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Sialidase Activity of Influenza A Virus in an Endocytic Pathway Enhances Viral Replication

Takashi Suzuki,[1,2]†* Tadanobu Takahashi,[1,2]† Chao-Tan Guo,[1,2,3] Kazuya I.-P. Jwa Hidari,[1,2] Daisei Miyamoto,[1,2] Hideo Goto,[2,4] Yoshihiro Kawaoka,[2,4,5,6] and Yasuo Suzuki[1,2]*

Department of Biochemistry, University of Shizuoka, School of Pharmaceutical Sciences and COE Program in the 21st Century, Shizuoka, Japan[1]; CREST, Japan Science and Technology Agency, Saitama, Japan[2]; Institute of Bioengineering, Zhejiang Academy of Medical Sciences, Hang Zhou, China[3]; Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo,[4] and International Research Center for Infectious Diseases,[5] Tokyo, Japan; and Department of Pathobiological Sciences, University of Wisconsin—Madison, Madison, Wisconsin[6]

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N2 neuraminidase (NA) genes of the 1957 and 1968 pandemic influenza virus strains possessed avian-like low-pH stability of sialidase activity, unlike most epidemic strains. We generated four reverse-genetics viruses from a genetic background of A/WSN/33 (H1N1) that included parental N2 NAs of 1968 pandemic (H3N2) and epidemic (H2N2) strains or their counterpart N2 NAs in which the low-pH stability of the sialidase activity was changed by substitutions of one or two amino acid residues. We found that the transfectant viruses bearing low-pH-stable sialidase (WSN/Stable-NAs) showed 25- to 80-times-greater ability to replicate in Madin-Darby canine kidney (MDCK) cells than did the transfectant viruses bearing low-pH-unstable sialidase (WSN/Unstable-NAs). Enzymatic activities of WSN/Stable-NAs were detected in endosomes of MDCK cells after 90 min of virus internalization by in situ fluorescent detection with 5-bromo-4-chloro-indole-3-yl-α-*N*-acetylneuraminic acid and Fast Red Violet LB. Inhibition of sialidase activity of WSN/Stable-NAs on the endocytic pathway by pretreatment with 4-guanidino-2,4-dideoxy-*N*-acetylneuraminic acid (zanamivir) resulted in a significant decrease in progeny viruses. In contrast, the enzymatic activities of WSN/Unstable-NAs, the replication of which had no effect on pretreatment with zanamivir, were undetectable in cells under the same conditions. Hemadsorption assays of transfectant-virus-infected cells revealed that the low-pH stability of the sialidase had no effect on the process of removal of sialic acid from hemagglutinin in the Golgi regions. Moreover, high titers of viruses were recovered from the lungs of mice infected with WSN/Stable-NAs on day 3 after intranasal inoculation, but WSN/Unstable-NAs were cleared from the lungs of the mice. These results indicate that sialidase activity in late endosome/lysosome traffic enhances influenza A virus replication.

Influenza A viruses possess two viral surface glycoproteins, hemagglutinin (HA), which is able to bind to cell surface sialoglycoconjugates (33), and neuraminidase (NA), which has sialidase enzymatic activity that removes sialic acid from the cell surface glycoconjugates and facilitates the budding of progeny virions from the host cell surface (18). The biological importance of viral NA is not only its enzymatic property of removing the virus receptor in the budding stage but also its role in determination of host range restriction on replication and virulence for the virus-infected host (3, 6, 9, 14, 23).

Human influenza A viruses with N2 subtypes have caused two pandemic outbreaks, in 1957 and 1968. In a previous study, we found that the 1957 H2N2 and 1968 H3N2 pandemic strains as well as duck viruses but not most of the epidemic strains isolated after 1968 possessed sialidase activity under low-pH conditions (27, 28). Molecular analysis of the chimeric NAs expressed in 293T cells showed that consensus amino acid regions responsible for low-pH stability did not exist between the 1957 and 1968 pandemic strain NAs but that substitutions of both amino acid Arg to Lys at position 344 and amino acid Phe to Leu at position 466 in the low-pH-stable A/Hong Kong/1/68 (H3N2) NA and a single substitution of amino acid Leu to Phe at position 466 in the low-pH-unstable A/Texas/68 (H2N2) NA resulted in significant change in the low-pH stability of the sialidase activities (29).

In the present study, we therefore generated four influenza viruses that included parental N2 NAs of pandemic A/Hong Kong/1/68 (H3N2) or epidemic A/Texas/68 (H2N2) and their counterpart N2 NAs in which the low-pH stability of the sialidase activities were changed by substitutions of one or two amino acid residues at positions 344 and 466 by using a plasmid-based reverse-genetics system from a genetic background of A/WSN/33 (H1N1). By characterization of the reverse-genetics viruses and in situ fluorescent detection of their sialidase activity, we showed that the low-pH stability of influenza A virus sialidase is functionally linked to virus entry via the endocytic pathway and contributes to the ability of virus replication in both Madin-Darby canine kidney (MDCK) cells and lungs of mice.

* Corresponding author. Mailing address: Department of Biochemistry, University of Shizuoka, School of Pharmaceutical Sciences, Shizuoka 422-8526, Japan. Phone: 81 54 264 5722. Fax: 81 54 264 5723. E-mail for T. Suzuki: Suzukit@u-shizuoka-ken.ac.jp.

† These authors contributed equally to this work.

MATERIALS AND METHODS

***N*-Acetylneuraminic acid derivatives.** 4-Guanidino-2,4-dideoxy-*N*-acetyl-neuraminic acid (zanamivir) was provided by Glaxo Smith Kline (Tokyo, Japan). 5-Bromo-4-chloro-indole-3-yl-α-*N*-acetylneuraminic acid (X-Neu5Ac) was pro-

vided by K. Ikeda (Shizuoka, Japan). 2′-(4-Methylumbelliferyl)-α-D-*N*-acetylneuraminic acid (4-MUα-Neu5Ac) was obtained from Sigma-Aldrich (St. Louis, MO).

Cells and antibodies. 293T cells were cultured in high-glucose Dulbecco's modified Eagle's minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO). MDCK cells were cultured in Eagle's minimum essential medium (MEM) supplemented with 5% heat-inactivated fetal bovine serum. Anti-N2 NA monoclonal antibodies (SIZ-1, UO-2, K-3, and A-4) were prepared from culture supernatants of each hybridoma established by a procedure described previously (13).

Cloning of NA genes. Viral RNA was isolated from virus particles with TRIzol reagent (Invitrogen Corp., Carlsbad, CA), which was used according to the manufacturer's instructions. Full-length cDNAs of the NA genes were amplified by using a reverse transcription-PCR kit (version 2.0; Takara Bio Inc., Otsu, Japan). The full-length PCR products of the NA genes from A/Texas/68 (H2N2) and A/Hong Kong/1/68 (H3N2) were subcloned into the plasmid expression vector pCAGGS/MCS (17) to generate pCATx68NA and pCAHK68NA and their counterpart constructs in which the low-pH stability of the sialidase activities were changed by substitutions of one or two amino acid residues at positions 344 and 466 as described previously (29).

Generation of transfectant influenza A viruses. The 12-plasmid system for generation of the virus A/WSN/33 (H1N1) was used essentially as reported previously (15, 16). NA cDNAs from pCAHK68NA, pCATx68NA, and their counterpart constructs were amplified by PCR and subcloned into the two BsmBI sites of the pHH21 vector (designated pPolI-HK68NA, pPolI-Tx68NA, pPolI-CHKNA, and pPolI-CTxNA). Each transfectant virus (H1N2) (WSN-HK68NA, WSN-Tx68NA, WSN-CHKNA, or WSN-CTxNA) was generated by transfection to 293T cells with the 11 plasmids added to pPolI-HK68NA, pPolI-Tx68NA, pPolI-CHKNA, or pPolI-CTxNA. The virus stocks (1×10^5 to 1×10^7 PFU/ml) were grown in MDCK cells after plaque purification on the cells. Confirmation of engineered NA in each of the recovered transfectant virus stocks was done by nucleotide sequencing of reverse transcription-PCR products derived from viral RNA. Each recovered virus was plaque purified and used for infection experiments using mice within two passages.

Low-pH stability of sialidase activities of transfectant viruses. The amount of each transfectant virus was determined by Western blotting techniques with anti-N2 NA monoclonal antibodies (a cocktail mixed with SIZ-1, UO-2, K-3, and A-4) after sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% gel. P50 virus (H1N2) (10 μg) was used as a control for sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Five microliters of each virus was mixed with an equal amount of 20 mM acetate buffer (pH 4.0 or 5.0) or 20 mM phosphate buffer (pH 6.0) and stored at 37°C for 5 min. The low-pH stability of each transfectant virus sialidase was examined by using 4-MUα-Neu5Ac as previously described (28, 29).

Virus growth in MDCK cells. MDCK cells in wells that were 3.5 cm in diameter were infected in duplicate with WSN-HK68NA, WSN-CHKNA, WSN-Tx68NA, or WSN-CTxNA at an multiplicity of infection (MOI) of 0.002. After incubation for 1 h, the inocula were removed and the cells were washed once with phosphate-buffered saline (PBS). Cultures were grown in 1.5 ml of MEM for 13, 19, 24, 37, 48, and 72 h postinfection. The supernatant was collected at selected times. MDCK cells in 12-well plates were incubated with 0.5 ml of 10-fold serial dilutions of the supernatant for 1 h at 34.5°C. Infected monolayers were overlaid with 2 ml of a solution of MEM containing acetylated trypsin (1 μg/ml) and 0.8% agarose. After incubation for 3 days at 34.5°C, plaques were fixed with an ethanol-acetic acid (5:1; vol/vol) solution and stained with 0.5% amide black in an ethanol-acetic acid-water (45:10:45; vol/vol/vol) solution. Small plaques were detected by immunostaining as described previously (26) using mouse anti-N2 monoclonal antibodies and horseradish peroxidase-labeled goat anti-mouse immunoglobulin G plus M (IgG+M) antibody. The virus titers were determined on the basis of numbers of PFU after treatment with acetylated trypsin (1 μg/ml) at 37°C for 30 min before the assay.

Release of progeny viruses from MDCK cells. Confluent MDCK cells were infected with the transfectant viruses at an MOI of 1 PFU/cell. The culture media without acetylated trypsin were harvested at 13 and 24 h postinfection, followed by treatment with acetylated trypsin (1 μg/ml) at 37°C for 30 min, and then virus titers in the media were determined by plaque assays on MDCK cells. In parallel, MDCK cells equally infected for 13 or 24 h were treated with 25 mU of *Vibrio cholerae* sialidase (Sigma-Aldrich, St. Louis, MO) and 10 mU *Arthrobacter ureafaciens* sialidase (Sigma-Aldrich, St. Louis, MO) per ml of medium for 1 h at 37°C to release all budded viruses from the cell surface. Virus titers in the media were also determined by plaque assays on MDCK cells. Infections for plaque assays were performed on ice to inhibit the bacterial sialidases. Virus release at 13 or 24 h postinfection was calculated as a percentage of virus titers regarded as the maximum virus yield with treatment of the bacterial sialidases, as reported before (32).

Hemadsorption assay. Confluent monolayers of MDCK and COS7 cells in 24-well plates (Corning Costar Corp., Cambridge, MA) were infected with 250 μl of MEM containing the transfectant viruses at an MOI of 0.02 PFU/cell at 37°C. After 30 min, the inoculum was removed from each well, and the monolayers were washed three times with PBS and incubated for 13 h at 34°C in 350 μl of MEM containing or not containing 5 μM zanamivir. The monolayers in each well were incubated on ice for 5 min. After the monolayers were washed three times with PBS, a 1% (vol/vol) suspension of guinea pig erythrocytes was added to each well, and the monolayers were incubated for 15 min on ice. Unadsorbed erythrocytes were removed by repeated gentle washing with cold PBS. Bound erythrocytes were lysed by adding 400 μl of distilled water, and absorbance of the clarified supernatant was measured at 545 nm.

Fluorescence microscopy. MDCK cells grown on Teflon-printed glass slides (ADCELL; Erie Scientific Comp., Portsmouth, NH) were adsorbed with each transfectant virus at an MOI of 100 PFU/cell on ice for 90 min. After incubation for 5 or 90 min at 37°C, the cells were fixed and permeabilized with cold methanol for 5 min. To visualize the in situ enzymatic activity of influenza virus NA adsorbed on or after invasion into cells, cells at 5 or 90 min postinfection were incubated with 0.01 mM X-Neu5Ac and Fast Red Violet LB (1 μg/ml) (ICN Biomedicals Inc., Aurora, Ohio) in 0.1 M sodium acetate buffer (pH 5.0) for 1 h at 37°C (20). Endosomes were detected by a monoclonal antibody against early endosome antigen 1 (EEA1) (BD Biosciences Clontech, Palo Alto, CA). The secondary antibody used was fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG+M antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Cellular nuclei were detected with 1 μg/ml 4′,5-diamino-2-phenylindole, dihydrochloride (DAPI) (Dojindo Co., Kumamoto, Japan). To establish the sialidase activity of influenza virus, the fixed cells were incubated with 5 μM zanamivir in 0.1 M sodium acetate buffer (pH 5.0) for 5 min before incubation with X-Neu5Ac and Fast Red Violet LB. The cells were observed with an LSM 510 laser scanning confocal microscope (Carl Zeiss, Germany).

Fluorescence-activated cell sorter (FACS) analysis. MDCK cells were adsorbed with each transfectant virus (MOI of 10 or 20 PFU/cell) on ice for 90 min. The cells were harvested with treatment of 0.125% trypsin-PBS just after adsorption and after 90 min postinfection at 37°C. The cells just after adsorption on ice were washed with PBS and then fixed with 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS for 5 min. The cells at 90 min postinfection were permeabilized with 0.05% Triton X-100 in PBS for 5 min and fixed with 4% paraformaldehyde in PBS for 5 min after being washed with PBS three times. Viral sialidase activities in the cells after 90 min postinfection were detected with X-Neu5Ac and Fast Red Violet LB as described above. The transfectant viruses adsorbed to the cells were stained with anti-P50 (H1N2) rabbit polyclonal antibody for 30 min followed by FITC-labeled goat anti-rabbit IgG antibody for 30 min. After the cells were washed with PBS three times, their fluorescence intensity was analyzed with the 488-nm line of an argon laser on an EPICS XL system II (Beckman Coulter, Inc., Fullerton, CA).

Zanamivir treatment of influenza virus under the conditions of endocytosis. MDCK cells cultured to confluence in 12-well plates were treated with 500 μl of MEM containing 5 μM zanamivir and 0.001% DEAE-dextran (Sigma-Aldrich, St. Louis, MO) for 15 min at 37°C. The cells were cooled on ice and incubated with 200 μl of 20,000 to 40,000 PFU of each transfectant virus for 90 min on ice. After being washed with MEM three times, the cells were incubated with 500 μl of MEM containing 5 μM zanamivir and 0.001% DEAE-dextran for 3.5 h at 37°C. After being washed with MEM three times, the cells were incubated with 500 μl of MEM for 18 h at 34.5°C. In parallel, an experiment without treatment with zanamivir was performed as a control. Virus titers in the media were determined by plaque assays on MDCK cells. To determine the number of viral-antigen-positive cells in each well, the cells were fixed with cold methanol for 5 min and incubated with mouse anti-N2 monoclonal antibodies at room temperature for 30 min. After the plates had been washed three times with PBS, the wells were incubated with horseradish peroxidase-labeled goat anti-mouse IgG+M antibody for 30 min at room temperature. The number of viral-antigen-positive cells in each well were detected by incubation with 0.5 ml of immunostaining reagent as described previously (26) and calculated from that of the cells per square centimeter and the area of a well. The effect of treatment with zanamivir is shown as a percentage relative to the counterpart control. The infectivities of the viruses upon treatment with zanamivir were calculated from numbers of PFU per number of viral-antigen-positive cells.

Inoculation to mice and virus titrations. Six- to 7-week-old male C57B6 mice were purchased from Japan LSC, Inc. Groups of three mice were each housed in individual cages within separate negative-pressure high-containment biosafety level 3 stainless steel isolators. Mice anesthetized with ether were inoculated

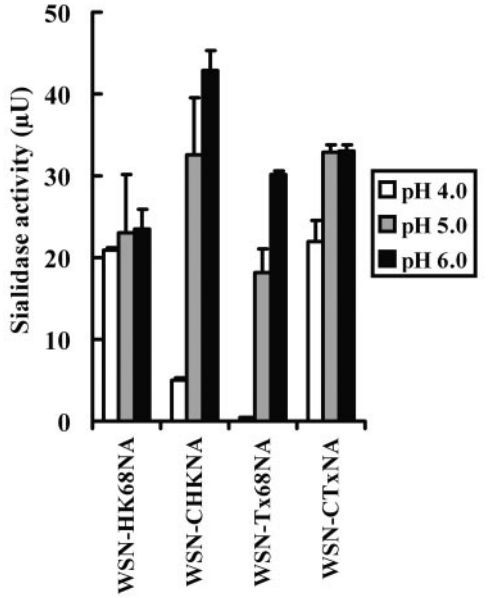

FIG. 1. Low-pH stability of sialidase activities of transfectant viruses generated by a reverse-genetics system. The amount of each transfectant virus (WSN-HK68NA, WSN-CHKNA, WSN-Tx68NA, and WSN-CTxNA) tested was determined by Western blotting techniques with anti-N2 NA monoclonal antibodies. The low-pH stability of sialidase activities of the viruses at pH 4.0, 5.0, or 6.0 was examined by using 4-MUα-Neu5Ac as described in Materials and Methods.

intranasally with 100 μl of 10^4 PFU of each transfectant virus isolate. All mice were sacrificed on day 3 after infection. MDCK cells in 12-well plates were incubated with 0.5 ml of log dilutions of the tissue homogenates in MEM for 1 h at 34.5°C. The virus titers in each tissue were determined by plaque-forming assays as described above.

RESULTS

Characterization of influenza A viruses bearing either low-pH-stable sialidase or low-pH-unstable sialidase. To investigate the effect of the low-pH stability of the sialidase activity on the infection of influenza A virus in vitro and vivo, we generated four influenza viruses (WSN-HK68NA, WSN-CHKNA, WSN-Tx68NA, and WSN-CTxNA) that included parental N2 NA (HK68NA or Tx68NA) of pandemic A/Hong Kong/1/68 (H3N2) or epidemic A/Texas/68 (H2N2) or their counterpart N2 NAs (CHKNA or CTxNA) in which the low-pH stability of the sialidase activity was changed by using a plasmid-based reverse-genetics system from a genetic background of A/WSN/33 (H1N1) (15, 16). The low-pH stability of the sialidase activities of the counterpart N2 NAs was changed by substitutions of one or two amino acid residues at positions 344 and 466 as reported previously (29). The low-pH stability of each reverse-genetics virus sialidase was determined by a fluorometric assay using 4-MUα-Neu5Ac (Fig. 1). The sialidase activities of WSN-Tx68NA, which included NA of epidemic A/Texas/68 (H2N2), and WSN-CHKNA, which included the counterpart NA of pandemic A/Hong Kong/1/68 (H3N2), decreased after incubation at pH 5.0 and were almost lost at pH 4.0 in comparison with what occurred after incubation at pH 6.0. On the other hand, WSN-HK68NA, which included NA of pandemic A/Hong Kong/1/68 (H3N2), and WSN-CTxNA, which included the counterpart NA of epidemic A/Texas/68 (H2N2), still retained their sialidase activities after incubation at pH 4.0.

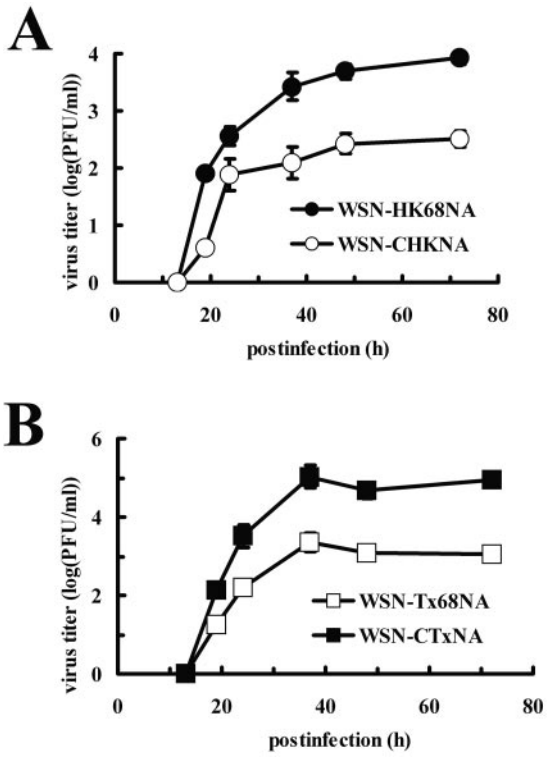

FIG. 2. Growth kinetics of the transfectant viruses on MDCK cells. MDCK cells were infected with each transfectant virus at an MOI of 0.002 PFU/cell, and the culture media were harvested at indicated times after infection. The virus titers at each time point are the averages and the standard deviations of results of duplicate experiments. (A) Growth curves of the transfectant viruses, including parental N2 NA of pandemic A/Hong Kong/1/68 (H3N2) (●; WSN-HK68NA) or including its counterpart N2 NA (○; WSN-CHKNA); (B) growth curves of the transfectant viruses, including parental N2 NA of epidemic A/Texas/68 (H2N2) (■); WSN-CTx68NA) or including its counterpart N2 NA (□; WSN-CTxNA).

Effect of the low-pH stability of sialidase activity on replication of influenza A viruses in MDCK cells. MDCK cells were infected with each transfectant virus at an MOI of 0.002 PFU/cell. Supernatants were collected from 13 to 72 h postinfection, and plaque assays were performed for each sample in duplicate (Fig. 2). WSN-HK68NA and WSN-CTxNA showed a 25- to 80-times-greater ability to replicate in MDCK cells than did WSN-CHKNA or WSN-Tx68NA.

It is known that the sialidase activity of influenza virus NA acts as a receptor-destroying enzyme by catalyzing the removal of sialic acids from the host cell surface and therefore promotes the release of progeny viruses from host cells (18). To determine whether the low-pH stability of viral sialidase affects the release of progeny viruses from the host cell surface, we determined the release of progeny viruses from virus infected-MDCK cells by treatment with bacterial sialidases as described previously (32). MDCK cells were infected with transfectant viruses at an MOI of 1 PFU/cell. Before the culture media were harvested at 13 or 24 h postinfection, virus-infected cells were treated with *Vibrio*

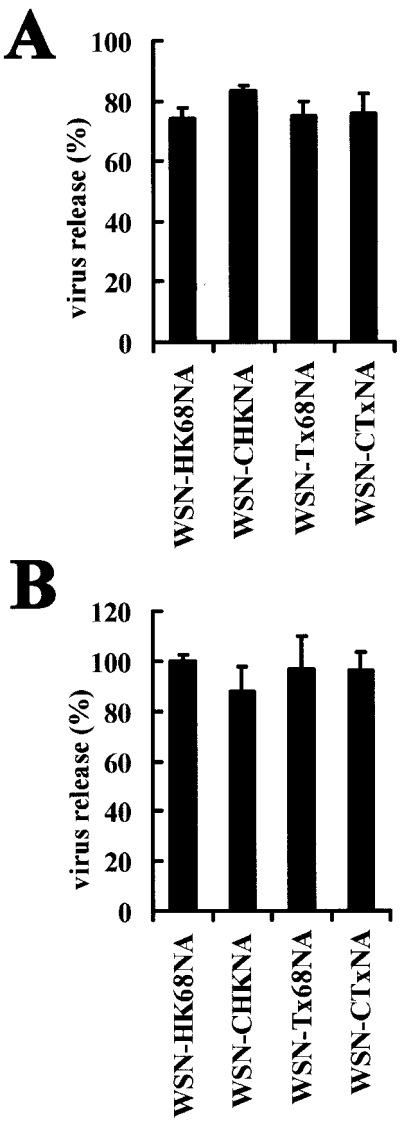

FIG. 3. Release of transfectant viruses from MDCK cells. MDCK cells were infected with each transfectant virus at an MOI of 1 PFU/cell. As controls, MDCK cells, which were infected with each transfectant virus under the same conditions, were treated with bacterial sialidases for 1 h at 37°C to release all budded viruses from the cell surface. Virus titers in each supernatant were determined by a plaque assay on MDCK cells. The amount of virus released at 13 h (A) or 24 h (B) postinfection was calculated as a percentage of virus titers with treatment of bacterial sialidases.

cholerae and *Arthrobacter ureafaciens* sialidase mixtures at 37°C for 1 h to entirely release the viruses budded from the cell surface. The titers of the progeny viruses, which were released from MDCK cells by treatment with bacterial sialidases after 13 or 24 h postinfection, were not significantly different from the progeny viruses released in the absence of bacterial sialidases (Fig. 3). Thus, a difference in the low-pH stability of the viral sialidases had no effect on the process of the release of progeny viruses.

Plaque formation of transfectant viruses on MDCK cells. The plaque size of each transfectant virus on MDCK cells was examined. The sizes of WSN-HK68NA and WSN-CTxNA plaques were much lager than those formed by WSN-CHKNA and WSN-Tx68NA (Fig. 4). Theses results indicate that low-pH-stable sialidases of the viruses promoted expansion of the sizes of virus plaques formed on MDCK cells but had no relation to either the relative specific activities of the sialidases or the release of progeny viruses from the cell surface as described above.

In situ fluorescent detection of viral sialidase activity. The low-pH condition under endocytosis, which is known as an internalization pathway for influenza A virus, is necessary for the acquisition of the fusogenic capacity of virus HA into the cell membrane (22, 24). In the present study, the low-pH stability of influenza virus sialidase gave rise to a distinct difference in virus replication and plaque size on MDCK cells, and we therefore suspected that this property is associated with some intracellular acidic compartments, such as lysosome, endosome, and the *trans* Golgi network. We applied fluorescent cytochemical detection using X-Neu5Ac and Fast Red Violet LB (20) to detect the sialidase activity of influenza virus under the condition of endocytic trafficking. MDCK cells were inoculated with each transfectant virus at an MOI of 100 PFU/cell on ice. After incubation for various periods at 37°C, the sialidase activity of incoming virus particles in the cells was detected by laser scanning confocal microscopy (Fig. 5A).

It has been reported that influenza viruses reach the early endosome after approximately 10 min of infection and the late endosome with a low pH after 40 to 60 min (24). The sialidase activities of all viruses tested were detected with EEA1 in early endosomes of cells after 5 min of virus internalization. However, the sialidase activity of WSN-Tx68NA or WSN-CHKNA was not observed in any area of the cells after 90 min of virus internalization. Interestingly, the sialidase activities of both WSN-HK68NA and WSN-CTxNA were still clearly detected in cytoplasmic areas of the cells after 90 min of virus internalization. Moreover, the sialidase activities after virus internalization were completely inhibited by treatment with 5 μM zanamivir (30, 31), a specific inhibitor of influenza virus NA (Fig. 5B). The results showed that influenza viruses bearing low-pH-stable sialidase maintained high levels of sialidase activity even under low-pH conditions (generally pH 5.0 or less) of lysosomes and late endosomes (1, 12) in the cells. We also examined the sialidase activities of transfectant viruses in Golgi regions of the cells after 7 h of virus internalization but could not find a point of obvious differences among the viruses (data not shown). The results indicate that the low-pH-stable sialidases of viruses probably function in lysosomes and late endosomes of the cells through the endocytic pathway rather than in the Golgi regions through the exocytic pathway, in terms of the cellular acidic compartments. To evaluate a difference in the sialidase activities of the transfectant viruses bearing either low-pH-stable sialidase or low-pH-unstable sialidase on the endocytic pathway, we determined the adsorption of the viruses to MDCK cells and their sialidase activities under the endocytic pathway by FACS analysis with indirect immunofluorescence using an anti-H1N2 polyclonal antibody and the in situ detection for sialidase activity described above. The fluorescence intensities of cells that adsorbed with WSN-HK68NA, WSN-CHKNA, WSN-Tx68NA, or WSN-CTxNA on

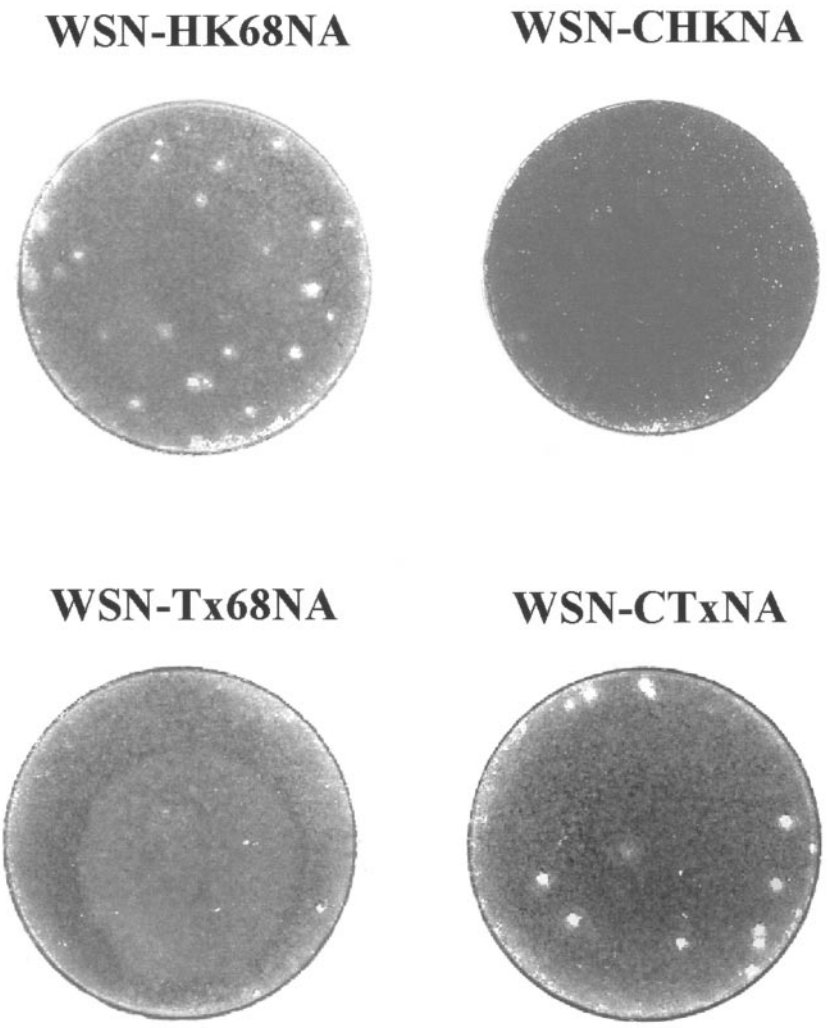

FIG. 4. Plaque formation by the transfectant viruses on MDCK cells. MDCK cell monolayers were infected with each transfectant virus. Infected monolayers were overlaid with 2 ml of a solution of MEM containing acetylated trypsin (1 μg/ml) and 0.8% agarose. After incubation for 3 days at 34.5°C, plaques were fixed and stained with 0.5% amide black solution.

ice showed no meaningful differences among the viruses in comparison with mock infections (Fig. 6A).

On the other hand, the sialidase activities under the endocytosis pathway of MDCK cells that had been infected with WSN-HK68NA, WSN-CHKNA, WSN-Tx68NA, or WSN-CTxNA showed a distinct difference between the viruses bearing low-pH-stable sialidase and low-pH-unstable sialidase. As expected, both WSN-HK68NA and WSN-CTxNA, which included low-pH-stable sialidase, exhibited significant sialidase activities under endocytosis, but neither WSN-Tx68NA nor WSN-CHKNA, which included low-pH-unstable sialidase, exhibited a detectable level of the sialidase activity in comparison with mock infections (Fig. 6B). Quantitative FACS analysis also indicates that the low-pH stability of the virus sialidases confers a high level of enzymatic activity even under low-pH conditions of endocytic trafficking.

Suppression of infectious progeny virus by inhibition of the sialidase activities of influenza A viruses bearing low-pH-stable sialidase under endocytosis. It is known that influenza virus is capable of completely releasing viral ribonucleoprotein into the cellular nucleus after 4 h of virus infection at 37°C (24). To elucidate the biological significance of the sialidase activities of influenza A viruses bearing low-pH-stable sialidase under endocytosis, MDCK cells were infected with each transfectant virus on ice and then treated with zanamivir at 37°C for 3.5 h. After treatment with zanamivir, the cells were washed thoroughly to exclude residual zanamivir, which inhibits any progeny virus release from the cell surface. At 18 h postinfection, virus titers in the media were determined by plaque assays on MDCK cells, and the number of infected cells was counted by immunostaining with N2 monoclonal antibody. The progeny viruses in the media of the cells infected with WSN-HK68NA or WSN-CTxNA were reduced to 15.7% or 25.1%, respectively, by treatment with zanamivir. In contrast, the virus titers in the media of the cells infected with WSN-CHKNA or WSN-Tx68NA under the same conditions showed a 92.8% or 97.5% reduction, respectively (Fig. 7A). Progeny virus titers per the viral-antigen-positive cells were calculated to estimate the effect of zanamivir on viral replication among four transfectant viruses (Fig. 7C). Inhibition of the sialidase activities of the

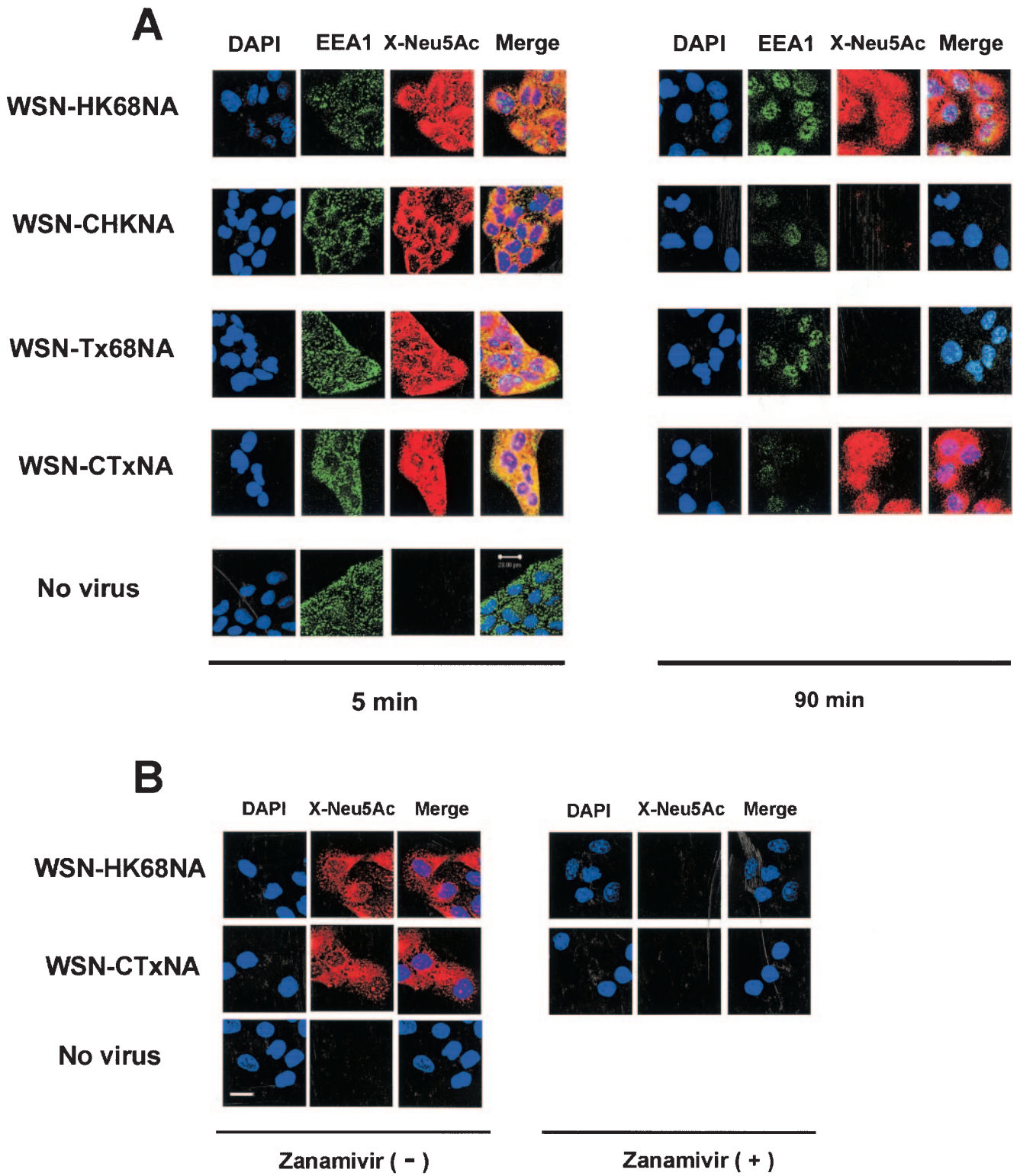

FIG. 5. In situ fluorescent detection of sialidase activity of the transfectant virus NA in MDCK cells. (A) MDCK cells grown on glass coverslips were adsorbed with each transfectant virus at an MOI of 100 PFU/cell on ice for 90 min. After incubation at 37°C for 5 min or 90 min, the cells were fixed and permeabilized with cold methanol for 5 min. (B) MDCK cells were adsorbed with WSN-HK68NA or WSN-CTxNA in the same way. After incubation at 37°C for 90 min, the cells were fixed and incubated with or without 5 μM zanamivir in 0.1 M sodium acetate buffer (pH 5.0) for 5 min. The cells were observed with a laser scanning confocal microscope. Viral sialidase activities (red) were detected with X-Neu5Ac and Fast Red Violet LB as described in Materials and Methods. Early endosomes (green) were detected by a monoclonal antibody against EEA1 and FITC-conjugated goat anti-mouse IgG+M antibody. The nuclei (blue) were visualized by DAPI staining.

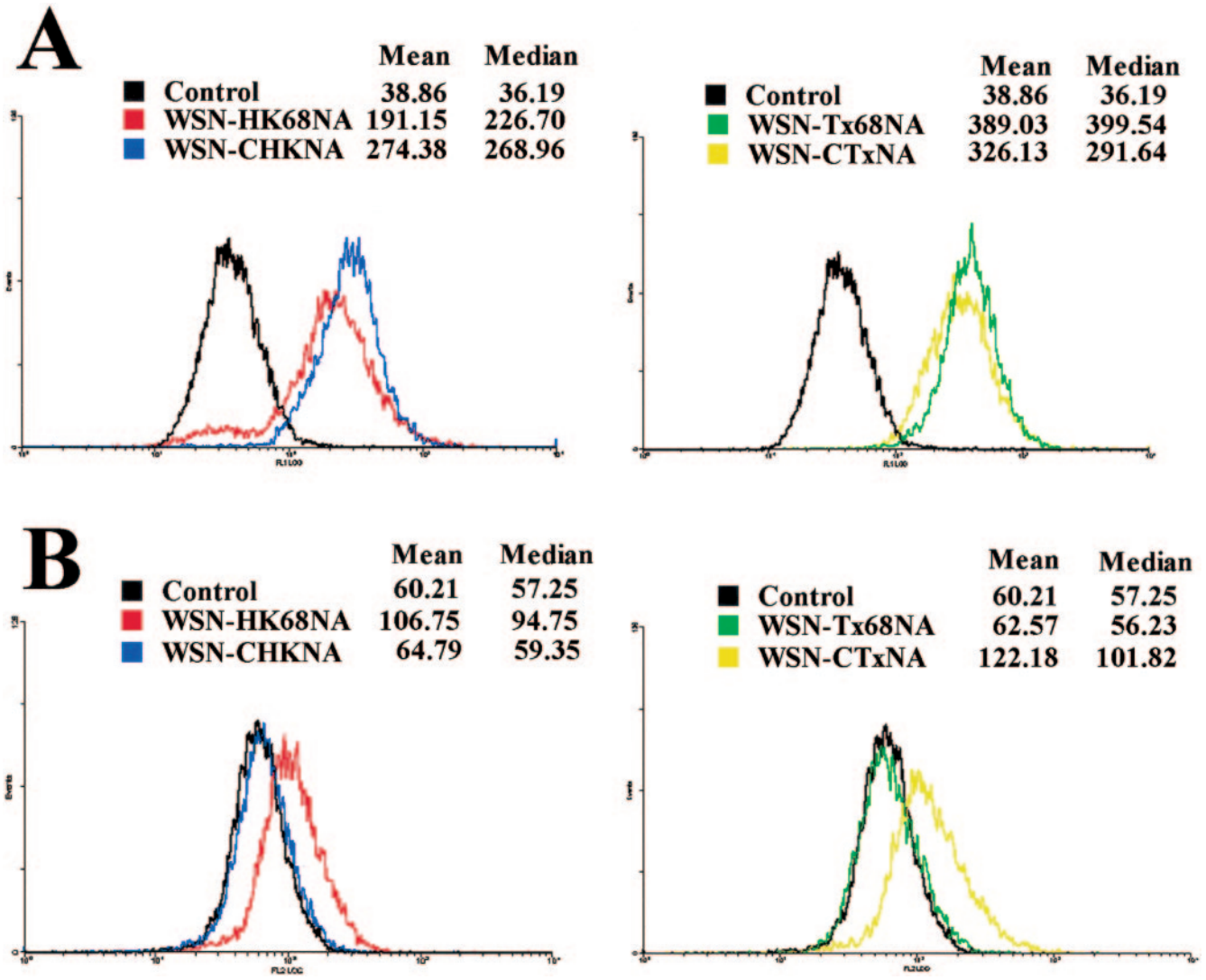

FIG. 6. Comparison of the levels of adsorption of transfectant viruses to MDCK cells and their sialidase activities under the endocytosis pathway by FACS analysis. MDCK cells were adsorbed with each transfectant virus on ice for 90 min. The cells were fixed and permeabilized just after adsorption and after 90 min postinfection at 37°C as described in Materials and Methods. (A) The transfectant viruses adsorbed to the cells were detected with anti-H1N2 polyclonal antibody. (B) Viral sialidase activities in the cells at 90 min postinfection were detected with X-Neu5Ac and Fast Red Violet LB. Each experiment was performed twice; representative data are shown. The mean and median fluorescence intensities are shown in the panels. WSN-HK68NA, red; WSN-CHKNA, blue; WSN-Tx68NA, green; WSN-CTxNA, yellow; mock, black.

viruses bearing low-pH-stable sialidase under endocytic trafficking resulted in a significant decrease of progeny virus from infected cells. However, the replication levels of the viruses bearing low-pH-unstable sialidase were not affected by treatment with zanamivir. In contrast, the inhibition of the viral sialidases had no effect on the number of virus-infected cells (Fig. 7B). To confirm that the transfectant viruses had susceptibilities to zanamivir, MDCK cells infected with each transfectant virus were treated with zanamivir after 4 h of virus infection at 37°C. The growth of the viruses tested after 18 h of virus infection were remarkably inhibited by zanamivir (Fig. 7D). The results indicate that the maintenance of the sialidase activity of influenza virus under endocytic trafficking considerably contributes to virus replication in MDCK cells.

Effect of the low-pH stability of sialidase activity on the process of the removal of sialic acid from HA in the Golgi regions of virus-infected cells. NA is also known to prevent the aggregation of progeny virions by removing sialic acid from *N*-linked oligosaccharides of newly synthesized HA and NA (18). The effects of the low-pH stability of viral sialidase on the process of the removal of sialic acid from HA in the Golgi regions and on the plasma membranes of MDCK and COS7 cells were examined by inhibition of zanamivir on the hemadsorption activity of the virus-infected cells as previously described (10). The hemadsorption activities of both MDCK and COS7 cells infected with WSN-HK68NA, WSN-CHKNA, WSN-Tx68NA, or WSN-CTxNA showed that NA of each transfectant virus removed sialic acid from approximately half

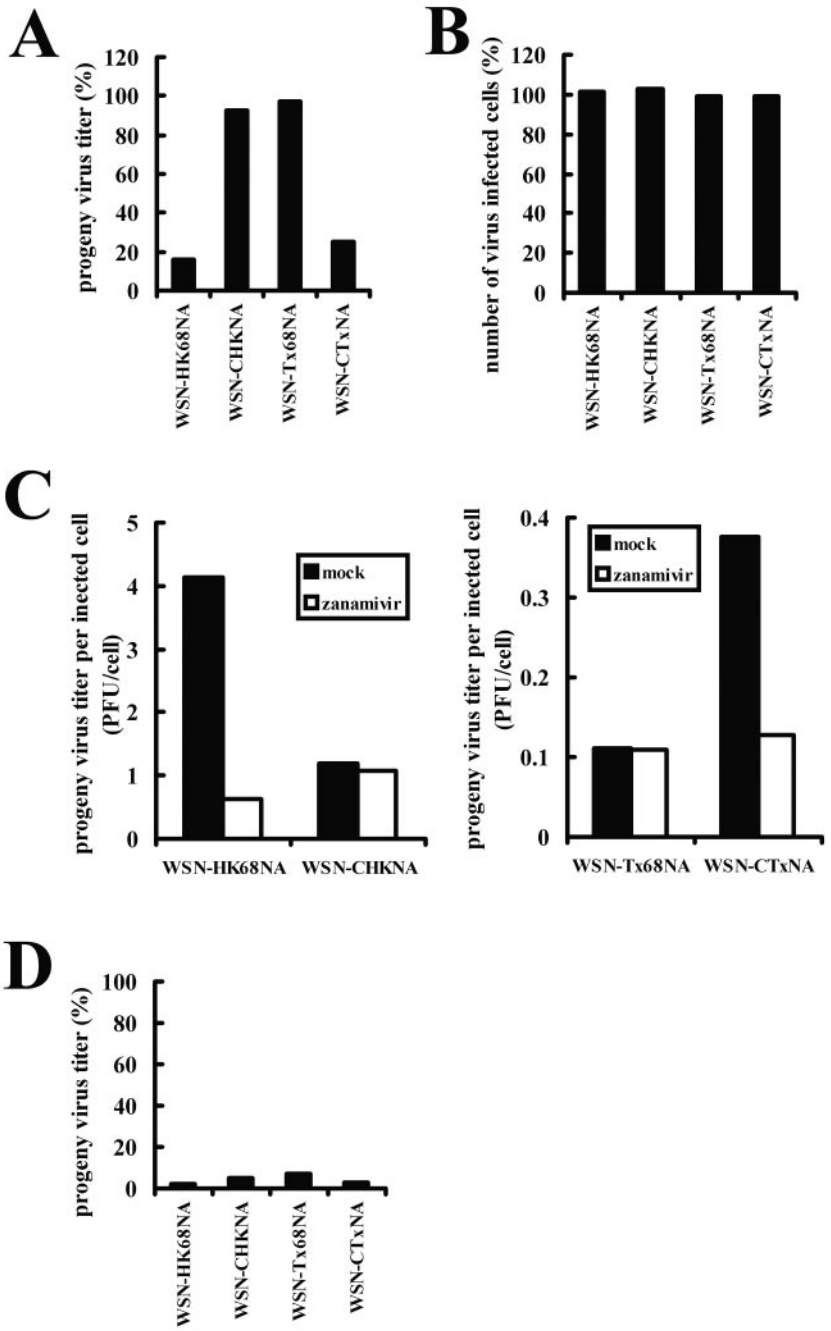

FIG. 7. Suppression of infectious progeny virus by inhibition of sialidase activities of transfectant virus NAs under endocytosis. MDCK cells were treated with 5 μM zanamivir and 0.001% DEAE-dextran up to 3.5 h postinfection. After treatment with zanamivir, the cells were washed thoroughly to exclude residual zanamivir. The culture media (A) were harvested at 18 h after the infection of each transfectant virus, and the cells were fixed with cold methanol. As controls, MDCK cells were treated with zanamivir after 4 h of virus infection, and the media (D) were harvested at 18 h after infection. Progeny virus titers in the media (A and D) and the viral-antigen-positive cells (B) are shown as percentages of that of each counterpart control without zanamivir. Progeny virus titers per viral-antigen-positive cell (C) were calculated to estimate the effect of zanamivir on viral replication among the four transfectant viruses.

of the newly synthesized HA in the Golgi regions of MDCK cells, similar to what occurred with COS cells. The data indicate no significant differences among the viruses from what occurred in the presence of zanamivir (Fig. 8).

Effect of low-pH-stable sialidase on the replication of influenza A virus in mice. We investigated whether the low-pH stability of influenza virus sialidase affects the replicative potential of influenza A virus in mice. We determined virus titers in various organs of three additional mice after intranasal inoculation with 10^4 PFU of each of the transfectant viruses (Table 1). The average titers of WSN-HK68NA and WSN-CTxNA were approximately 9.2×10^6 and 7.2×10^4 PFU/g

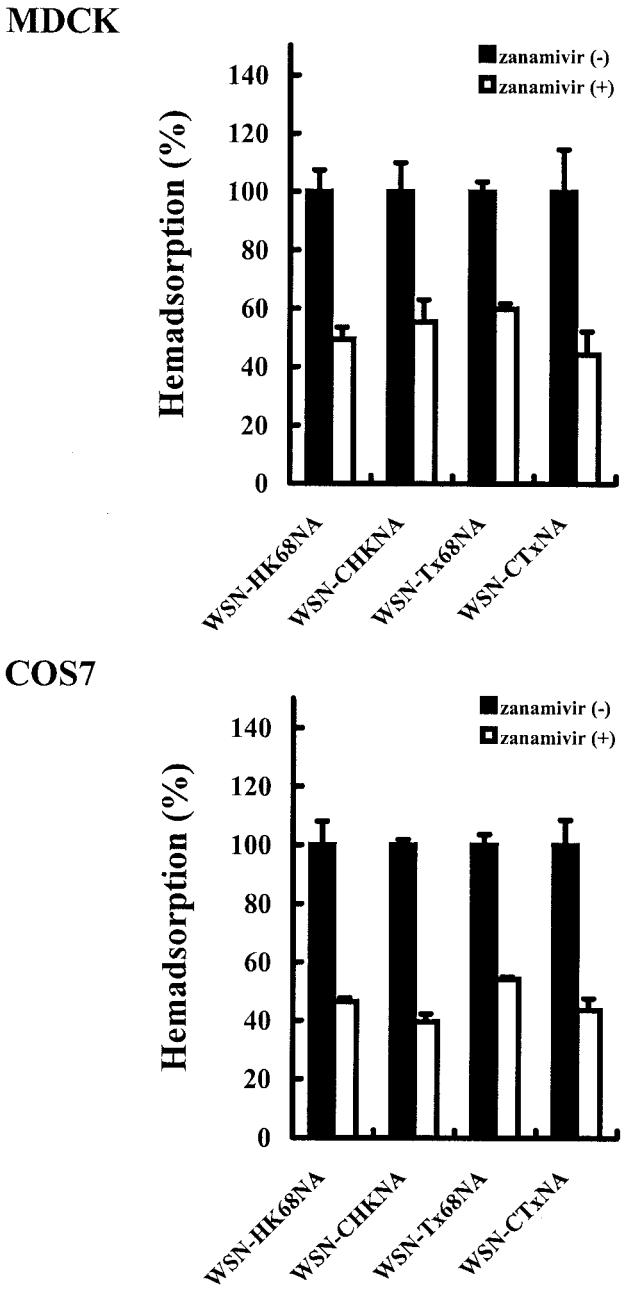

FIG. 8. Effect of zanamivir on hemadsorption of MDCK and COS7 cells infected with the transfectant viruses. MDCK and COS7 cells were infected with each transfectant virus. After incubation with 5 μM zanamivir for 13 h, hemadsorption of the cells was determined by absorbance at 545 nm and is shown as a percentage relative to the hemadsorption of cells incubated without zanamivir. Each value is the mean from triplicate experiments.

tissue, respectively, in lungs on day 3 after inoculation. Replication of the viruses was limited to the lungs. In contrast, WSN-Tx68NA and WSN-CHKNA infection were rapidly cleared in all organs tested on day 3 after inoculation. The results indicate that the low-pH stability of influenza A virus sialidase should promote the ability of a virus to replicate in lungs.

TABLE 1. Determination of virus titers in tissues of mice inoculated intranasally with the transfectant viruses

Transfectant virus	Mouse lot	Virus titer (log PFU/g) in[a]:				
		Lung	Brain	Kidney	Liver	Spleen
WSN-HK68NA	1	6.10	—	—	—	—
	2	7.27	—	—	—	—
	3	6.89	—	—	—	—
WSN-CHKNA	1	—	—	—	—	—
	2	—	—	—	—	—
	3	—	—	—	—	—
WSN-Tx68NA	1	—	ND	ND	ND	ND
	2	—	ND	ND	ND	ND
	3	—	ND	ND	ND	ND
WSN-CTxNA	1	4.87	ND	ND	ND	ND
	2	4.77	ND	ND	ND	ND
	3	4.95	ND	ND	ND	ND

[a] Mice were inoculated intranasally with 100 μl of 10^4 PFU of each transfectant virus. All mice were sacrificed on day 3 after infection. The virus titers in each tissue were determined by the plaque-forming assay. —, no virus could be detected. ND, not determined.

DISCUSSION

We found that sialidase activities in the 1957 and 1968 pandemic influenza A virus strains, A/Singapore/1/57 (H2N2), A/Japan/305/57 (H2N2), A/Aichi/2/68 (H3N2), and A/Hong Kong/1/68 (H3N2), as well as in duck viruses were maintained under low-pH conditions and that consensus amino acid regions responsible for low-pH stability did not exist in pandemic NAs but that two amino acid substitutions in the low-pH-stable A/Hong Kong/1/68 (H3N2) NA and a single substitution in the low-pH-unstable A/Texas/68 (H2N2) NA resulted in a significant change in low-pH stability (27, 29).

In this study, we therefore generated four reverse-genetics viruses (WSN-HK68NA, WSN-CHKNA, WSN-Tx68NA, and WSN-CTxNA) from the genetic background of A/WSN/33 (H1N1) and examined whether the low-pH stability of virus sialidase activity has an effect on virus replication in MDCK cells and in mice. Virus growth kinetics and plaque size on MDCK cells showed that WSN-Tx68NA and WSN-CHKNA (with alterations in two amino acids of A/Hong Kong/1/68 NA), which had low-pH-unstable sialidase, exhibited a highly attenuated phenotype in virus replication on MDCK cells, in comparison with WSN-HK68NA and WSN-CTxNA (with an alteration of one amino acid in A/Texas/68 NA), which had low-pH-stable sialidase. Surprisingly, in the mouse lung, WSN-HK68NA and WSN-CTxNA exhibited the ability to replicate, but WSN-Tx68NA and WSN-CHKNA were cleared from the lungs. In a mouse model of H5N1 and WSN virulence, virus was isolated from systemic organs, such as the brain, spleen, kidney, and liver (2, 4). In this study, the transfectant viruses were isolated from the lung but not other organs. A/WSN/33 (H1N1) HA does not have a multibasic cleavage site such as H5 HA. Goto and Kawaoka showed that A/WSN/33 (H1N1) virus NA can sequester plasminogen in serum, providing a mechanism for the enhancement of WSN HA cleavage. In contrast, other virus NAs, including A/Hong Kong/1/68 (H3N2) NA, in which the low-pH stability of sialidase activity has been found, could not promote WSN HA cleavage in the presence of FBS or either bovine or human plasminogen (3). We also demonstrated that transfectant viruses bearing low-

pH-stable sialidase enhanced the replicative ability in MDCK cells by using a medium without FBS. Our results indicate that the low-pH stability of the virus sialidase contributes to the promotion of virus replication in the lung.

To determine whether the low-pH stability of virus sialidase is associated with some intracellular acidic compartments, such as lysosome, endosome, and the *trans* Golgi network, we used an in situ fluorescent assay with X-Neu5Ac and Fast Red Violet LB to detect the enzymatic activity of influenza virus sialidase in cultured cells. We demonstrated, by using laser scanning confocal microscopy and FACS analysis of virus-infected MDCK cells, that the sialidase activity of the transfectant viruses bearing low-pH-unstable sialidase under endocytosis decreased to the mock level. In contrast, the transfectant viruses bearing low-pH-stable sialidase maintained the enzyme activity even under low-pH conditions of endosomal compartments. Inhibition of sialidase activity of the viruses bearing low-pH-stable sialidase under endocytic trafficking resulted in a significant decrease of progeny virus from infected cells. However, the replication level of the viruses bearing low-pH-unstable sialidase was not affected by treatment with zanamivir. These findings indicate that the continuous sialidase activity of influenza virus bearing low-pH-stable NA during the endocytic pathway contributed to an increase in progeny virus titers from infected cells.

We demonstrated by hemadsorption assays of transfectant virus-infected cells that the difference in the low-pH stabilities of the viral sialidases had no effect on the process of the removal of sialic acid from newly synthesized HA in the Golgi regions. In the *trans* Golgi network, proton channel activity of newly synthesized M_2 protein, which causes equilibration of the acidic pH of the lumen of the Golgi apparatus with the cytoplasm (5, 21), may prevent inactivation of the low-pH-unstable sialidase.

Recently, Matrosovich et al. reported that influenza virus NA was important for the initiation of influenza virus infection in cultures of primary human tracheobronchial and nasal epithelial cells (11). In that study, the NA inhibitor oseltamivir carboxylate remarkably reduced the infection of not only avian influenza viruses but also 1996 and 1997 human influenza viruses, in which low-pH-unstable sialidases are probably maintained. On the other hand, our results showed that inhibition of the sialidase activities of viruses bearing low-pH-stable sialidase under endocytic trafficking resulted in a significant decrease in progeny virus from infected MDCK cells. However, the replication level of the viruses bearing low-pH-unstable sialidase was not affected by treatment with zanamivir. Human airway epithelial cells but not MDCK cells secrete mucins that protect them from pathogenic insult (25). These differences may indicate that influenza virus NA functions mainly in the removal of decoy receptors on mucins at the initial stage of influenza virus infection in cultures of primary human tracheobronchial and nasal epithelial cells. In addition, pH conditions of the endosomal compartments in MDCK cells and mouse pulmonary tissues may be different from that of primary human tracheobronchial and nasal epithelial culture cells.

We investigated whether the maintenance of the sialidase activity of influenza virus under endocytic trafficking enhances hemagglutinin-mediated fusion (7) and release of viral ribonucleoproteins. However, we could not find distinct differences among the viruses in the transport of viral RNPs to the nucleus by observation with a laser scanning confocal microscope using an anti-NP monoclonal antibody (data not shown). It has been reported that influenza virus NA functioned as an inducer of apoptosis in MDCK cells and human monocyte U937 cells (14, 19) and that virus-induced apoptosis executed by caspase 3 activation was a crucial event for efficient influenza virus propagation (34). In addition, lysosomal sialidase of cultured human colon cancer cells was up-regulated in the process of apoptosis (8). Our findings therefore indicate that efficient influenza virus propagation in the mouse lung and MDCK cells infected with the transfectant viruses bearing the low-pH-stable sialidase may be related to apoptosis induced by the virus sialidase maintained under low-pH conditions of the endosomal compartments. This hypothesis is supported by the results of our recent study in which FACS analysis using annexin V showed that apoptosis induced in human monocyte U937 cells infected with each transfectant virus is obviously different between low-pH-stable and -unstable sialidases (unpublished data). We speculate that the low-pH stability of sialidase activity maintained in avian (duck)-like NA of human pandemic influenza viruses facilitates viral pathogenicity and transmission. Additional studies of viral propagation should be carried out in the future.

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