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Influenza A Virus Neuraminidase Limits Viral Superinfection▽

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Enveloped viruses use multiple mechanisms to inhibit infection of a target cell by more than one virion. These mechanisms may be of particular importance for the evolution of segmented viruses, because superinfection exclusion may limit the frequency of reassortment of viral genes. Here, we show that cellular expression of influenza A virus neuraminidase (NA), but not hemagglutinin (HA) or the M2 proton pump, inhibits entry of HA-pseudotyped retroviruses. Cells infected with H1N1 or H3N2 influenza A virus were similarly refractory to HA-mediated infection and to superinfection with a second influenza A virus. Both HA-mediated entry and viral superinfection were rescued by the neuraminidase inhibitors oseltamivir carboxylate and zanamivir. These inhibitors also prevented the removal of α-2,3- and α-2,6-linked sialic acid observed in cells expressing NA or infected with influenza A viruses. Our data indicate that NA alone among viral proteins limits influenza A virus superinfection.

Influenza A viruses are enveloped, segmented, negative-stranded RNA viruses. They are comprised of eight RNA segments encoding at least 10 distinct proteins (33, 36, 37). Three of these proteins are expressed on the plasma membrane and incorporated in the envelope of the budding virion: hemagglutinin (HA), neuraminidase (NA), and an ion channel (M2). In addition, the budding virion includes the M1 matrix protein and eight ribonucleoprotein (RNP) complexes, comprising each of the eight RNA segments associated with viral proteins, including the viral nucleocapsid and the three components of the transcriptase complex (PA, PB1, and PB2). Release of the virion from the cell requires NA, which cleaves sialic acids (SA) that would otherwise bind HA and prevent the virion from escaping the virus-producing cell (28, 41). The HA of released virions binds SA on a target cell. The SA-bound virion is endocytosed into an acidified endosomal compartment. There, the M2 ion channel facilitates acidification of the virion and dissociation of the RNP complexes from the M1 matrix protein (38, 39). The low pH of the endosome induces conformational changes in HA that promote mixing of viral and endosomal lipids, membrane fusion, and entry of the RNP complexes (3, 44, 55).

NA, like HA and M2, is incorporated into the envelope of the budding virion, which has led investigators to focus on the role of virion-associated NA in virion release (19, 53). The original discovery of NA's role as the determinant of the "receptor-destroying" activity of free virus in hemagglutination reactions has reinforced this focus on virion-associated NA (7, 41). Previous studies have also identified a role for virion-bound NA in cleaving SA in the extracellular space (24). To date, no function has been described for NA expressed on the virus-producing cell. The roles of cell surface and virion-expressed NA are of some interest due to the current clinical use of two NA inhibitors for treatment of influenza A virus infection. Oseltamivir carboxylate (the active form of Tamiflu) and zanamivir (Relenza) are SA analogs that interfere with the sialidase activity of NA (28).

Enveloped viruses can prevent the entry of additional virions into infected cells, usually by expressing proteins that interfere with expression of the viral receptor. Notably, cell-expressed HA-neuraminidases of several paramyxoviruses mediate such superinfection exclusion by removing the SA receptor from the cell surface (15, 27). Other mechanisms of superinfection exclusion have also been described. For example, the envelope glycoproteins of alpha- and gammaretroviruses limit superinfection through receptor interference by directly engaging the receptor in the producer cell (1). Human immunodeficiency virus type 1 (HIV-1) encodes the nef protein, which promotes internalization and degradation of the HIV-1 receptor CD4 (9, 21). In addition to limiting superinfection, receptor down-regulation can inhibit premature intracellular fusion, prevent reinfection by a budding virion, or facilitate virion release (4). Superinfection has an additional potential consequence for segment viruses, such as influenza A virus, because RNA segment reassortment requires infection of the same cell by two viruses (54). To date, it has not been determined whether, and by what mechanism, influenza A viruses inhibit superinfection.

Here, we examined the ability of the three cell surface-expressed influenza A virus proteins to inhibit infection mediated by the HA protein. We show that NA from multiple isolates, but not HA or M2 protein, efficiently inhibited infection of retroviruses pseudotyped with a range of HA molecules. Cells infected with either H1N1 or H3N2 influenza A virus were similarly refractory to HA-mediated entry and to superinfection with a second influenza A virus. Both oseltamivir carboxylate and zanamivir rescued the efficiency of HA-mediated entry and influenza A virus superinfection. Cells expressing NA, or infected with influenza A virus, had markedly lower levels of α-2,3- and α-2,6-linked SA, and SA expres-

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sion was restored by both NA inhibitors. Our data indicate that NA, alone among viral proteins, limits influenza A virus superinfection and does so by removing surface SA from the virion-producing cell.

MATERIALS AND METHODS

Cells and reagents. Human 293T and Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection [ATCC]) were maintained in Dulbecco's minimal essential medium (DMEM) (Invitrogen). Human lung epithelial A549 cells were grown in RPMI medium (Invitrogen). Both media were supplemented with 10% fetal bovine serum (Atlanta Biologicals), 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Oseltamivir carboxylate was a generous gift of K. S. Shia (National Health Research Institute, Taiwan). Zanamivir hydrate was purchased from Sequoia Research Products, United Kingdom.

Plasmids and constructs. The pPolISapIRib (pPSR) vector-based reverse-genetic system for influenza A virus A/PR/8/34 (H1N1) was a generous gift from G. G. Brownlee and E. Fodor (Oxford University) (8, 47). The pHH21 vector-based reverse-genetic system for influenza A virus A/Udorn/72 (H3N2) was kindly provided by R. A. Lamb (Northeastern University) (32, 50). Oseltamivir-resistant pPSR-NA H260Y (H274Y with N2 numbering) (12) and amantadine-sensitive pPSR-M, M2 A27V, I28V, and N31S mutations (48) were generated by the QuikChange method (Stratagene). pCAGGS.MCS-based plasmids that encode A/South Carolina/1/1918 (H1N1) HA protein [pCAGGS-H1(SC)] or A/Brevig Mission/1/1918 (H1N1) NA protein [pCAGGS-N1(SC)] were generous gifts from P. Palese (Mount Sinai School of Medicine) (11, 51). pCAGGS-H7(FPV), which encodes HA proteins for A/FPV/Rostock/34 (H7N1), was kindly provided by X. Yang (Dana Farber Cancer Institute) (56). A codon-optimized plasmid that contains the sequence of A/Hong Kong/481/1997 (H5N1) M2 protein [pcDNA3.1-M2(HK)] was a generous gift from T. Mirzabekov (MSM Technologies). Coding regions of influenza A virus A/PR/8/34 (H1N1) HA or NA protein were amplified using plasmids pPSR-HA and pPSR-NA as templates. The PCR products were cloned into the NheI/KpnI restriction sites of pcDNA3.1 (Invitrogen) [pcDNA3.1-N1(PR)] and the EcoRI/XhoI sites of pCAGGS.MCS [pCAGGS-N1(PR) and pCAGGS-H1(PR)] (20, 34). Enzymatically inactive pCAGGS-N1(PR) E262D was created using the QuikChange method (22). Plasmids that encoded codon-optimized NA and HA proteins for influenza A virus A/Thailand/2(SP-33)/2004(H5N1) were created by recursive PCR. The PCR products were cloned into NheI/KpnI [pcDNA3.1-N1(Thai)], or AgeI/BamHI [pcDNA3.1-H5(Thai)] restriction sites of pcDNA3.1. Coding regions of C-terminally c-*myc*- or C9-tagged NA proteins and N-terminally c-*myc*-tagged HA proteins were amplified using plasmids encoding nontagged proteins as templates. The PCR products were cloned into the EcoRI/XhoI (NA plasmids) or SacI/XhoI (HA plasmids) restriction sites of pCAGGS.MCS. The region encoding an N-terminally c-*myc*-tagged M2 protein was amplified and cloned into the NheI/KpnI restriction sites of pcDNA3.1.

Influenza A viruses. Influenza A viruses A/PR/8/34 (H1N1) and Alice strain (H3N2) were purchased from the ATCC. Alice strain is a recombinant between A/PR/8/34 and A/England/42/72 (H3N2), with HA and NA segments derived from the latter strain (23). Various recombinant A/PR/8/34 (H1N1) and A/Udorn/72 (H3N2) viruses, carrying different combinations of an amantadine-resistant/sensitive M segment and an oseltamivir-resistant/sensitive NA segment, were generated following a previously described method (8). The recombinant influenza A viruses used in this study included amantadine-sensitive, oseltamivir-resistant influenza A virus A/PR/8/34 (H1N1) and amantadine-resistant, oseltamivir-sensitive influenza A virus A/Udorn/72 (H3N2). All viruses were propagated in MDCK cells cultured in DMEM supplemented with 0.2% bovine serum albumin (Sigma) and 2 μg/ml l-1-tosylamido-2-phenylethyl chloromethyl ketone (TPCK)-treated trypsin (Worthington) at 34°C for 48 h (42). The culture supernatant was filtered through a 0.45-μM syringe filter (Nalgene), and viruses were titered by standard plaque assay procedures (14).

Pseudotyped retroviruses. 293T cells plated at 70% confluence in T75 culture flasks were transfected using the calcium phosphate method (6) with 10 μg plasmid DNA encoding Moloney murine leukemia virus (MLV) gag and pol, 10 μg pQCXIX-based (BD Biosciences) green fluorescent protein (GFP)-expressing vector, 12 μg plasmid pcDNA3.1-N1(PR), and 3 μg of a pCAGGS.MCS-based plasmid encoding various influenza A virus HA proteins [H1(PR), H5(Thai), and H7(FPV)] (25, 26). For producing MLV-GFP pseudotyped with H1(SC), 12 μg pCAGGS-H1(SC) and 3 μg pCAGGS-N1(SC) were used. Vesicular stomatitis Indiana virus G protein (VSV-G)- and amphotropic MLV envelope glycoprotein (MLV-env)-pseudotyped MLV-GFP were included as control viruses (25, 26). Following transfection, 293T cells were washed once with phosphate-buffered saline and maintained in serum-containing medium [H5(Thai)-, H7(FPV)-, VSV-G-, and MLV-env-pseudotyped viruses] or in serum-free DMEM supplemented with 0.2% bovine serum albumin [H1(SC) and H1(PR) pseudotypes]. After 24 h [H5(Thai)- and H7(FPV)-pseudotyped MLV-GFP] or 48 h ([H1(SC), H1(PR), VSV-G, and MLV-env pseudotypes], the culture supernatant was harvested and filtered through a 0.45-μm syringe filter. Before cell transduction, the viral supernatant of H1(SC) and H1(PR) pseudotypes was incubated with 16 μg/ml TPCK-treated trypsin for 1 hour at 25°C and then mixed with trypsin-neutralizing solution (Cambrex). 293T cells in 24-well poly-D-lysine (Sigma)-coated plates or A549 cells in 12-well plates with 70% confluence were then incubated with 200 μl (293T cells) or 500 μl (A549 cells) viral supernatant for 1 hour. Forty-eight hours after infection, the cells were harvested, fixed with 1% formaldehyde (Polysciences Inc.), and analyzed by flow cytometry.

Transfection and flow cytometry. 293T cells were transfected with plasmid encoding various influenza A virus HA, NA, or M2 proteins using the calcium phosphate method (6). Transfection of A549 cells with plasmids encoding NA protein variants was performed using Lipofectamine 2000 (Invitrogen). In parallel with pseudotype infection or SA expression assays, protein cell surface expression was determined. c-*myc*-tagged proteins (NA, HA, and M2) were labeled with 5 μg/ml of the murine anti-c-*myc* antibody 9E10 (National Cell Culture Center). C9-tagged proteins were stained with 5 μg/ml of the murine anti-C9 antibody 1D4 (National Cell Culture Center). *R*-phycoerythin (PE)-conjugated goat anti-mouse immunoglobulin G (IgG) (Sigma) was used as the secondary antibody for both 9E10 and 1D4. After being labeled, the cells were washed three times with staining buffer, fixed with 1% formaldehyde, and analyzed by flow cytometry.

SA expression. 293T cells, plated in six-well plates to 90% confluence, were incubated with infectious influenza A virus A/Udorn/72 (H3N2) or A/PR/8/34 (H1N1) at a multiplicity of infection (MOI) of 1 for 1 h. Five hours later, cells were harvested for SA staining. During the total 6-h period, the cells were treated with 1 μM oseltamivir carboxylate, 1 μM zanamivir, or medium alone. To measure SA levels, infected cells were labeled for 1 hour with 10 μg/ml biotinylated *Maackia amurensis* lectin II (which preferentially recognizes α-2,3-linked SA; Vector Laboratories) or with 5 μg/ml biotinylated *Sambucus nigra* lectin (which preferentially recognizes α-2,6-linked SA; Vector Laboratories) (43) and stained with 5 μl (in 50 μl staining buffer) allophyocyanin-conjugated streptavidin (BD Biosciences) for an additional hour. The cells were washed with staining buffer, fixed with 1% formaldehyde, and analyzed by flow cytometry. 293T cells, plated in six-well plates, were transfected with 1 μg of vector alone or with plasmids encoding C-terminally C9-tagged influenza virus N1(PR), N1(SC), or N1(Thai) proteins. The cells were then treated with 1 μM oseltamivir carboxylate, 1 μM zanamivir, or medium alone. Two days after transfection, SA staining was performed using the procedures described above.

Superinfection assays. 293T cells, plated to 90% confluence in six-well plates, were incubated with either H1N1 (A/PR/8/34) or H3N2 (Alice strain) virus at an MOI of 1 for 1 hour. Five hours later, the cells were incubated with a second virus, infectious H3N2 (Alice strain) or H1N1 (A/PR/8/34) virus, respectively, at an MOI of 1 for an additional hour. Over the entire infection period, the cells were treated with 1 μM oseltamivir carboxylate, 1 μM zanamivir, or medium alone. After infection, the cells were grown in serum-containing medium overnight without inhibitor and then treated with 1 U/ml *Clostridium perfringens* neuraminidase (Sigma) to remove adhered viral particles for 2 h before being harvested. The infected cells were then labeled with 1 μg/ml murine anti-influenza virus H1 IgG2a (C179) and anti-influenza virus H3 IgG1 (F49; Takara Bio) (35, 52) and stained with 170 ng/ml PE-conjugated goat anti-mouse IgG2a and 1 μg/ml fluorescein isothiocyanate-conjugated goat anti-mouse IgG1 antibodies (Pierce Biotechnology). The cells were fixed with 1% formaldehyde and analyzed by flow cytometry. For superinfection assays using A549 cells, cells were plated to 90% confluence in six-well plates and incubated with H3N2 (A/Udorn/72) virus at an MOI of 1.5 for 1 hour. The cells were then treated with 1 μM oseltamivir carboxylate, 1 μM zanamivir, or medium alone for 5 h; washed three times in phosphate-buffered saline; and incubated with a second H1N1 (A/PR/8/34) virus at an MOI of 5 for another hour. Sixteen hours later, A549 cells were treated with 1 U/ml *C. perfringens* neuraminidase for 4 h, labeled with the antibodies described above, and analyzed by flow cytometry.

To evaluate superinfection of highly pathogenic avian influenza virus (HPAIV) HA protein-pseudotyped MLV-GFP, 293T cells, plated to 90% confluence in 24-well plates, were incubated with influenza A virus A/PR/8/34 (H1N1) or Alice strain (H3N2) at an MOI of 1 for 1 hour. One hour before or 2, 4, 6, or 8 h after the initial incubation with infectious viruses, the cells were incubated with H5(Thai)-, H7(FPV)-, or VSV-G-pseudotyped MLV-GFP for an

TABLE 1. Origins of influenza A virus proteins used in these studies[a]

Name used	Influenza A virus origin
Plasmids expressing single viral proteins	
H1(PR)	A/PR/8/34 (H1N1)
H1(SC)	A/South Carolina/1/1918 (H1N1)
H5(Thai)	A/Thailand/2(SP-33)/2004 (H5N1)
H7(FPV)	A/FPV/Rostock/34 (H7N1)
M2(HK)	A/Hong Kong/481/97 (H5N1)
N1(PR)	A/PR/8/34 (H1N1)
N1(SC)	A/Brevig Mission/1/1918 (H1N1)
N1(Thai)	A/Thailand/2(SP-33)/2004 (H5N1)
MLV-GFP pseudoviruses	
H1(SC)	HA: A/South Carolina/1/1918 (H1N1) NA: A/Brevig Mission/1/1918 (H1N1)
H1(PR)	HA: A/PR/8/34 (H1N1) NA: A/PP/8/34 (H1N1)
H5(Thai)	HA: A/Thailand/2(SP-33)/2004 (H5N1) NA: A/PP/8/34 (H1N1)
H7(FPV)	HA: A/FPV/Rostock/34 (H7N1) NA: A/PP/8/34 (H1N1)
MLV-env	Env: amphotrophic murine leukemia virus
VSV-G	G: vesicular stomatitis Indiana virus

[a] Complete names of viruses from which NA, HA, and M2 genes expressed in cells and used to generate MLV-GFP pseudoviruses were derived are given. Entry proteins of VSV and MLV, used as controls in several experiments, are also listed. As indicated, pseudovirus names are based on their respective entry proteins.

additional hour. During the entire infection period, the cells were treated with 1 μM oseltamivir carboxylate, 1 μM zanamivir, or medium alone. After this infection period, the cells were maintained in regular medium without inhibitor. Two days later, the 293T cells were harvested, fixed with 1% formaldehyde, and analyzed by flow cytometry.

Viral reassortment assays. MDCK cells at 80% confluence in six-well plates were incubated with recombinant amantadine-resistant, oseltamivir-sensitive influenza A virus A/Udorn/72 (H3N2) at an MOI of 1 for 1 hour. Three hours later, the cells were incubated with a second virus, recombinant amantadine-sensitive, oseltamivir-resistant influenza A virus A/PR/8/34 (H1N1), at an MOI of 1 for an additional hour. During the entire 5-hour infection period, the cells were treated with medium alone or with 1 μM oseltamivir carboxylate. The infected cells were then grown in medium containing 1 μM oseltamivir carboxylate, 2.5 μg/ml amantadine, and 2 μg/ml TPCK-treated trypsin. Sixteen hours later, the viral supernatant was filtered through a 0.45-μM syringe filter and incubated with 293T cells for 1 hour. Sixteen hours later, the infected 293T cells were labeled with Alexa 647 (Pierce)-conjugated anti-H3 IgG1 (F49), Alexa 488 (Pierce)-conjugated anti-H1 IgG2a (H36-4-5.2), biotin (Dojindo)-conjugated anti-N1 IgG2a (NA-112-S2.4), and peridinin chlorophyll *a* protein-conjugated streptavidin (BD Biosciences). Anti-influenza A virus antibodies H36-4-5.2 and NA-112-S2.4 were generous gifts from W. Gerhard (Wistar Institute) (10, 29, 45). The labeled cells were then fixed with 1% formaldehyde and analyzed by flow cytometry.

RESULTS

Retroviruses pseudotyped with the HPAIV H7 have been described (25, 40). Here, we generated pseudoviruses by cotransfecting 293T cells with plasmids expressing HA, retroviral proteins, and a packaged GFP reporter gene. In order to facilitate virion release, an NA-expressing plasmid was also included (Table 1 shows the origins of HA and NA used for production of pseudoviruses). HPAIVs have a polybasic site at the HA1/HA2 junction that is processed by furin-like proteases in the virus-producing cell (44, 46). Human influenza viruses typically lack this site and are cleaved by extracellular proteases (17, 18, 30). We generated four pseudoviruses expressing HA from two human viruses and two HPAIVs (Table 1 shows the nomenclature and viral origins of pseudoviruses and influenza A virus proteins). Human H1(SC) or H1(PR) pseudovirus was activated by trypsin before incubation with target cells, whereas trypsin was not used to activate avian H5(Thai) and H7(FPV) pseudoviruses.

To initially explore the roles of three candidate viral proteins in limiting influenza A virus superinfection, we expressed various HA, NA, or M2 proteins in 293T cells and assessed their effects on pseudovirus entry. We hypothesized that HA might exclude superinfection through receptor interference, that NA might enzymatically remove the SA receptor, and that M2 could disrupt the endosomal compartment where fusion occurs. As shown in Fig. 1A, neither H5(Thai) HA nor M2(HK) inhibited pseudovirus infection. In contrast, expression of the N1(Thai) NA prevented efficient entry mediated by several HA proteins. Entry of retroviruses pseudotyped with the VSV-G protein or with the MLV envelope glycoprotein was unaffected by expression of any influenza A virus protein tested. Each viral protein was expressed comparably, as indicated by flow cytometry using an anti-tag antibody (Fig. 1B). Expression of NA molecules, N1(SC) and N1(PR), from two human influenza A viruses were similarly capable of inhibiting entry mediated by HA, but not by control entry proteins (Fig. 1C and D). Expression of three additional HA proteins from human or avian viruses had no effect on pseudovirus entry (Fig. 1E and F). These data indicate that NA expressed on the cell surface inhibited HA-mediated entry.

We investigated the role of the enzymatic activity of NA in inhibiting HA-mediated infection. Two clinically used NA inhibitors, oseltamivir carboxylate and zanamivir, were examined for the ability to restore pseudovirus entry into NA-expressing cells. As little as 1 nM oseltamivir carboxylate enhanced the entry of three HA-pseudotyped viruses but had no effect on control pseudoviruses (Fig. 2A). In most cases, at 1 μM oseltamivir carboxylate, HA-mediated entry was similar to that of untreated cells lacking NA. This level is close to the peak plasma concentration (1.2 to 1.9 μM) of oseltamivir carboxylate obtained after administration of 75 mg Tamiflu twice daily (http://www.fda.gov/cder/foi/label/1999/21087lbl.pdf). Quantitatively similar results were observed using zanamivir (Fig. 2B). Neither NA inhibitor affected pseudovirus entry of cells lacking NA (Fig. 2C and D). The role of the NA activity in limited HA-mediated entry was confirmed using an enzymatically inactive N1(PR) variant, E262D, in A549 lung epithelial cells (Fig. 2E) or 293T cells (not shown). The expression of E262D was comparable to that of wild-type N1(PR) (Fig. 2F). We conclude that the ability of cell-expressed NA to inhibit HA-mediated entry is determined by its enzymatic activity.

We examined the effect of NA expression and influenza A viral infection on cell surface expression of two forms of SA, α-2,3 linked or α-2,6 linked, distinguished by the galactose carbon to which they bond (49). Expression of N1(PR), N1(SC), or N1(Thai) substantially reduced α-2,6-linked SA compared with mock-transfected cells or those expressing the

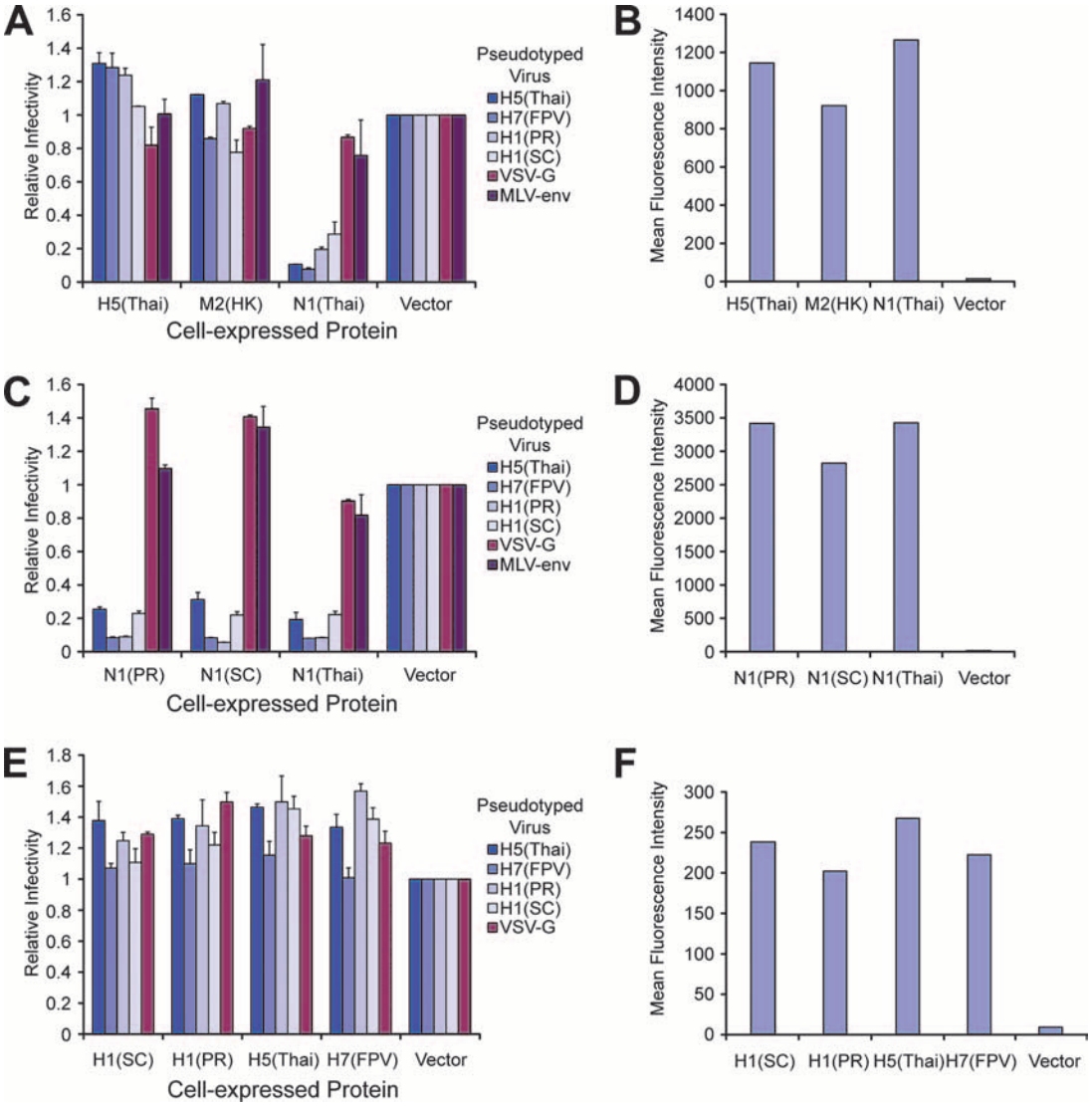

FIG. 1. Influenza A virus NA expression inhibits HA-mediated entry. (A) MLV-GFP pseudotyped with the indicated influenza A virus HA proteins, VSV-G protein, or amphotropic MLV envelope glycoprotein were incubated for 1 hour with 293T cells transfected with vector alone or plasmids expressing the indicated c-*myc*-tagged influenza A virus proteins. Pseudovirus infection was determined by flow cytometry and normalized to that of cells transfected with vector alone. (B) In parallel, protein cell surface expression was assayed using an aliquot of the same cells analyzed in panel A. 293T cells were labeled with murine anti-c-*myc* IgG (9E10) and PE-conjugated secondary antibodies. The labeled cells were analyzed by flow cytometry. (C) An experiment similar to that in panel A, except that NA proteins from several different influenza A virus isolates were characterized. (D) An experiment similar to that in panel B, except that the expression levels of various NA proteins were determined. (E) An experiment similar to that in panel A, except that HA proteins from several different influenza A virus isolates were characterized. (F) An experiment similar to that in panel B, except that the expression levels of various HA proteins were determined. The error bars represent standard deviations.

inactive N1(PR) variant E262D (Fig. 3A). NA expression also reduced α-2,3-linked SA levels (Fig. 3B). In each case, SA expression was restored by 1 μM oseltamivir carboxylate or zanamivir. Cell surface expression levels of the NA proteins were similar and were not affected by either NA inhibitor (Fig. 3C). To determine if cells infected with influenza A virus showed a similar decrease in SA expression, we incubated 293T cells with infectious A/PR/8/1934 (H1N1) or A/Udorn/72 (H3N2) for 1 hour. Six hours after infection, cell surface α-2,3- and α-2,6-linked SA expression decreased similarly to that of cells transfected to express NA (Fig. 3D and E). Again, NA inhibitors restored SA expression. These data indicate that NA expression and influenza A virus infection remove cell surface SA. Loss of SA presumably accounts for the decreased HA-mediated entry observed in Fig. 1 and 2.

To assess the role of NA activity in limiting superinfection of replicating influenza A viruses, we incubated 293T cells sequentially with infectious A/PR/8/34 (H1N1) and Alice strain (H3N2) viruses in the presence of oseltamivir carboxylate, zanamivir, or medium alone. The following day, the cells were treated with bacterial neuraminidase to remove bound virions, labeled with H1- and H3-specific antibodies, and analyzed by

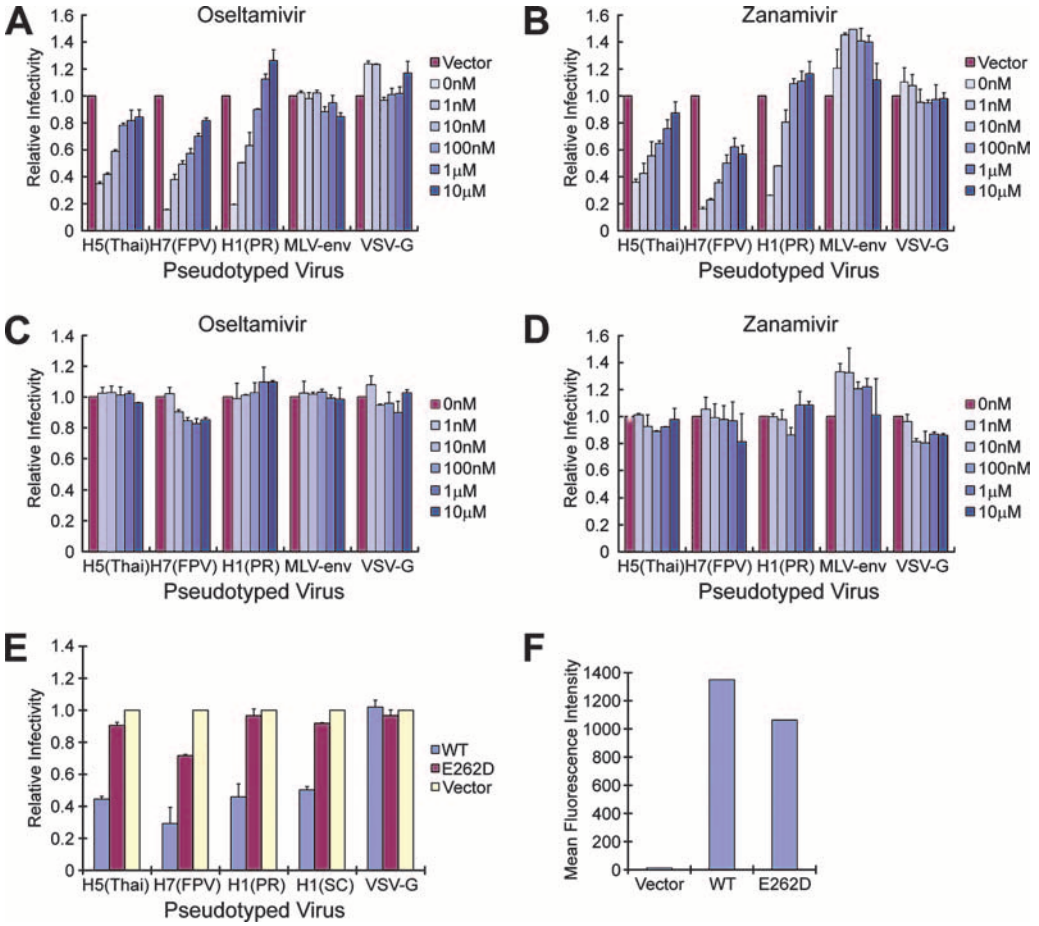

FIG. 2. Inhibition of HA-mediated entry requires enzymatic activity of NA. (A) 293T cells were transfected with vector alone or with a plasmid encoding NA of A/PR/8/34. NA-expressing cells were then treated with the indicated concentrations of oseltamivir carboxylate and infected with various MLV-GFPs pseudotyped with the indicated influenza A virus or control entry proteins. Pseudovirus infection was determined by flow cytometry and normalized to that of cells transfected with vector alone. (B) An experiment similar to that in panel A, except that cells were treated with the indicated concentrations of zanamivir. (C) Control experiments similar to that in panel A, except that entry of MLV-GFP pseudotypes was measured using 293T cells transfected with vector alone. (D) Control experiment similar to that in panel B, except that entry of MLV-GFP pseudotypes was measured using 293T cells transfected with vector alone. (E) A549 cells were transfected with vector alone or with plasmids encoding C9-tagged wild-type (WT) A/PR/8/34 NA protein or an enzymatically inactive variant (E262D mutant) thereof. Entry of MLV-GFP pseudotyped with the indicated entry proteins was assayed as in Fig. 1A. Pseudovirus infection was determined by flow cytometry and normalized to that of cells transfected with vector alone. (F) An experiment similar to that in Fig. 1B, except that murine anti-C9 IgG (1D4) was used to recognize these C9-tagged NA variants. The error bars represent standard deviations.

flow cytometry. Both oseltamivir carboxylate and zanamivir substantially increased the number of cells recognized by both antibodies, indicating that cells were infected with both H1N1 and H3N2 viruses (Fig. 4A). Similar results were obtained when the order of viruses was reversed (Fig. 4B). When cells were incubated with H1N1 or H3N2 virus alone, no anti-HA antibody cross-reactivity was observed (data not shown). Under these conditions, NA inhibitors had no effect on the expression of HA molecules. NA inhibitors also enhanced superinfection in the lung epithelial cell line A549. Cells were incubated with A/Udorn/72 (H3N2) for 1 hour, treated with NA inhibitors or medium alone for 5 hours, and washed. The cells were then incubated with A/PR/8/34(H1N1) for 1 hour. The following day, cell surface H1 and H3 levels were analyzed by flow cytometry, as in Fig. 4B. Both oseltamivir carboxylate and zanamivir enhanced the cell surface expression of H1 in H3-positive cells (Fig. 4C). Collectively, the data in Fig. 4 show that NA activity limits the superinfection of infectious influenza A viruses.

We next sought to determine whether entry mediated by HAs from two HPAIVs was similarly suppressed by NA expressed in cells infected with infectious human influenza A viruses. H5 or H7 pseudovirus was incubated 1 hour before or 1, 3, 5, or 7 h after incubation for 1 hour with infectious A/PR/8/34 (H1N1) or Alice strain (H3N2) virus in the presence or absence of oseltamivir carboxylate or zanamivir (the experiment is shown in Fig. 5A). H5 and H7 pseudovirus entry into H1N1- or H3N2-infected cells was markedly suppressed within 2 hours of exposure of the cells to infectious virus in the absence of NA inhibitors (Fig. 5B and C). Both NA inhibitors restored HA-mediated entry to levels observed when pseudovirus was incubated with cells before live-virus infection. Infectious virus and NA inhibitors had no effect on entry mediated by the VSV-G protein. Our data indicate that entry mediated

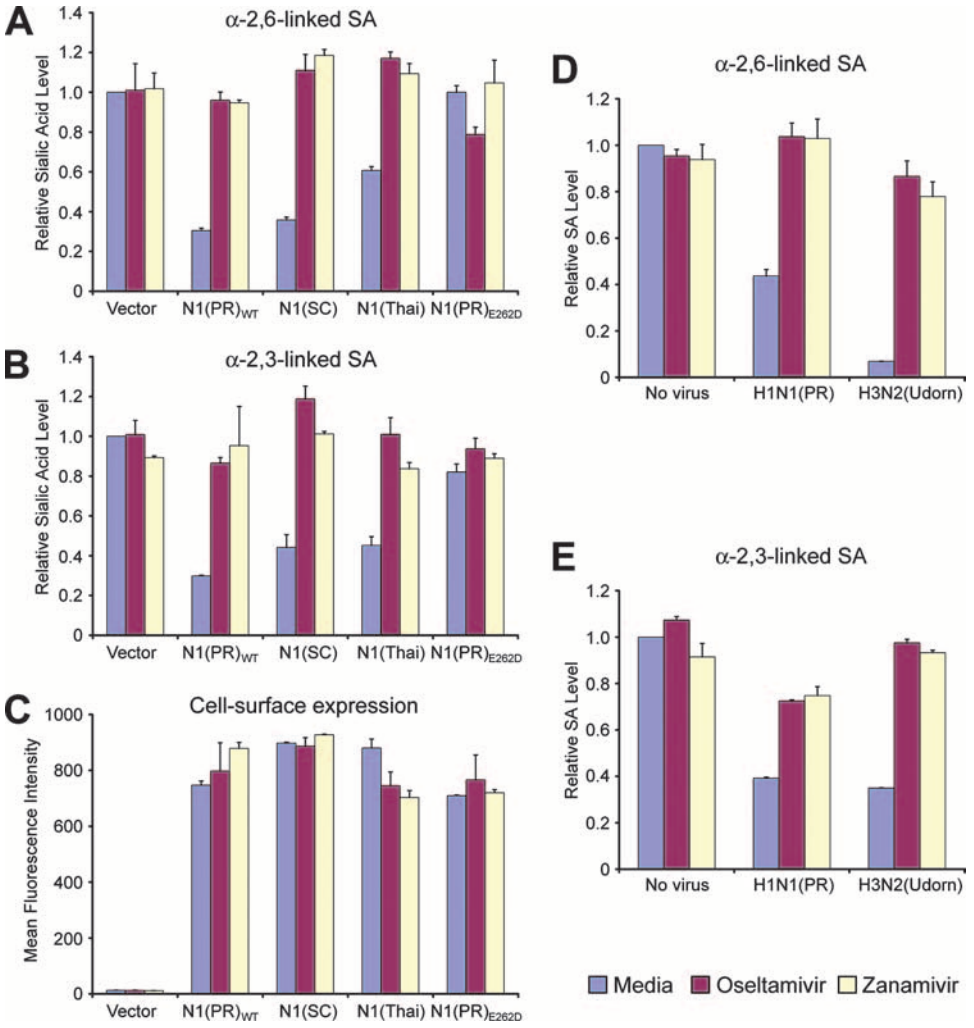

FIG. 3. Cell-expressed NA and infectious influenza A viruses remove SA from the cell surface. (A) 293T cells were transfected with plasmids expressing the indicated C9-tagged influenza A virus NA proteins or with vector alone and incubated with 1 μM of the indicated NA inhibitor or with medium alone. Two days posttransfection, the cells were labeled with a biotinylated lectin preferentially recognizing α-2,6-linked SA. Surface SA levels were measured by flow cytometry using fluorophore-conjugated streptavidin. Relative SA surface expression is shown as mean fluorescence intensity normalized to that of vector alone. (B) An experiment performed like that in panel A, except that cells were labeled with a lectin preferentially recognizing α-2,3-linked SA. (C) Cell surface expression of NA variants was assayed as in Fig. 2F. (D) 293T cells incubated with infectious influenza A/PR/8/34 (H1N1) or A/Udorn/72 (H3N2) virus, as indicated, were maintained in medium alone or containing 1 μM of the indicated NA inhibitors. After 6 hours, the cells were labeled with a biotinylated lectin preferentially recognizing α-2,6-linked SA. Surface SA levels were measured by flow cytometry using fluorophore-conjugated streptavidin. (E) An experiment similar to that in panel D, except that the cells were labeled with a lectin preferentially recognizing α-2,3-linked SA. The error bars represent standard deviations.

by HPAIV HA molecules is inhibited by the expression of NA in cells infected with either of two human influenza A viruses.

DISCUSSION

Many, perhaps most, enveloped viruses have mechanisms by which they resist superinfection, for example, by expressing a gene that down-regulates the viral receptor (e.g., HIV-1 nef) or by receptor interference mediated by the viral fusion protein (e.g., alpha- and gammaretroviruses) (1, 9, 21). In addition, the HA neuraminidases of several paramyxoviruses have been shown to interfere with attachment of SA-dependent viruses (15, 27). Here, we demonstrated that influenza A virus NA is necessary and sufficient among viral proteins to prevent subsequent superinfection of an infected cell. The enzymatic activity of NA is essential for this superinfection exclusion, and superinfection exclusion correlates with a loss of SA expression in the infected cell. Previous studies of NA have focused on its role on the virion surface, where it may facilitate virion release and digestion of SA in the respiratory mucus (24, 41). Here, we have clearly shown a biological consequence of viral neuraminidase expressed within the cell.

Receptor down-regulation can contribute in many ways to viral replication, and therefore, it is still unclear whether exclusion of superinfection per se is advantageous to influenza A viruses. For example, removal of receptor can reduce the extent of premature intracellular fusion events in HIV-1-infected

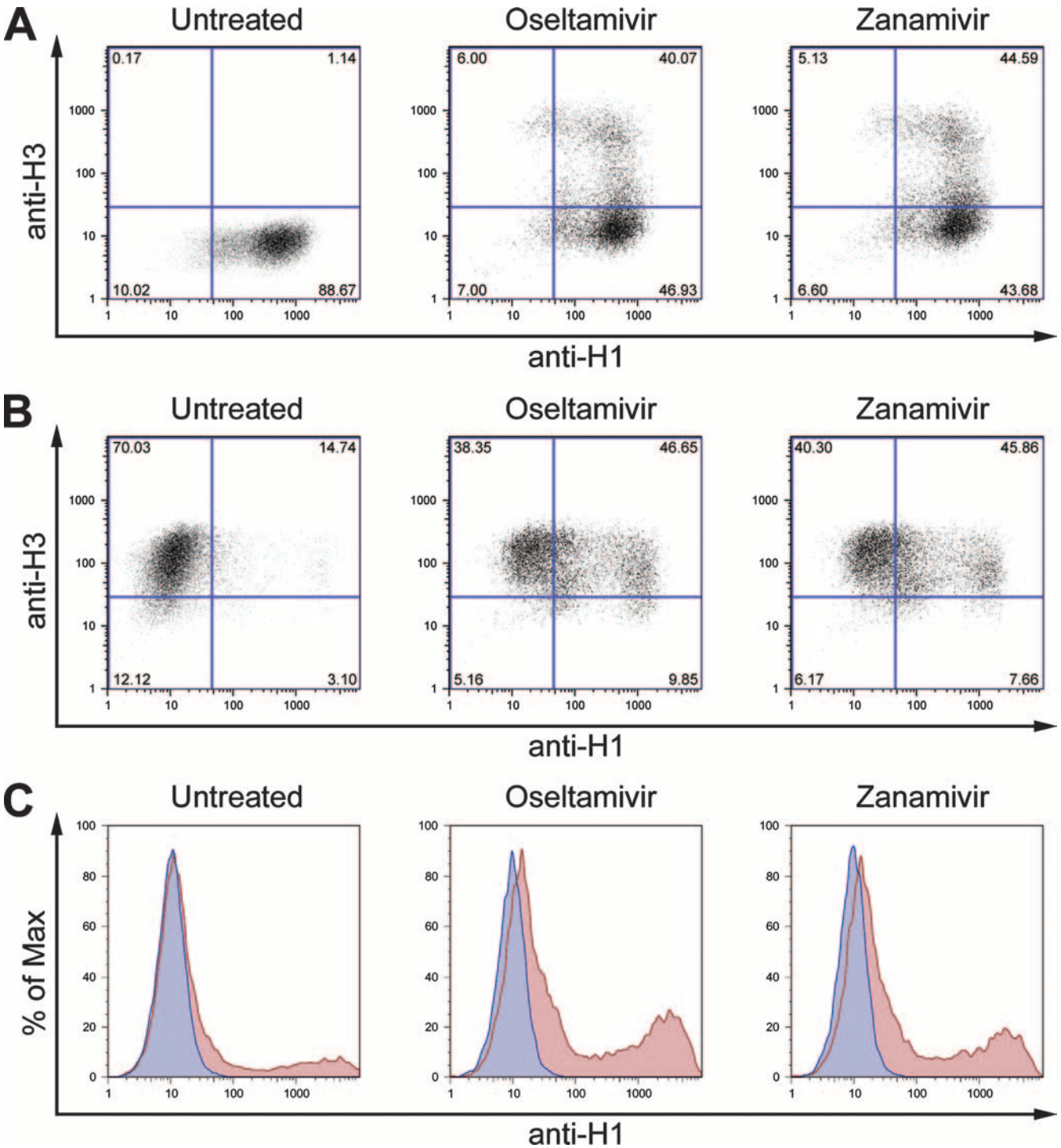

FIG. 4. NA inhibitors promote influenza A virus superinfection. (A) 293T cells were incubated with infectious H1N1 (A/PR/8/34) virus for 1 hour. Five hours later, the cells were incubated with infectious H3N2 (Alice strain) virus, again for 1 hour. During the entire 7-hour period, the cells were treated with medium alone, 1 μM oseltamivir carboxylate, or 1 μM zanamivir, as indicated. Infected cells were labeled with murine anti-influenza H1 IgG2a (C179) (horizontal axis) and murine anti-influenza H3 IgG1 (F49) (vertical axis), followed by appropriate PE- and fluorescein isothiocyanate-conjugated secondary antibodies. The labeled cells were analyzed by flow cytometry. This experiment is representative of three with similar results. (B) An experiment similar to that in panel A, except that the order of viruses has been reversed. (C) A549 cells were incubated with infectious H3N2 (A/Udorn/72) virus for 1 hour; treated with 1 μM oseltamivir carboxylate, 1 μM zanamivir, or medium alone for 5 h; and incubated with medium alone (blue) or with H1N1 (A/PR/8/34) virus (red) for another hour. Sixteen hours later, the infected cells were labeled with antibodies described in the legend to panel A. Histograms of H1 expression of H3-positive cells are shown.

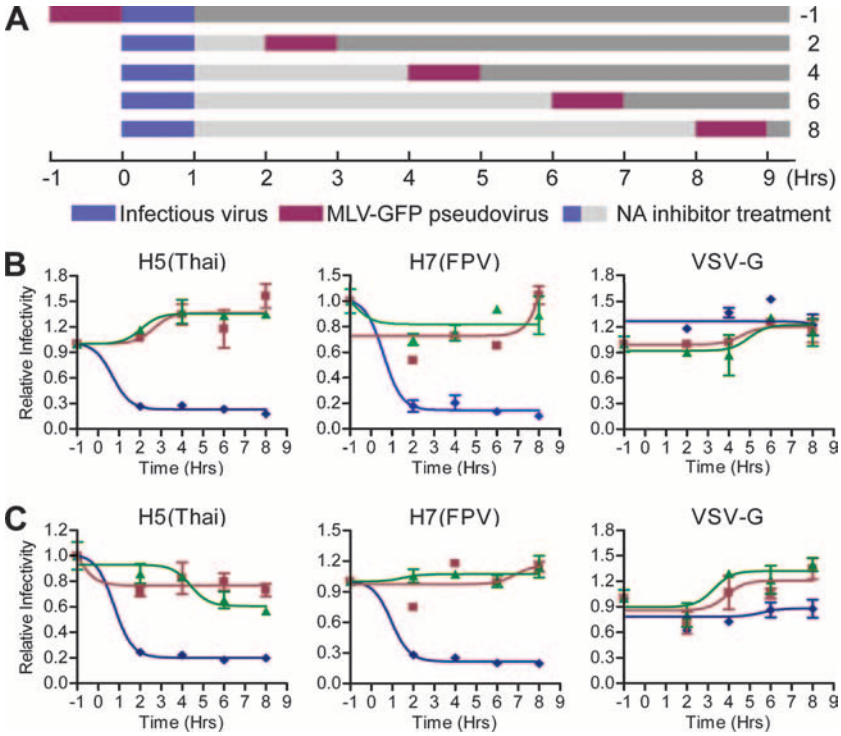

FIG. 5. NA inhibitors promote entry of MLV-GFP pseudotyped with HPAIV HA proteins. (A) A diagram of the experimental approach used in panels B and C is shown. 293T cells were incubated with infectious H1N1 (A/PR/8/34) or H3N2 (Alice strain) virus in the presence of medium alone, 1 μM oseltamivir carboxylate, or 1 μM zanamivir. After 1 hour, the virus was removed and the medium was replaced with medium containing NA inhibitor at the same concentration. MLV-GFP pseudotyped with the various HA molecules or with the VSV-G protein was incubated with cells 1 hour before or at the indicated time after infection with the infectious influenza A virus. Virus entry, measured as GFP fluorescence and normalized to that of pseudotyped virus added before incubation with infectious virus, was determined by flow cytometry. Blue indicates the incubation period with infectious viruses. Red indicates the incubation period with MLV-GFP pseudoviruses. Blue and light gray represent the total period of exposure to NA inhibitors in experiments where inhibitor was present. The numbers on the right indicate the start times of pseudovirus incubation relative to the start time of incubation with infectious virus. (B) Results of virus entry described in the legend to panel A. 293T cells were infected with H1N1 (A/PR/8/34) virus in the presence of medium alone (blue diamonds), 1 μM oseltamivir carboxylate (red squares), or 1 μM zanamivir (green triangles). (C) An experiment similar to that in panel B, except that the cells were incubated with H3N2 (Alice strain) virus. The error bars represent standard deviations.

cells (4). Removal of SA by cell-associated NA may itself contribute to virion release in the manner generally attributed to virion-associated NA. Finally, removal of SA can prevent reinfection of the virus-producing cell by recently assembled virions. In addition to these functions for receptor down-regulation, it may also be adaptive for a virus to exclude superinfection to retain full use of cellular resources and to stabilize the viral genome by limiting the frequency of reassortment of viral segments.

Reassortment of viral segments is an especially important mechanism in influenza A virus evolution (31, 37, 54). Reassortment can generate novel influenza A viruses, sometimes with phenotypes distinct from either original virus. Two major influenza A virus pandemics, in 1957 and 1968, arose from reassortment of human and avian viruses. Infection of a cell by two virions is necessary for reassortment of viral segments (2, 31). If NA limits the frequency with which two viruses infect a common cell, it may also limit the frequency of reassortment. Therefore, variation in the efficiency with which a given NA digests α-2,3- or α-2,6-linked SA may impact the frequency of reassortment between two viruses. Analysis of these efficiencies, and of the SA specificities of NA and HA of circulating isolates, may provide insight into past and future influenza A virus evolution.

We have shown here that, in tissue culture, NA inhibitors could enhance the frequency with which two viruses infect the same cell. An additional potential implication of our studies is that, under some conditions, NA inhibitors might also increase the frequency of reassortment of two viruses. Usually, inhibitor-promoted superinfection would not result in the production of a reassorted virus because the inhibitor would inhibit the release of any such virus. Moreover, in general, NA inhibitors tend to lower the viral load so as to reduce the likelihood that two viruses will infect a common cell. In a tissue culture setting, however, we have been able to demonstrate markedly enhanced reassortment in the presence of oseltamivir carboxylate between an NA inhibitor-sensitive H3N2 virus and an H1N1 virus expressing a naturally occurring oseltamivir resistance mutation in NA (Fig. 6). This enhanced reassortment likely occurs because oseltamivir carboxylate prevents the first, sensitive virus from removing SA from the cell surface, whereas the resistant NA of the superinfecting virus can facilitate the release of reassorted viruses. The physiological relevance of this tissue culture observation is not yet clear,

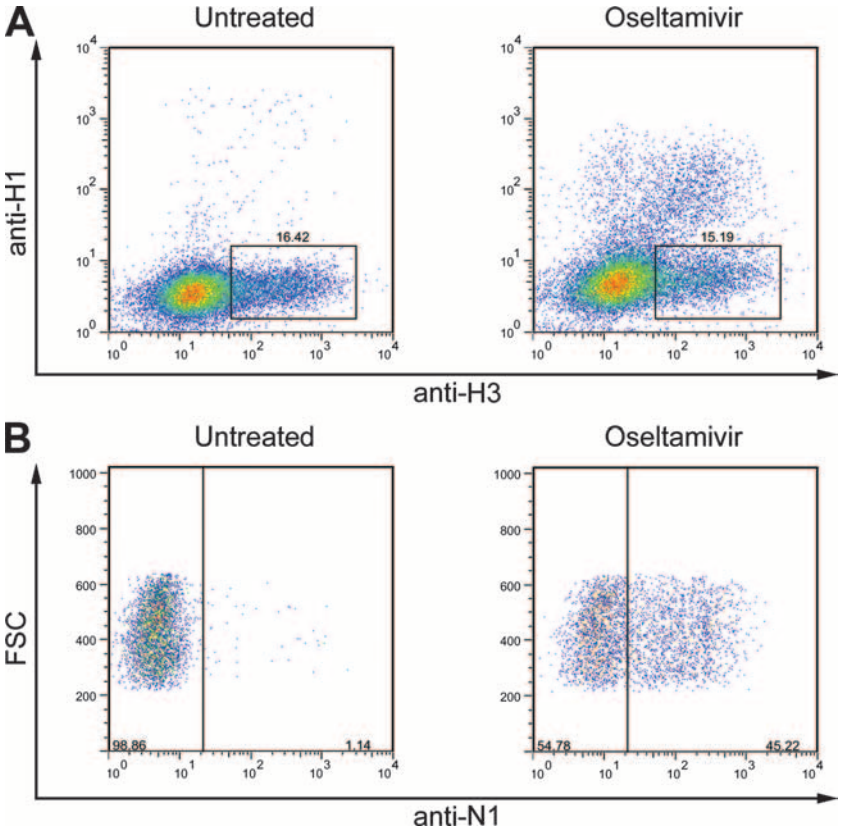

FIG. 6. NA inhibitors can promote reassortment between inhibitor-sensitive and inhibitor-resistant influenza A viruses. MDCK cells were incubated for 1 hour with a recombinant amantadine-resistant, oseltamivir-sensitive H3N2 (A/Udorn/72) virus. Four hours later, the cells were incubated for 1 hour with a second virus, recombinant amantadine-sensitive, oseltamivir-resistant H1N1 (A/PR/8/34) virus. The cells were maintained in the presence or absence of 1 μM oseltamivir carboxylate over the 6-hour period of infection. The cells were washed and then incubated for 16 h in the presence of 2.5 μg/ml amantadine and 1 μM oseltamivir carboxylate to limit replication of input viruses. The cell supernatants were harvested and incubated with 293T cells for 1 hour. The 293T cells were then maintained in regular medium for 16 h. The 293T cells were labeled with Alexa 647-conjugated murine anti-H3 IgG1 (F49), Alexa 488-conjugated murine anti-H1 IgG2a (H36-4-5.2), biotinylated murine anti-N1 IgG2a (NA-112-S2.4), and peridinin chlorophyll *a* protein-conjugated streptavidin. The labeled cells were then analyzed by flow cytometry for H1, H3, and N1 expression. (A) H3 expression plotted against H1 expression. The rectangular boxes indicate H3-positive, H1-negative cells gated for analysis in panel B. (B) N1 expression of H3-positive, H1-negative cells plotted against cell forward scatter. N1-positive, H3-positive, H1-negative cells indicate the presence of reassorted H3N1 virus. Note the presence of H3N2 virus in initially untreated cells (left), reflecting sufficient removal of SA at the time of oseltamivir and amantadine treatment, permitting H3N2 release. Note also the presence of reassorted H3N1 virus expressed from cells initially treated with oseltamivir (right), but not untreated cells (left).

particularly because resistant viruses are often attenuated (5, 13, 16) and because many other factors contribute to reassortment frequencies (31). Our data nonetheless suggest that additional animal studies of reassortment in the presence of NA inhibitors may be warranted.

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