the heterogeneity (A/G) at nucleotide position 271 in the F gene, at amino acid position 91 in the F protein (threonine/alanine), and G-variant cloned virus was more fusogenic but rep-
licated to lower cumulative titers in vitro and was less neurovirulent in vivo [19]. Using the same strain, Ser to Asp substitution at position 466 of HN resulted in de-
creased receptor binding and neuraminidase activity, related to biological differences as well as F gene mutation [20].

As for the fusogenicity of F protein of mumps virus, mutational analysis of fusion-negative and -positive strains in COS7 cells demonstrated that the amino acid at position 195 of the F protein was critical for varying fusogenicity [21]. In this study, amino acid position 383 of the mumps virus F protein was closely related to the fusogenicity in B95a cells. Positions 195 and 383 are not located in the well-known functional domains where the biological function has already been determined. Russell et al. [22] reported that amino acid positions 443, 447 and 449 of the F protein of SV5, a localized seg-
ment upstream of HR2, exhibited a key regulatory switch between the native 6-helix bundle structure and fusogenic conformations. Therefore, the amino acid at position 383 of the mumps virus F protein is adjacent to HR2, and so a similar regulatory function for the stabil-
ity of the 6-helix bundle structure was considered.

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