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BRIEF REPORT

An NA-deficient 2009 pandemic H1N1 influenza virus mutant can efficiently replicate in cultured cells

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Abstract We identified a novel neuraminidase (NA)-deficient virus that was a 2009 pandemic influenza H1N1 virus mutant. The mutant virus had a deletion of 1,009 nt in the NA gene and lacked an enzymatic domain. Although the yield of the NA-deficient virus was limited, it formed large plaques when applied to MDCK cell cultures, indicating that the virus was able to spread to adjacent cells. Furthermore, the NA-deficient virus was eluted from chicken erythrocytes at 37 °C, even in the presence of the antiviral drug peramivir. Spread of this NA-deficient virus may pose a potential threat to anti-influenza therapies.

Influenza A viruses commonly infect a large variety of hosts, including humans, pigs, horses and birds. Two of the eight RNA segments of the genome encode the surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), which have 16 and 9 subtypes, respectively [2]. The HA protein is involved in virus attachment to cell-surface receptors and mediates entry of the virus into the cell by membrane fusion. The NA protein is required to release progeny virions and to prevent them from aggregating. This enzyme removes terminal sialic acids from oligosaccharide side chains, where HA binds [3].

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The pandemic influenza A (H1N1) virus [A(H1N1)pdm09], containing a combination of swine, avian and human virus gene segments, emerged in humans in Mexico City in March 2009 and quickly spread worldwide [1, 17]. To date, only a small number of oseltamivir-resistant A(H1N1)pdm09 strains containing a single amino acid change (His to Tyr) at position 275 within NA (H275Y) have been detected [13] and reported among seasonal H1N1 viruses worldwide since the 2007–2008 influenza season [20]. In Japan, a surveillance study conducted during the 2008–2009 influenza season showed that oseltamivir resistance was evident in 99.7 % of H1N1 virus isolates [18].

We previously showed that the HA genes of 70 A(H1N1)pdm09 strains isolated from a single student population in 2009 in Tobetsu, Hokkaido, clustered into three groups [7]. Of these isolates, two strains exhibited lower sensitivity to oseltamivir than other strains. Morlighem et al. conducted a phylogenetic analysis of 253 samples based on the NA gene of Japanese A(H1N1)pdm09. They identified an N248D mutation in NA, which allowed discrimination of isolates between the early (May 2009) and peak (October 2009 to January 2010) phases of infection [13]. In the present study, we describe for the first time the isolation of an NA-deficient mutant of A(H1N1)pdm09, and its subsequent characterization.

One of the two isolates with low sensitivity to oseltamivir, A/Hokkaido/T64/2009 (H1N1) (T64), was found to form plaques of various sizes on Madin-Darby canine kidney (MDCK) cells, indicating that it was composed of a mixed population of virions. We analyzed several plaques and successfully obtained an NA-deficient mutant, designated T64LP4, which formed large uniform-sized plaques (Fig. 1A). A full-genome amplification technique [8] demonstrated that T64LP4 lacked the NA gene (Fig. 1B).

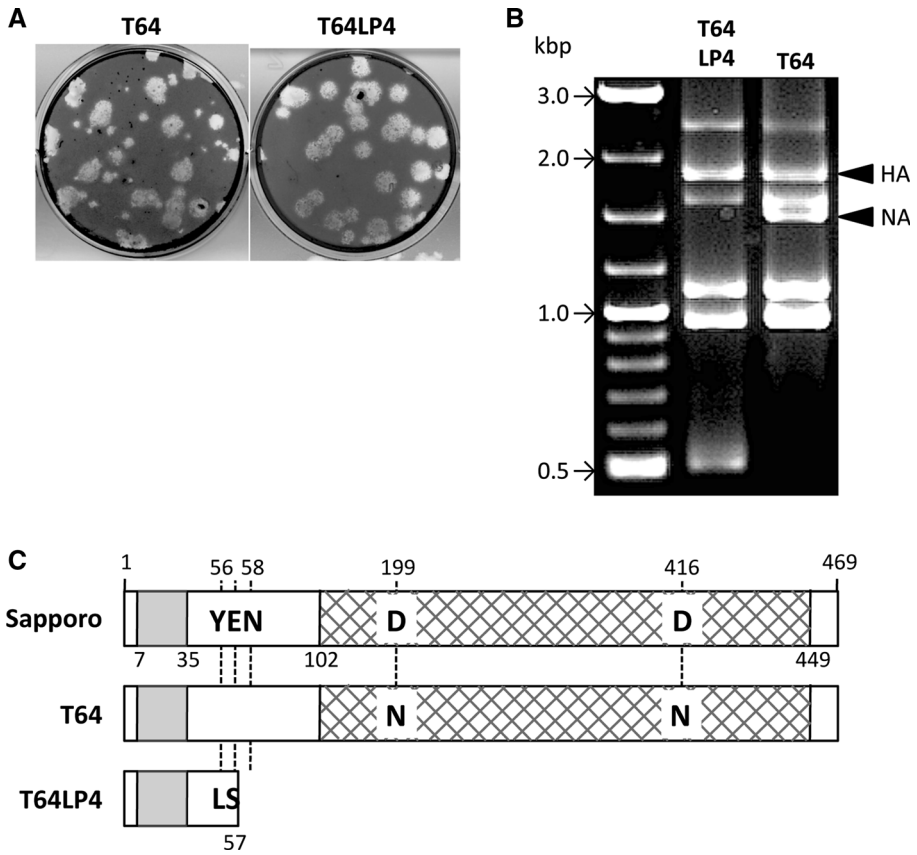

Fig. 1 Characterization of the T64 parental strain and T64LP4 mutant strain. (**A**) Plaque morphology of T64 and T64LP4 strains on MDCK cells. Virus was inoculated onto MDCK cells grown in 6-well plates and incubated at 37 °C for 1 h. Cells were then overlaid with Dulbecco's modified Eagle's medium (DMEM) supplemented with 1 % Avicel RC-591 (FMC BioPolymer), 50 μg/mL gentamicin, and 5 μg/mL trypsin and incubated at 35 °C. Crystal violet staining was used to visualize plaques at 2 days postinfection. (**B**) Amplification of the T64 and T64LP4 genomes. Each segment of the virus was amplified as described previously [8]. A 2-Log DNA ladder was used as a molecular weight marker. Arrowheads indicate bands corresponding to the HA and NA genes. (**C**) Schematic diagram of NA proteins and the 0.5-kb fragment. The 1.5-kb fragment derived from T64 and the 0.5-kb fragment from T64LP4 were sequenced directly using primers specific for the NA gene. Amino acid differences among Sapporo, T64, and T64LP4 strains are indicated. The transmembrane and enzymatic domains are indicated in gray and by hatched boxes, respectively

The amplification products included a prominent extra band that migrated faster than that of the NS gene. The nucleotide sequence of the 0.5-kb band coincided with 166 nt at the 5′-terminus and 235 nt at the 3′-terminus of the NA gene for the parental strain T64 (DDBJ Accession Nos. AB847956 and AB847955). Phylogenetic analysis of the gene indicated that T64LP4 was derived from the parental virus (data not shown). The 1,009-nt deletion generated an open reading frame (ORF) encoding a protein of 57 amino acids (aa), with 55 aa corresponding to the N-terminus of NA (Fig. 1C). The mutant NA comprised the transmembrane and 20-aa stalk regions, along with an additional 2 aa (Leu and Ser). The mutant NA lacked an enzymatic globular head domain. We also observed two aa changes in the mutant T64 NA (D199N and D416N), based on A/Sapporo/1/2009 (H1N1) (Sapporo), which was the first A(H1N1)pdm09 isolated in Hokkaido.

Growth kinetics in MDCK cells were compared between the parent T64 and mutant T64LP4 strains. The growth rate of T64LP4 was slower than that for T64 (Fig. 2), and the yield of T64LP4 was 1,000-fold lower than that of T64 at 32 h postinfection. After 32 h postinfection, *Clostridium perfringens* sialidase was added to the culture medium of T64- and T64LP4-infected cells. Although the enzyme had no effect on T64 titer, the T64LP4 titer increased more than 40-fold 2 h after sialidase was added. This finding indicated that the low yield of T64LP4 was partially due to a lack of NA activity.

Elution of T64 and T64LP4 virions from chicken erythrocytes was compared. Both types were eluted from erythrocytes after a 6-h incubation at 37 °C, although T64LP4 lacked the NA enzymatic domain (Fig. 3). Peramivir inhibited elution of T64 and A/Denmark/524/2009 (H1N1) (Den/524). No inhibition was observed for T64LP4

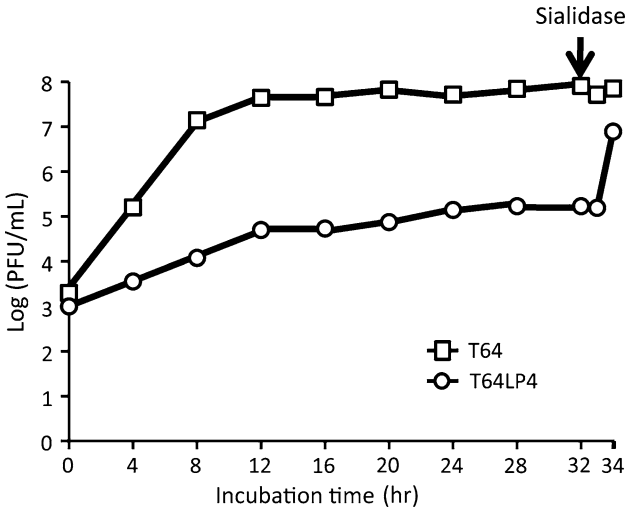

Fig. 2 One-step growth of T64 and T64LP4 viruses in MDCK cells. Monolayers of MDCK cells were infected at a multiplicity of infection of approximately 6 PFU/cell and incubated at 35 °C. At 32 h postinfection, *C. perfringens* sialidase (New England Biolabs) was added to a final concentration of 30 mU/mL. At the indicated time points, supernatants were harvested, and virus titers were determined using plaque assays

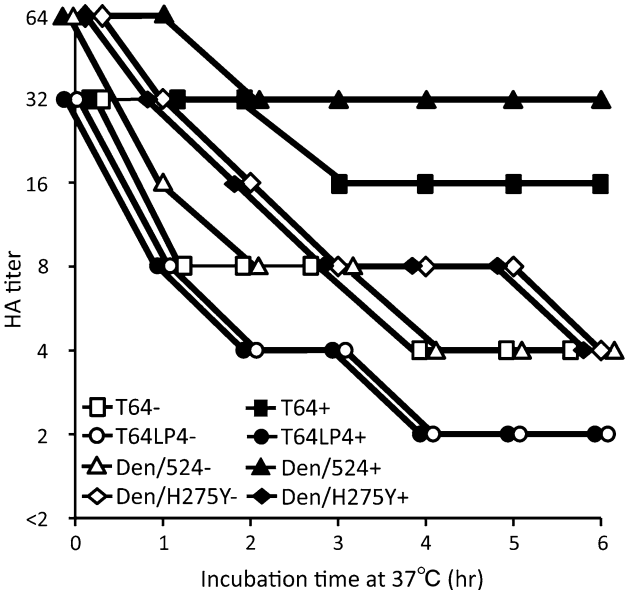

Fig. 3 Virus elution assays for T64 and T64LP4 using chicken erythrocytes. Hemagglutination titers of the viruses were determined on ice using the microtiter method [21]. Peramivir (Shionogi) was added to each well at a final concentration of 100 nM. Microtiter plates were incubated at 37 °C for 1 h, and titers were determined at 1-h intervals. Open and solid symbols indicate virus alone and virus with peramivir, respectively

or A/Denmark/527/2009 (H1N1) (Den/H275Y), which contained the H275Y mutation.

An optimal balance between receptor binding and receptor destruction by HA and NA respectively, is important for viral replication in cultured cells and experimental animals [5, 11, 15, 16, 19, 24]. Mismatched pairs of HA and NA can be rescued by adaptive mutations in one or both proteins after several cycles of infection in cell culture [4, 9, 10]. Oseltamivir-resistant viruses with the H275Y mutation exhibited decreased ratios of NA:HA activities compared with drug-sensitive viruses [23]. For T64 and T64LP4, two aa substitutions (K153E and M257I) were observed in HA (DDBJ accession Nos. AB665976 and AB847957). At positions 153-157, there is a prominent loop to the left of the receptor-binding site [22]. Substitution from a positively charged Lys to a negatively charged Glu affects the affinity for the negatively charged sialic acid moiety. It is therefore possible that T64LP4 has reduced receptor-binding affinity, thereby enabling it to be eluted from erythrocytes despite the fact that it lacks NA.

Peramivir treatment resulted in no observable effects on the growth of T64LP4- or Den/H275Y (data not shown). The S247N mutation of NA resulted in a moderate reduction to oseltamivir and zanamivir sensitivity, as has also been observed in A(H1N1)pdm09 isolates [6]. When the H275Y mutation is combined with the S247N mutation, the virus becomes highly resistant to oseltamivir. Although the yield of T64LP4 was decreased by approximately 1,000-fold compared with the parental strain, it formed large plaques on MDCK cells. This finding indicates that NA is not essential for cell-to-cell infection. Mori et al. reported that influenza viruses can spread in an NA-independent manner to adjacent cells [12]. Furthermore, bacterial sialidase can improve the yield of T64LP4. Upper respiratory tract bacteria such as *Streptococcus pneumoniae* release sialidase, and even human saliva contains sialidase [14]. It is therefore postulated that T64LP4 can efficiently replicate in the upper respiratory tract of humans. Such an NA-deficient influenza virus will more than likely be resistant to all NA inhibitors and pose a potential threat to anti-influenza therapies. To control influenza epidemics, surveillance and extensive studies on NA-deficient clinical isolates are urgently required.

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