Receptor Tyrosine Kinase Inhibitors Block Multiple Steps of Influenza A Virus Replication

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Received 16 September 2010/Accepted 22 December 2010

Host signaling pathways play important roles in the replication of influenza virus, but their functional effects remain to be characterized at the molecular level. Here we identify two receptor tyrosine kinase inhibitors (RTKIs) of the tyrphostin class that exhibit robust antiviral activity against influenza A virus replication in cultured cells. One of these (AG879) is a selective inhibitor of the nerve growth factor receptor and human epidermal growth factor receptor 2 (TrkA/HER2) signaling; the other, tyrphostin A9 (A9), inhibits the platelet-derived growth factor receptor (PDGFR) pathway. We find that each inhibits at least three postentry steps of the influenza virus life cycle: AG879 and A9 both strongly inhibit the synthesis of all three influenza virus RNA species, block Crm1-dependent nuclear export, and also prevent the release of viral particles through a pathway that is modulated by the lipid biosynthesis enzyme farnesyl diphasate synthase (FPPS). Tests of short hairpin RNA (shRNA) knockdown and additional small-molecule inhibitors confirmed that interventions targeting TrkA can suppress influenza virus replication. Our study suggests that host cell receptor tyrosine kinase signaling is required for maximal influenza virus RNA synthesis, viral ribonucleoprotein (vRNP) nuclear export, and virus release and that specific RTKIs hold promise as novel anti-influenza virus therapeutics.

Influenza virus imposes substantial burdens on public health by causing annual epidemics and occasional pandemics of acute respiratory disease that may lead to potentially severe and deadly complications, such as pneumonia. Antiviral therapeutics are a critical tool in combating influenza virus infections, especially in years when the vaccine strain does not match well with the circulating virus, when vaccines are unavailable at the early pandemic stage, or when vaccines are in short supply. Development of novel anti-influenza virus drugs is urgent, as variant strains resistant to all currently available drugs have been isolated and are expected to evolve rapidly (7, 8, 26, 44, 59). Targeting host cell signaling pathways or other host factors required for influenza virus replication offers an alternative strategy for antiviral drug development. Recent proteomic screening using small interfering RNA (siRNA) libraries has identified hundreds of host factors that may promote influenza virus replication (3, 16, 22, 24, 49), but the challenge of validating, characterizing, and interdicting their respective activities through pharmacological means remains.

Influenza A virus is an enveloped, negative-strand RNA virus with a segmented RNA genome (38). Influenza virus enters cells through receptor-mediated endocytosis after binding to sialylated receptors (50). After internalization, the low-pH environment in endosomes triggers fusion of viral and endosomal membranes and facilitates the release of viral ribonucleoprotein (vRNP) complexes into the cell cytoplasm (58). The released vRNPs then enter the nucleus, where viral RNA (vRNA) replication and transcription occur (38). Newly synthesized vRNPs are exported from the nucleus via the cellular Crm1-mediated nuclear export pathway (1, 12, 28, 55). Virus budding is mediated mainly by the viral M1 protein, which interacts with viral integral membrane proteins (HA, NA, and M2) and vRNP complexes at the plasma membrane (5, 33). The final release of virions from the cell surface requires the neuraminidase activity of viral NA protein (37, 39). Despite extensive studies, many aspects of influenza virus replication are incompletely understood, including the roles of host signaling pathways and cellular factors at each step of the virus life cycle. Identification of small-molecule compounds targeting any of these processes can yield biological insights as well as potential new therapies. For example, amantadine was found to block virus uncoating (4, 29), and viruses resistant to amantadine were found to harbor mutations in the ion channel region of the M2 transmembrane domain, suggesting both that the viral M2 protein is the target of amantadine (17) and that M2 ion channel activity is essential for virus uncoating. Viral HA protein was also found to influence amantadine sensitivity, implying an interaction between HA and M2 (17).

Receptor tyrosine kinases (RTKs) are a group of growth factor receptors that, upon ligand binding, undergo autophosphorylation at Tyr residues (18, 48, 52). These phosphorylated tyrosines then recruit Src homology 2 (SH2)- and phosphotyrosine-binding (PTB) domain-containing proteins that activate or link to downstream signaling pathways, such as the Ras/ERK/MAPK, PI3K/Akt, and JAK/STAT pathways (40, 48). Together, the complex signaling network triggered by RTKs leads to regulation of cell growth, migration, metabolism, and differentiation. Due to their critical roles in the development...
and progression of various cancers, RTKs have recently been studied extensively as targets for anticancer therapeutics. Host signaling through RTKs and other tyrosine kinases has also been shown to play important roles in virus replication. The tyrosine kinase inhibitor genistein was found to block replication of HIV-1, herpes simplex virus type 1 (HSV-1), and arena-virus (51, 53, 61), for example, and Src family kinases are known to be important for assembly and maturation of dengue virus and West Nile virus (6, 19). The Raf/MEK/ERK (42) and PI3K/Akt (9, 10, 15) pathways downstream of RTKs play important roles in influenza virus replication. It has been shown that Raf/MEK/ERK signaling is required for the nuclear export of influenza virions (42). The functional mechanism by which the PI3K pathway affects influenza virus replication is unclear, however. One recent report indicates that epidermal growth factor receptor (EGFR) signaling promotes influenza A virus uptake by cells (11).

In this study, we identify two specific RTK inhibitors (RTKIs), known as AG879 and tyrphostin A9 (A9), that have strong antiviral activity against influenza A virus, and we demonstrate that they both inhibit the Crm1-dependent nuclear export of the vRNP complex, viral RNA synthesis, and virus release. We show that diverse interventions targeting TrkA can impede influenza virus replication, thus validating this specific RTK as a candidate drug target. Our findings provide mechanistic insights into the potential roles of host RTK signaling in facilitating influenza virus replication, and they also suggest that specific RTKIs could be developed as potential anti-influenza virus therapeutics.

MATERIALS AND METHODS

Cells and viruses. 293T cells (human kidney epithelial cells) and A549 cells (human lung epithelial cells) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% heat-inactivated fetal bovine serum. Madin-Darby canine kidney (MDCK) cells were maintained in Eagle’s minimal essential medium supplemented with 5% fetal bovine serum. After infection with influenza A virus, MDCK cells were grown in L-15 medium containing 15 mM HEPES (pH 7.5), nonessential amino acids, 0.75 g of NaHCO₃ per liter, and 0.125% (wt/vol) bovine serum albumin. Influenza virus strains A/WSN/33 (A/WSN) and A/PR/834 (A/PR8) were grown in 10-day-old embryonated chicken eggs, and their titers were determined by plaque assay on MDCK cells. The WSN-LUC reporter viruses were generated as previously described (25). Their titers were determined by plaque assay on MDCK cells.

Identification of two RTKIs, AG879 and A9, with anti-influenza virus activity. Using the WSN-LUC reporter virus system that we have previously described (25), we screened a small library of 80 kinase inhibitors for anti-influenza virus activity. MDCK cells on 96-well plates were infected with WSN-LUC virus at an MOI of 0.5 for 1 h, washed with PBS, and treated with DMSO vehicle control, respective compounds at 10 μM, or ribavirin at various concentrations as a positive control. The LUC activity in the infected MDCK cells was measured and normalized against that for the DMSO control (Fig. 1A, lane 1). As shown in a representative screen (Fig. 1A), most of the tested compounds showed no inhibitory effects on LUC activity in the WSN-LUC-infected cells, whereas the nonspecific

RESULTS

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antiviral compound ribavirin inhibited LUC expression in a dosage-dependent manner (Fig. 1A, lanes 22 to 24). Notably, two inhibitors (Fig. 1A, lanes 16 and 17) significantly decreased LUC activity, suggesting that they each strongly suppress virus infectivity or viral gene expression. To determine whether any compound inhibited virus yield, we used the supernatants from these treated, WSN-LUC-infected MDCK cells as described for panel A to infect fresh MDCK cells, in which LUC activity was measured and normalized to that for the DMSO control (lane 1). Lane 16, AG879; lane 17, A9; lanes 22 to 24, ribavirin at 5, 10, and 20 ng/ml, respectively. (C) Determination of cytotoxicity of the RTKIs by MTT assay. A549 cells were incubated with various concentrations of the respective RTKIs or the vehicle control DMSO for 48 h prior to the MTT assay. (D) Dose-dependent inhibition of A/WSN/H1N1 virus in A549 cells by AG879 and A9. A549 cells were infected with A/WSN virus at an MOI of 0.01 and treated with various concentrations of the respective RTKIs or the vehicle control DMSO. Virus yields at 48 hpi were quantified by plaque assay. Error bars indicate standard deviations (SD) (n = 3).
pared to results for vehicle control DMSO and negative control AG494, both AG879 and A9 showed dose-dependent inhibition of influenza virus yield in A549 cells. Note that the virus yield with AG879 at 27 μM was ~0 PFU and is therefore undetectable in Fig. 1D and that the strong virus inhibition produced by A9 at 9 μM could partly be due to cytotoxicity at that concentration (Fig. 1C). Based on these studies of cytotoxicity and antiviral efficacy, we used A9 at 4 μM and AG879 and AG494 at 10 μM in all subsequent work. Taken together, these initial studies identified two specific RTKIs that can strongly inhibit both influenza A viral gene expression and virus yield.

**RTKIs AG879 and A9 effectively block influenza A virus replication in cell culture.** To verify their anti-influenza virus activities, we first examined whether AG879 and A9 can block replication of more than one influenza virus strain in diverse cell lines. MDCK or A549 cells were infected with A/WSN at an MOI of 0.01 in the presence of DMSO or various compounds (AG879, A9, or AG494). Virus titers in the supernatants at 48 hpi were quantified by plaque assays. Compared to results for DMSO or AG494 controls, AG879 and A9 efficiently blocked A/WSN replication >4,000-fold in MDCK cells and >200-fold in A549 cells (Fig. 2A), suggesting that their anti-influenza virus activity is not restricted to a single cell type. We also found that, in addition to blocking laboratory-adapted A/WSN virus, AG879 and A9 lowered virus yields of a different influenza A virus strain (A/PR8) >100-fold in A549 cells 18 h after infection at an MOI of 0.1 (Fig. 2B).

We further characterized the inhibitory effects of AG879 and A9 in a single-round replication assay, distinct from the multiple-round assay described above. A549 cells were infected with A/WSN at a high MOI (MOI of 2), washed three times with PBS, and then maintained in medium containing DMSO or various compounds. The virus titer in the supernatants at various time points was quantified (Fig. 2C). Newly synthesized viruses were detected as early as 8 hpi in DMSO- or A494-treated cells and increased in number exponentially at later times. In contrast, production of new viral particles (over background) in AG879- or A9-treated cells was not detectable until 12 hpi and lagged behind results for the controls by >1 log at later times. Consistent with those findings, viral NP protein was highly expressed in the DMSO-treated cells at 8 hpi but undetectable in the AG879-treated cells when analyzed by Western blotting (Fig. 2D).

RTKIs are not the only inhibitors of host signaling that can block influenza virus replication. Previous studies have identified several host signaling inhibitors with anti-influenza virus activity, including the MEK inhibitor U0126 and the NF-κB inhibitor Bay11-7082 (Bay11). To compare their antiviral potencies, we infected A549 cells with A/WSN virus at an MOI of 0.1 for 18 h in the presence of DMSO or various compounds (AG879, A9, or AG494; Table 1). Virus titer was determined by plaque assay. (B) AG879 and A9 inhibit influenza A/PR8 virus replication. A similar virus yield inhibition assay was performed with AG879 cells infected with A/PR8 strain at an MOI of 0.1 for 18 h in the presence of DMSO or various inhibitors. (C) AG879 and A9 block single-round replication of influenza A virus. Virus titers were determined at various time points after A/WSN infection of AG494 cells at a high MOI (MOI of 2) in the presence of DMSO or various inhibitors. (D) Western blot analysis of viral NP proteins at 8 hpi in cells mock infected (mock) or infected with A/WSN virus (MOI of 1) and treated with DMSO or AG879. (E) RTKIs AG879 (10 μM) and A9 (4 μM) inhibited influenza virus replication much more strongly than MEK inhibitor U0126 (50 μM) and NF-κB inhibitor Bay11-7082 (Bay11) (10 μM). Pairwise statistical comparisons to the DMSO control group were performed using Student’s t test (***, P < 0.001; ****, P < 0.0001). Error bars indicate SD (n = 3).

**FIG. 2.** RTKIs AG879 and A9 effectively block influenza A virus replication in cell culture. (A) AG879 and A9 block the multiplication of influenza A/WSN virus in both MDCK and A549 cells. Cells were infected with A/WSN at an MOI of 0.1 for 18 h in the presence of DMSO, AG879 (10 μM), tyrphostin A9 (4 μM), or AG494 (10 μM). Virus titer was determined by plaque assay. (B) AG879 and A9 inhibit influenza A/PR8 virus replication. A similar virus yield inhibition assay was performed with AG494 cells infected with A/PR8 strain at an MOI of 0.1 for 18 h in the presence of DMSO or various inhibitors. (C) AG879 and A9 block single-round replication of influenza A virus. Virus titers were determined at various time points after A/WSN infection of AG494 cells at a high MOI (MOI of 2) in the presence of DMSO or various inhibitors. (D) Western blot analysis of viral NP proteins at 8 hpi in cells mock infected (mock) or infected with A/WSN virus (MOI of 1) and treated with DMSO or AG879. (E) RTKIs AG879 (10 μM) and A9 (4 μM) inhibited influenza virus replication much more strongly than MEK inhibitor U0126 (50 μM) and NF-κB inhibitor Bay11-7082 (Bay11) (10 μM). Pairwise statistical comparisons to the DMSO control group were performed using Student’s t test (***, P < 0.001; ****, P < 0.0001). Error bars indicate SD (n = 3).

**AG879 and A9 inhibit the later stages of the influenza virus life cycle.** To characterize the specific step(s) of the influenza virus life cycle that is impeded by AG879 and A9, we examined the time course of their inhibitory effects. A549 cells were infected with A/WSN virus at an MOI of 1 and then, at various time points postinfection, were treated with DMSO or an inhibitor. We measured the titer of infectious viral particles released into the supernatant at 9 hpi, as a complete influenza virus life cycle (from the initial cell attachment to the final release of newly synthesized infectious viral particles) takes about 8 h (Fig. 2C). Both AG879 and A9 efficiently blocked virus production even when they were applied as late as 6 hpi (Fig. 3A), suggesting that these compounds are able to inhibit later stages of the virus life cycle. In addition, when applied at 1, 2, or even 4 hpi, each reduced virus production comparably to the degree achieved when applied at 1 h prior to infection (~1 hpi) (Fig. 3A), indicating that neither inhibitor blocks virus entry. A virus attachment assay, performed with and without inhibitor, demonstrated that AG879 and A9 did not
AG879 and A9 block nuclear export of influenza vRNPs by inhibiting the host Crm1-dependent pathway. Influenza virus RNA synthesis occurs in the nucleus. Incoming vRNPs, which contain NP-encapsidated viral genomic RNAs with associated viral polymerase proteins (PA, PB1, and PB2), must be imported to the nucleus for RNA transcription or replication to occur. Newly synthesized vRNPs are exported from the nucleus via the cellular Crm1-mediated nuclear export pathway (12, 28, 55) as a (Crm1-RanGTP)-NEP/NS2 and M1-vRNP complex (1) to be packaged into virions at the plasma membrane. To investigate the effects of AG879 and A9 on nucleocytoplasmic trafficking of vRNPs, we infected A549 cells with A/WSN at an MOI of 5 and then treated the cells with various inhibitors. U0126, a MEK inhibitor that has been shown to block influenza vRNP nuclear export (42), was included as a positive control. Localization of the NP protein was assessed by indirect immunofluorescence at various times postinfection. Representative images are shown in Fig. 4A. Under all conditions tested, the incoming vRNPs were detected mainly in the cytoplasm at 1.5 hpi, in both the cytoplasm and the nuclei at 2.5 hpi, and mostly within the nuclei at 4 hpi, suggesting that AG879 and A9 do not affect vRNP nuclear import early in virus infection. In contrast, at a later stage of virus replication (7 hpi), newly synthesized vRNPs were detected predominantly in the cytoplasm of cells treated with vehicle control DMSO or negative control AG494 but predominantly within the nuclei of those treated with U0126, AG879, or A9. To quantify these findings, we calculated the mean percentage of FITC-NP signal within the nucleus (nuc%) in 40 cells for each drug treatment at each time point (Fig. 4B); statistical analysis confirmed that only samples treated with U0126, AG879, and A9 at 7 hpi were significantly different from the DMSO control (P < 0.005). These data suggest that, like the MEK inhibitor U0126, AG879 and A9 prevent the nuclear export of vRNPs late in infection.

As influenza vRNP nuclear export not only depends on the cellular Crm1 pathway but also requires the viral proteins NEP/NS2 and M1, the nuclear retention of vRNPs following AG879 or A9 treatment might be due either to direct inhibition of the cellular Crm1 nuclear export pathway or to significantly decreased levels of viral protein expression (Fig. 2D) resulting from a possible blockade of viral RNA synthesis (see below for more details). We therefore examined the effect of AG879 and A9 on the nucleocytoplasmic trafficking of the HIV Rev protein, whose nuclear export depends on the Crm1 pathway (34). As shown in Fig. 4C, the rev-GFP fusion protein showed a predominant nuclear localization in control samples that had been treated with DMSO or AG494. The MEK inhibitor U0126 moderately increased the nuclear localization of rev-GFP, while AG879 and A9 led to a predominant nuclear retention. Statistical analysis of the proportion of the rev-GFP signal found within nuclei (nuc%) confirmed that nuclear localization of rev-GFP was significantly increased by U0126, AG879, or A9 treatment compared to that for controls (P = 0.008, P = 0.000001, and P = 0.000003, respectively). These findings strongly suggest that AG879 and A9 directly inhibit the cellular Crm1 nuclear export pathway, which may largely account for their ability to cause nuclear retention of influenza vRNPs (Fig. 4A).

AG879 and A9 strongly inhibit influenza virus RNA synthesis. We then evaluated whether AG879 and A9 can directly inhibit viral RNA synthesis, using a 5-plasmid assay (25) that is based on the reconstitution of all of the cis- and trans-acting elements required for influenza virus RNA replication and transcription. Compared to results for vehicle control DMSO and negative control AG494, the addition of AG879, A9, or ribavirin, a known inhibitor of influenza virus RNA synthesis, decreased the LUC activity expressed from either vRNA or cRNA templates by ~95% (Fig. 5A), strongly suggesting that the RTKs inhibit viral RNA synthesis. To validate this finding in virus-infected cells, we infected A549 cells with A/WSN at an MOI of 1 and then treated cells with various inhibitors at 1 hpi. Whole-cell RNA was isolated from the infected cells at 5 hpi and quantified for vRNA, cRNA, and mRNA using an established quantitative real-time RT-PCR assay (25). The levels of all three RNA species were found to be significantly decreased in AG879- and A9-treated cells, as indicated by increased threshold cycle (C_T) values (Fig. 5B).

Our previous studies have suggested that activation of NF-κB signaling promotes the efficient replication of influenza virus RNAs (25). Although NF-κB is also a downstream pathway of RTK signaling, AG879 and A9 are unlikely to block viral RNA synthesis via that pathway, as we have shown that NF-κB signaling is differentially involved in vRNA but not mRNA or cRNA synthesis (25), whereas AG879 and A9 block synthesis of all three viral RNA species (Fig. 5A and B). This is further evidenced by overexpression of NF-κB subunit p65, which we have shown can increase influenza vRNA synthesis and also rescue vRNA synthesis that has been...
AG879 and A9 inhibited the release of influenza virus particles. To determine whether the antiviral RTKIs affect virus assembly and release, we utilized a previously described assay (20) to quantify viruses that are either membrane-associated (assembled) or released into the supernatant (budded). A549 cells were infected with A/WSN at an MOI of 2. At 8 hpi, we washed these cells extensively with PBS and then added fresh medium containing DMSO or an inhibitor. After treatment for 15, 30, 45, or 60 min, we determined the titer of infectious viruses released to the supernatants, as well as that of the membrane-associated viruses, as described in Materials and Methods. At all time points, we recovered comparable amounts of membrane-associated infectious viruses in DMSO- and inhibitor-treated samples (Fig. 6A). In contrast, the titers of released extracellular viruses dropped significantly, by 80 to 90%, in cells treated with AG879 or A9 compared to those for controls (Fig. 6A). (Note that virus titer is shown on a log scale.) Our data suggest that the antiviral RTKIs block influenza virus release but do not impair its binding to the plasma membrane.

Wang et al. (54) have shown that an interferon (IFN)-induced protein, viperin, inhibits influenza A virus release from

\[ &\text{FIG. 4. RTKIs AG879 and A9 impair the nuclear export of influenza vRNPs by inhibiting the host Crm1-dependent pathway. (A) A549 cells} \\
&\text{were infected with A/WSN virus and treated with either DMSO vehicle control or chemical inhibitors. At various time points, cells were stained} \\
&\text{with anti-NP antibody, followed by FITC-conjugated secondary antibody, and observed under a fluorescence microscope. A representative image} \\
&\text{from each condition is shown. Three independent experiments were conducted. (B) Quantification of the percentage of NP-FITC nuclear signal} \\
&\text{(nuc%). Results shown are the average from 40 cells for each drug treatment at each time point. Statistical analysis was conducted as described} \\
&\text{in Materials and Methods, and statistical significance as determined by t test is shown. (C) A549 cells were transfected with the rev-GFP fusion} \\
&\text{protein expression vector and treated with DMSO or respective inhibitors for 4 h prior to observation under a fluorescence microscope. For each} \\
&\text{treatment, the average percentage of rev-GFP nuclear signal (nuc%) and the P value of pairwise statistical comparisons to the DMSO control} \\
&\text{group are shown.}
\]
the plasma membrane by affecting the membrane fluidity and formation of lipid rafts and that it acts through a pathway dependent upon farnesyl diphosphate synthase (FPPS), an enzyme essential for isoprenoid biosynthesis. Overexpression of FPPS was able to reverse viperin-mediated inhibition of virus production and restore normal membrane fluidity. To determine whether AG879 and A9 might also target FPPS, we examined the effect of FPPS overexpression on AG879- and A9-induced inhibition of virus release. A549 cells were transfected with either an FPPS expression vector or an empty vector control, infected with A/WSN, and then treated with DMSO or inhibitor for 30 min. As expected, AG879 and A9 significantly reduced virus release in the cells transfected with empty vector. However, overexpression of FPPS completely prevented this inhibition, restoring the levels of virus release from AG879- or A9-treated cells to those of the controls (Fig. 6B). This suggests that the two RTKIs may target FPPS to inhibit influenza virus particle release. As we did not observe any change in FPPS expression levels under the inhibitor treatment by Western blotting (data not shown), we speculate that AG879 and A9 may act by impairing FPPS function. It remains to be determined how RTK signaling regulates the activity of FPPS and how FPPS functions to facilitate influenza virus budding (5, 33).

TrkA is important for influenza virus replication. Although AG879 is a known inhibitor of TrkA and HER2 and A9 reportedly blocks PDGFR activation, we sought to determine whether their antiviral activities were actually due to inhibition of those RTKs rather than to off-target effects. To that end, we tested six additional small-molecule RTKIs, with reported

![FIG. 5. AG879 and A9 strongly inhibit the synthesis of all three viral RNA species independently of the NF-κB pathway. (A) AG879 and A9 inhibit viral RNA transcription from the cRNA or vRNA promoter, based on the FLuc-based 5-plasmid assay. A549 cells were transfected with expression plasmids of NP, PA, PB1, and PB2, together with a vNA-LUC or cNA-LUC reporter construct. DMSO or inhibitor was added to the cells at 8 h posttransfection and FLuc activity determined 16 h later. FLuc, firefly luciferase. (B) Synthesis of all three RNA species (vRNA, cRNA, and mRNA) was decreased in the virus-infected cells treated with AG879 and A9. The viral RNA level was quantified by real-time RT-PCR and is shown as C_T value. (C) Overexpression of NF-κB molecule p65 cannot reverse the inhibition of viral RNA transcription from the cRNA promoter by AG879 or A9. The 5-plasmid assay was conducted as described for panel A in cells transfected with empty vector plasmid or p65 expression vector. Compound treatment and LUC assay were similarly performed. Results shown are the averages from at least three independent experiments. Error bars indicate SD. Statistical analysis was conducted with Student’s t test (**, P < 0.01).](image)

![FIG. 6. AG879 and A9 inhibit virus release via FPPS. (A) Determination of infectious viruses associated with membranes or released to the supernatants. A549 cells were infected with A/WSN (MOI of 2) for 8 h and treated with either DMSO or compound for 15, 30, 45, and 60 min. Titers of infectious virus in the supernatants and in the cell lysates were determined. (B) FPPS overexpression fully restored the block of virus release by AG879 and A9. A virus release assay similar to that described for panel A was conducted in cells that had been transfected with empty vector or FPPS expression vector. Results shown are the averages from at least three independent experiments. Error bars indicate SD. Statistical analysis was conducted with Student’s t test (**, P < 0.01; ***, P < 0.001; ****, P < 0.0001).](image)
FIG. 7. TrkA signaling is important for influenza virus replication. (A) TrkA inhibitors significantly reduce influenza virus production in vitro. A549 cells were infected with A/WSN at an MOI of 0.1, in the presence of either vehicle control (DMSO alone) or various inhibitors: SKI-606 (10 μM), ZD1839 (10 μM), AG9 (4 μM), AG1296 (10 μM), AG879 (10 μM), TAK-165 (10 μM), GW441756 (10 μM), or K252a (2 μM). Virus production at 18 hpi was quantified by plaque assay. (B) TrkA-specific shRNAs decreased the TrkA expression level. A549 cells were transfected with the shRNA-expressing plasmid specific for either LUC or TrkA and then infected with influenza A virus. TrkA protein expression was detected 48 h after transfection by Western blot analysis using anti-TrkA antibody. (C) TrkA-specific shRNAs significantly decreased virus production. A549 cells transfected 48 h previously with either LUC- or TrkA-specific shRNA plasmid were infected with A/WSN at an MOI of 1. Virus production was quantified at 12 hpi. Results shown are the averages from at least three independent experiments. Error bars indicate SD. Statistical analysis was conducted with Student’s t test (***, P < 0.001; ****, P < 0.0001).

RTK SIGNALING AND INFLUENZA VIRUS

Host cell RTKs are growth factor receptors that regulate a variety of cellular activities related to growth, metabolism, and differentiation. In this study, we have shown that two small-molecule RTKIs, AG879 and A9, can each potently block influenza virus replication at multiple steps of the virus life cycle, impairing vRNP nuclear export, RNA synthesis, and virus release. AG879 and A9 are tyrphostin-class compounds that selectively antagonize the TrkA/HER2 and PDGFR pathways, respectively. By testing additional pharmacologic inhibitors and specific shRNA knockdown, we were able to verify the importance of at least one of these target RTKs (TrkA) in influenza A virus replication. These findings serve to extend prior studies that have also suggested important roles for RTK signaling in the influenza virus life cycle. Several independent genome-wide screens conducted to search for host factors involved in influenza virus replication have previously implicated particular RTKs and many of their downstream targets (3, 16, 22, 49, 56). In those studies, siRNA knockdown of at least 5 known RTKs, transforming growth factor receptor (TGFR) (49), fibroblast growth factor 1 (FGFR-1) through FGFR-4 (24), NTRK2/TrkB (24), EphB6 (22), and EphB2 (24), resulted in reduced influenza virus replication, supporting the functional role of RTK signaling in influenza virus replication. Another recent report (11) suggests, moreover, that EGFR signaling is important to promote influenza A virus uptake by infected cells. Thus, mounting evidence indicates that diverse pathways of RTK signaling may be required at multiple discrete steps of the influenza virus life cycle and so may present novel targets for antiviral drug development. To the extent that any given drug might target multiple host components and multiple steps of virus replication, drug-resistant viral variants are less likely to occur. It is worth noting that, given their low therapeutic indexes in vitro, AG879 and A9 are unlikely to be useful as antiviral agents themselves, but nonetheless they point the way to developing or discovering better antiviral drugs.

That RTK signaling is involved in the nuclear export of influenza vRNPs is not surprising, as one RTK signaling pathway, the Raf/MEK/ERK pathway, has previously been reported to be important for this process (42). Our data further reveal, however, that host RTK signaling is involved in directly regulating the host Crm1-dependent nuclear export pathway. Crm1 (also called exportin1, or Xpo1) is a major nuclear export receptor for proteins and for many RNAs (21); it forms trimeric transport complexes with RanGTP and export cargo molecules, a process promoted by the Ran-binding protein RanBP3. RTK signaling might be proposed to regulate Crm1 nuclear export through various mechanisms. Yoon et al. have shown that growth factor-mediated modulation of nuclear export occurs through phosphorylation of RanBP3 by RSK and Akt, which are the respective downstream targets of the Ras/TrkA-specific shRNA vectors showed significantly reduced virus replication, yielding titers ~1 log lower than those of control cells (Fig. 7C). Taken together, our findings using three TrkA-specific small-molecule inhibitors as well as shRNA knockdown strongly imply that signals emanating from TrkA are needed for maximal influenza A virus replication.

specificities for the Src (SKI-606), EGFR (ZD1839), PDGFR (AG1296), HER2 (TAK-165), and TrkA (K252a and GW441756) kinases. Each compound was used at a concentration (2 to 10 μM) reported to exert RTKI activity but which we found to be noncytotoxic by the MTT assay. As depicted in Fig. 7A, we found that the two TrkA inhibitors GW441756 and K252a each blocked influenza virus replication to a degree comparable to that of AG879 (i.e., ~2 log) at the concentrations tested. TAK-165, a HER2 inhibitor, had only modest efficacy, reducing virus production by 70%. The other compounds showed no appreciable antiviral activity. Thus, three different TrkA inhibitors (AG879, GW441756, and K252a) suppressed influenza A virus replication in this assay, whereas compounds targeting other host RTKs did not. Additional studies are needed to verify the antiviral target(s) of A9, however, as another reported PDGFR inhibitor (AG1296) did not block virus production in our assay.

We then used a pair of shRNA-expressing plasmids to specifically knock down TrkA expression in target cells prior to influenza virus infection. The TrkA-specific shRNA vectors significantly reduced TrkA protein expression in A549 cells, as determined by Western blotting, whereas a control LUC-specific shRNA had no effect (Fig. 7B). Upon infection with influenza A virus at an MOI of 1, cells transfected with the...
ERK/RSK and PI3K/Akt pathways (60). We have not yet evaluated whether AG879 or A9 can block the phosphorylation of RanBP3 or affect other components of the Crm1 nuclear export complex. In addition, interaction of Crm1 with cargo proteins can be regulated by cargo phosphorylation (21). Several viral protein components of influenza vRNPs are known to be phosphoproteins, including PA (47), NP (23, 43), M1 (13, 14, 23), and NEP/NS2 (46), and hyperphosphorylation of a mutant M1 protein has been shown to cause its aberrant nuclear retention (57). Conflicting results concerning the relationship between NP phosphorylation and nuclear export have been reported: one study reported that phosphorylated NP accumulated in the nucleus and cytoplasm with similar kinetics (41), suggesting that phosphorylation did not affect NP nucleocytoplasmic trafficking, whereas another study found that vRNPs isolated from the nucleus contained much more phosphorylated NP than those from the cytoplasm, consistent with differential nuclear export (2). Whether phosphorylation of vRNP components regulates nuclear export and whether AG879 and A9 cause nuclear retention of vRNPs by specifically blocking that process require further investigation.

A variety of host signaling pathways and other host factors have been implicated in regulating influenza virus RNA synthesis, but their underlying mechanisms are largely unknown. Several cellular factors that stimulate influenza virus RNA synthesis have been identified, including Hsp90 (31), the splicing-related factor AP56/BAT1 (30), and the chaperone TatsF1 (32). Recent proteomic screens using siRNA libraries have identified hundreds of candidate host factors that affect influenza virus replication (3, 16, 22, 24, 49), but exactly which host factors are functionally required for viral RNA synthesis and how they function await further research. We have previously shown that NF-kB signaling can differentially regulate influenza virus RNA synthesis by promoting vRNA but not mRNA or cRNA synthesis (25). Here we present evidence that host RTK signaling, by contrast, is important for the synthesis of all three influenza virus RNA species, through mechanisms that remain to be characterized.

Influenza virus particles are assembled and bud at the plasma membrane at sites that are enriched in cholesteryl and glycosphingolipids, forming lipid raft microdomains (5, 33). The eventual release of virus from the plasma membrane requires closure of the bud and separation of the virus particle from the host membrane, processes that are influenced by viral components as well as host factors. It has been shown that inhibitors of certain G proteins and protein kinases can inhibit influenza virus budding (20), suggesting an important role for host signaling in this process. An enzyme essential for isoprenoid biosynthesis, FPPS, appears to be critically involved in influenza virus budding, possibly owing to its role in the formation of lipid rafts (54). At least two different classes of influenza virus inhibitors, the IFN response protein viperin (54) and the antiviral RTKs AG879 and A9 (Fig. 6B), block influenza virus release via FPPS. Moreover, siRNA-mediated knockdown of FPPS significantly reduces influenza virus replication (54), confirming that FPPS is a potential target for developing anti-influenza virus drugs. Further studies will cast light on the functional mechanisms by which FPPS, as well as other host factors, affect influenza virus budding and release.

**ACKNOWLEDGMENTS**

We thank Y. Kawaoka (University of Wisconsin—Madison) for the influenza virus protein plasmids, P. Creswell (Yale University) for the FPPS expression plasmid, W. Greene (UCSF) for the plasmid expressing NF-kB molecule p65, A. Mergia (University of Florida) for the rev-GFP expression plasmid, and P. Edward (UCLA) for the anti-FPPS antibody. This work was supported by NIH grants AI067704 to Tristram G. Parslow and AI083409 to Yuying Liang.

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