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GENETIC DIVERSITY AND EVOLUTION

Identification of a Permissive Secondary Mutation That Restores the Enzymatic Activity of Oseltamivir Resistance Mutation H275Y

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ABSTRACT Many oseltamivir resistance mutations exhibit fitness defects in the absence of drug pressure that hinders their propagation in hosts. Secondary permissive mutations can rescue fitness defects and facilitate the segregation of resistance mutations in viral populations. Previous studies have identified a panel of permissive or compensatory mutations in neuraminidase (NA) that restore the growth defect of the predominant oseltamivir resistance mutation (H275Y) in H1N1 influenza A virus. In prior work, we identified a hyperactive mutation (Y276F) that increased NA activity by approximately 70%. While Y276F had not been previously identified as a permissive mutation, we hypothesized that Y276F may counteract the defects caused by H275Y by buffering its reduced NA expression and enzyme activity. In this study, we measured the relative fitness, NA activity, and surface expression, as well as sensitivity to oseltamivir, for several oseltamivir resistance mutations, including H275Y in the wild-type and Y276F genetic background. Our results demonstrate that Y276F selectively rescues the fitness defect of H275Y by restoring its NA surface expression and enzymatic activity, elucidating the local compensatory structural impacts of Y276F on the adjacent H275Y.

IMPORTANCE The potential for influenza A virus (IAV) to cause pandemics makes understanding evolutionary mechanisms that impact drug resistance critical for developing surveillance and treatment strategies. Oseltamivir is the most widely used therapeutic strategy to treat IAV infections, but mutations in IAV can lead to drug resistance. The main oseltamivir resistance mutation, H275Y, occurs in the neuraminidase (NA) protein of IAV and reduces drug binding as well as NA function. Here, we identified a new helper mutation, Y276F, that can rescue the functional defects of H275Y and contribute to the evolution of drug resistance in IAV.

KEYWORDS H275Y, Y276F, influenza A virus, neuraminidase

Influenza A virus (IAV) causes seasonal epidemics and recurrent pandemics (1). IAV has two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), which codetermine the subtype of IAV (e.g., H1N1 or H3N2). HA binds to sialic acid on the surface of host cells and mediates endocytosis of viral particles, while NA cleaves sialic acid to enable efficient release of newly budded viral particles. Disrupting the function of either NA or HA is effective at limiting IAV infections (2–6).

Oseltamivir is an orally administered NA competitive inhibitor (NAI). Oseltamivir remains the most widely used therapeutic against IAV and continues to be stockpiled

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in preparation for future pandemics. However, numerous oseltamivir resistance mutations have been detected in viruses isolated from patient samples (7, 8). The predominant oseltamivir resistance mutation in the H1N1 subtype is the histidine-to-tyrosine mutation at position 275 of NA (H275Y in the N1 numbering system used in this work). H275Y causes multiple H1N1 strains to become insensitive to oseltamivir (9, 10). Notably, oseltamivir-resistant H1N1 strains with H275Y circulated globally with high frequency (>50%) between 2007 and 2009 and have continued to exist in human hosts with a 1 to 10% frequency (11–14). Understanding the fitness of oseltamivir resistance mutations is important for predicting their contribution to future IAV genetic variation and the likely future effectiveness of NAIs.

The majority of oseltamivir resistance mutations cause fitness defects in the absence of drug pressure. Previously, we performed a high-throughput mutational scan that quantified the fitness effect of all single nucleotide mutations in the active site of NA, which identified fitness defects in the absence of drug for known oseltamivir resistance mutations (15). For example, I223M, H275Y, and N295S all showed ~30% fitness defects without drug pressure. Of note, both H275Y and N295S impaired the replication and transmission capability of IAV (9, 16–18), consistent with the fitness defects observed in our screen. Most oseltamivir resistance mutations accumulate at the active site of NA and disrupt binding to substrate as well as drug, thus reducing enzyme activity. Many of the resistant mutations, including H275Y, also decrease net enzyme activity by perturbing the expression level of NA (19, 20). Reduced enzyme activity of NA in turn leads to attenuated infectivity and/or transmission in the absence of drug pressure. In sum, the fitness cost of oseltamivir resistance mutations hinders their propagation.

Permissive or compensatory mutations can compensate for the fitness cost of oseltamivir resistance mutations. Secondary mutations are able to compensate for the reduced net enzyme activity and restore the replication efficiency. Studies by Bloom and colleagues found that R194G, R222Q, and V234M were able to increase the surface expression of NA with H275Y in the seasonal H1N1 strains and rescue fitness (19). R222Q was also shown to improve the binding affinity (K_m) and catalytic efficiency (V_{max}) of NA with H275Y (21). Three additional permissive mutations identified from phylogenic analyses of natural isolates were also found to buffer the fitness defect of H275Y, though to a lesser extent (22). These permissive mutations exhibited high frequency in H1N1 when H275Y was prevalent from 2007 to 2009 (21, 22), providing a plausible explanation for competent growth and transmission of H275Y. Although the vast majority of circulating viruses in 2012 to 2013 were NAI sensitive, approximately 99% of current circulating pandemic H1N1 2009 (pH1N1) carry two other mutations, V241I and N369K, which are permissive for the H275Y substitution (23–25). Experiments in ferret models also demonstrated that V241I and N369K rescued the replication and transmission defect of a H1N1 strain with H275Y (23, 26). High-throughput screening also identified E215D as an additional mutation compensating for the fitness defect of H275Y in several primary isolates (27). These observations indicate the continued potential for the emergence of a highly fit H1N1 with resistance to NAIs depending on the prevalence of permissive mutations. Further investigation of the mechanism of permissive mutations is thus necessary to understand the evolutionary potential of IAV in the presence of selection pressure from NAIs.

In this study, we determined the potential of Y276F as a permissive mutation to rescue the fitness defect of H275Y. Y276F is a hyperactive mutation that enhances the enzyme function of NA from A/WSN/33 by approximately 70% (15). We hypothesized that Y276F may restore the fitness defect of oseltamivir resistance mutations by increasing net NA activity. We determined the experimental fitness, enzyme activity, surface expression, and sensitivity to oseltamivir for a panel of oseltamivir resistance mutations with or without Y276F. In the A/WSN/33 strain, we found that Y276F restores the fitness, enzyme activity, and surface expression for H275Y, but not other resistance mutations, indicating a specific epistatic interaction between Y276F and H275Y instead

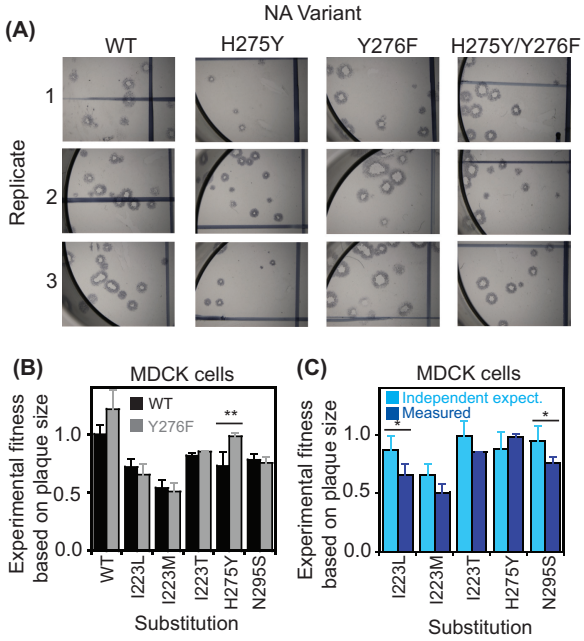

FIG 1 Plaque size assessment of the experimental fitness of oseltamivir-responsive mutations with or without Y276F. (A) Representative images from replicate plaque assays used to estimate fitness. All variants and replicates were performed in parallel. (B) The fitness based on plaque size on MDCK cells of NA with mutations in an otherwise WT background (black) or with Y276F (gray). The plaque size of mutant virus was normalized to that of the WT virus. Error bars show standard deviations ($n = 3$). **, $P < 0.05$, based on Student's t test. (C) Fitness of double mutants from independent expectations (light blue) or from plaque size measurements on MDCK cells (dark blue). Error bars represent standard deviations (SD) ($n = 3$, with propagated errors for independent expectations). *, $P < 0.1$, based on Student's t test.

of a general buffering role of Y276F. In addition to the A/WSN/33 strain, we also found that Y276F rescues net enzyme activity of H275Y in a more modern strain (A/California/04/09 NA). Taken together, the results of our study show that Y276F can restore the enzyme activity and fitness of H275Y NA.

RESULTS

Y276F rescues the fitness defect of H275Y. We sought to determine the relative fitness of the oseltamivir resistance mutations in the wild type (WT) or Y276F mutated genetic background. In the absence of Y276F, all of the oseltamivir resistance mutations exhibited 30 to 50% fitness defects (Fig. 1A and B; Table 1), consistent with our previous high-throughput screening. In contrast, Y276F provided a fitness advantage compared to WT of about 20% ($P < 0.05$), also consistent with our previous high-throughput studies (15). Of note, Y276F has not been observed at high frequency in H1N1 isolates, indicating distinctions between lab and natural evolution that are further detailed in Discussion. Y276F did not change the fitness of most resistant mutations in the absence of drug pressure. Among the resistant mutations we analyzed (I223M, I223T, I223L, H275Y, and N295S), H275Y was the only one for which fitness was clearly increased by Y276F (Fig. 1B).

Our measurements of fitness for single and double mutations provided an opportunity to investigate epistatic effects among Y276F and oseltamivir resistance mutations. We quantified epistasis by comparing the fitness effects of double mutants to the effects of each mutation individually. We considered the fitness effects of each mutant independent if the double mutant was equal to the product of the fitness effects of single mutants. In contrast, positive epistasis means that the double mutant is more fit than predicted based on the fitness effects of the single mutants, and negative epistasis is when the double mutant is less fit than predicted. Most of the drug-resistant

TABLE 1 Relative plaque size measurements for A/WSN/33 viruses with different NA variants on MDCK cells

		Size (μM)	
Experiment[a]	No. of plaques	Mean	SD
WT-1	18	58.5	16.6
WT-2	26	54.9	15.8
WT-3	30	64.3	11.2
Y276F-1	23	61.9	18.13
Y276F-2	19	80.9	19.21
Y276F-3	37	73.3	18.69
H275Y-1	12	35.2	7.45
H275Y-2	23	48.4	10.98
H275Y-3	12	45.6	9.32
I223L-1	26	37.7	13.45
I223L-2	26	43.9	10.25
I223L-3	26	45.9	14.29
I223M-1	13	33.8	10.2
I223M-2	24	34.4	10.51
I223M-3	26	27.4	7.49
I223T-1	20	48.3	13.01
I223T-2	17	49.7	16.22
I223T-3	37	46.9	15.34
N295S-1	32	43.2	10.71
N295S-2	23	49.2	14.01
N295S-3	32	46.5	11.65
Y276F-H275Y-1	30	56.4	18.39
Y276F-H275Y-2	29	60.0	21.82
Y276F-H275Y-3	29	58.0	11.67
Y275F-I223L-1	15	32.9	10.6
Y275F-I223L-2	32	40.2	11.04
Y275F-I223L-3	27	43.4	12.29
Y275F-I223M-1	8	28.4	6.86
Y275F-I223M-2	9	27.2	6.39
Y275F-I223M-3	12	35.3	13.17
Y275F-I223T-1	26	49.7	13.1
Y275F-I223T-2	26	50.4	12.43
Y275F-I223T-3	35	50.7	14.61
Y275F-N295S-1	26	41.7	11.77
Y275F-N295S-2	38	45.1	10.04
Y275F-N295S-3	38	47.4	10.94

[a]NA variant and repeat number.

mutants showed negative epistasis with Y276F (Fig. 1C). In contrast, Y276F shows a pattern of positive epistasis with H275Y. These analyses indicate that the impacts of Y276F on fitness depend on the genetic background.

We further investigated the impacts of H275Y and Y276F on viral fitness by assessing viral replication kinetics by reverse transcription-quantitative PCR (RT-qPCR). Fitness assessed by viral replication in MDCK cells is similar to what was estimated by plaque size (Fig. 2A). The RT-qPCR studies showed statistically significant decreases in fitness for the H275Y enzyme compared to WT and increases for the Y276F enzyme relative to WT at 24 h postinfection. We also saw statistically significant positive epistasis between H275Y and Y276F from the replication data (Fig. 2B). RT-qPCR data follow the same trends as the plaque size data in MDCK cells, with less measurement variation and therefore greater ability to discern statistically significant differences. We also examined the fitness effects of H275Y and Y276F in A549 cells by measuring viral replication kinetics by RT-qPCR (Fig. 2C and D). The A549 cell line is derived from a human lung carcinoma and thus is more representative of cells infected by IAV in humans. A549 cells and MDCK cells differ in the fraction of sialic acid-galactose (SA-Gal) linkages in the α2,3 and α2,6 configurations. We observed statistically significant differences in

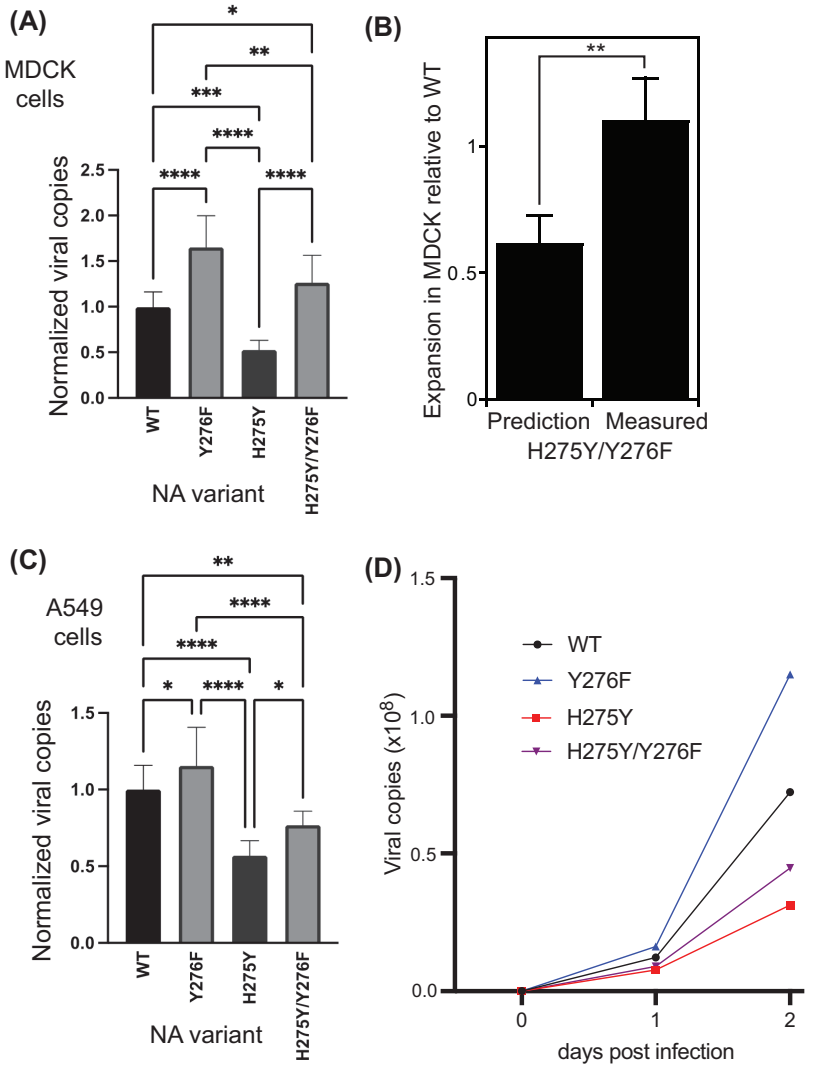

FIG 2 Viral expansion assessment of the experimental fitness effects of H275Y and Y276F in MDCK and A549 cells. (A) Relative abundance of influenza A/WSN/33 WT, Y276F, H275Y, and H275Y/Y276F NA from MDCK cell culture supernatants collected 24 h following infection with an MOI of 0.01. (B) Epistasis analysis of H275Y/Y276F. Predicted expansion based on independent effects of each individual mutation is lower than experimental measurement of this variant ($P = 0.01$, single-tail t test with errors propagated for independent prediction). (C) Relative abundance of influenza A/WSN/33 WT, Y276F, H275Y, and H275Y/Y276F NA in A549 cell culture supernatants collected 24 h following infection with an MOI of 0.1. Viral copies in supernatants were quantified by RT-qPCR of influenza A virus M segment, and the viral copy number of each was normalized to the average value of WT (5.2×10^8 or 2.1×10^8 copies/mL for two independent MDCK cell experiments; 1.2×10^8 or 1.7×10^8 copies/mL for two independent A549 cell experiments). Error bars in panels A and C represent the standard deviations for 5 wells from two independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ (ordinary one-way ANOVA). (D) Viral growth kinetics of WSN/33 variants in A549 cells. Each point is the average value of IAV RNA quantified by RT-qPCR for replicate wells from a representative experiment.

viral expansion in A549 cells that follow the same trends as in MDCK cells. These results suggest that the fitness rescue of H275Y by Y276F is observed in both host cells.

Y276F restores the enzymatic activity and surface expression of H275Y NA. Having established that Y276F can rescue the fitness defect of the H275Y enzyme, we examined the mechanism of this rescue. We first investigated how these mutations impacted enzyme activity in A/WSN/33 viral particles. Consistent with previous results, all of the oseltamivir resistance mutations showed reduced NA activity in the WT genetic background, indicating that the fitness defect of these mutations resulted from defects in enzyme activity (Fig. 3A). Conversely, Y276F alone exhibited an increase

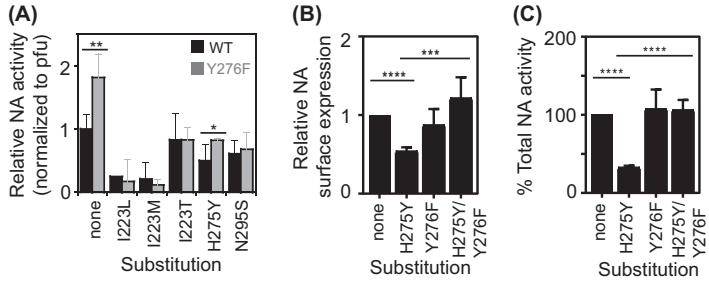

FIG 3 Enzyme activity of NA with oseltamivir resistance mutations with or without Y276F. (A) Enzyme activity of variants in a WT genetic background (black) or with Y276F (gray). The enzyme activity of NA was determined by a fluorometric assay (MUNANA) and normalized to the viral titer. Relative NA activities were determined by normalizing to WT. Error bar indicate SD ($n = 3$). **, $P < 0.05$, based on Student's t test. (B) Relative surface expression of WT, H275Y, Y276F, and H275Y/Y276F NA determined by expressing NA in 293T cells. Relative expression was determined based on mean fluorescence from flow cytometry normalized to WT. SD are shown ($n = 5$). ***, $P < 0.005$, and ****, $P < 0.0001$, based on an unpaired Student's t test. (C) Percent total NA enzymatic activity for WT, H275Y, Y276F, and H275Y/Y276F NA, determined by expressing NA in 293T cells, followed by MUNANA assay and normalization to WT. Error bar, show SD ($n = 5$). ****, $P < 0.0001$, based on unpaired Student's t test.

in NA activity of approximately 70% compared to WT (Fig. 3A). The enzyme activity of most drug-resistant mutations with Y276F was slightly worse than or similar to that seen with these mutations in a WT background. Thus, Y276F failed to rescue the enzyme activity of most drug-resistant NA mutants. The ability of Y276F, but not most drug resistance mutations studied here, to increase enzyme activity of WT provides an explanation for the observed negative epistasis among these mutations.

The total enzyme activity of NA can be modulated by changing its quantity (surface expression) or proficiency (e.g., k_{cat}/K_m), so we sought to distinguish these two possibilities. We determined the relative surface expression and enzyme activity of A/WSN/33 NA variants (WT, H275Y, Y276F, and H275Y/Y276F) expressed in 293T cells. H275Y resulted in roughly 50% less surface expression of NA than WT (Fig. 3B) and enzyme activity that was further reduced (Fig. 3C), indicating that H275Y also reduced enzyme proficiency. Y276F had slightly less surface expression than WT but slightly more enzyme activity. This finding indicates that Y276F NA is a more proficient enzyme than WT in the A/WSN/33 strain. The H275Y/Y276F double mutant showed about 20% more surface expression of NA than WT and enzyme activity similar to that of WT, indicating slightly reduced enzyme proficiency. The surface expression and activity of Y276F NA did not differ with statistical significance ($P > 0.1$) from those of H275Y/Y276F NA. Together, these results indicate that Y276F restored the function of H275Y NA primarily through enhancing surface expression.

Y276F has little effect on oseltamivir resistance. We then examined the sensitivity of IAV to oseltamivir in the WT or Y276F genetic background. We determined 50% enzyme inhibitory concentration (IC_{50}) values for oseltamivir inhibition of NA enzyme activity (Fig. 4 and Table 2). Drug resistance mutations all exhibited increased IC_{50}s relative to WT, consistent with the ability of these variants to avoid inhibition by oseltamivir. On its own, Y276F exhibited an oseltamivir profile similar to that of WT. All drug resistance mutations also exhibited similar oseltamivir profiles with or without Y276F, indicating that Y276F does not have a strong impact on the affinity of NA for oseltamivir.

Y276F rescues H275Y defects in a modern strain of IAV. H275Y has been observed at low frequency (1 to 3%) in modern circulating IAV strains (25), and we were interested to know if the Y276F mutation would also be permissive in a more modern and clinically relevant strain than A/WSN/33. We evaluated the effect of H275Y and Y276F in the A/California/04/2009 strain by expressing single and double mutants of NA in 293T cells. H275Y showed roughly 50% less surface expression of NA compared to WT NA (Fig. 5A) and a similar reduction in enzyme activity (Fig. 5B), indicating similar enzyme proficiency. Y276F increased surface expression by about 10% relative to WT

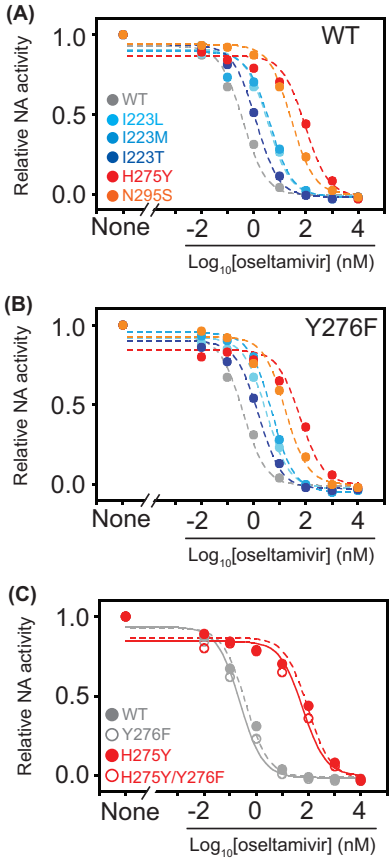

FIG 4 Inhibition of NA variants by oseltamivir. (A and B) Enzyme activity of viruses with increasing doses of oseltamivir (dose-response curve) in the WT genetic background (A) or with Y276F (B). (C) Dose-response curve for WT and H275Y in the WT or Y276F background.

and enzyme activity by about 15%, indicating that enzyme proficiency was slightly increased. The H275Y/Y276F double mutant showed a 5% reduction in surface expression compared to WT, indicating that the Y276F mutation largely rescued the expression defect of H275Y NA. The H275Y/Y276F double mutant exhibited enzyme activity similar to WT and clearly higher activity than H275Y. These results indicate that Y276F restored the enzyme activity for H275Y in the A/California/04/2009 strain primarily by improving the surface expression of NA.

DISCUSSION

In this study, we found that Y276F can rescue the fitness defect of H275Y by restoring surface expression and net enzyme activity of NA from A/WSN/33 and A/California/04/09 NA bearing the H275Y drug resistance mutation. Moreover, the H275Y/Y276F double mutant remained insensitive to oseltamivir inhibition in the A/WSN/33 strain.

TABLE 2 IC_{50}s of substitutions with Y276 (WT) or F276

	IC_{50} (μM) (fold change)	
Substitution	**Y276**	**F276**
WT	0.33 (1)	0.21 (0.65)
I223L	3.2 (9.9)	3.0 (9.3)
I223M	3.4 (10)	5.0 (15)
I223T	1.1 (3.4)	1.4 (4.2)
H275Y	150 (460)	77 (230)
N295S	29 (90)	17 (52)

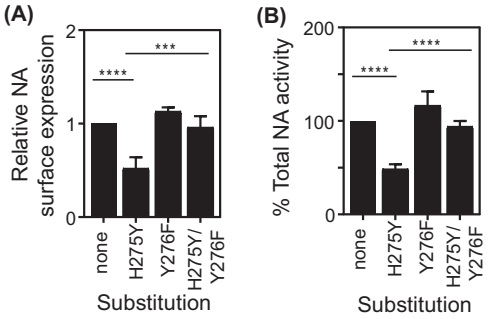

FIG 5 Impacts of H275Y and Y276F in pH1N1 A/California/04/2009 strain. (A) The relative surface expression of NA variants (WT, H275Y, Y276F, and H275Y/Y276F) was assessed based on expression in 293T cells and flow cytometry. (B) Enzyme determined by MUNANA assay and normalization to WT. Error bars show SD ($n = 5$). ***, $P = 0.004$, and ****, $P < 0.0001$, based on unpaired Student's t test.

These results indicate that Y276F may serve as a permissive mutation of H275Y, allowing robust growth of H275Y comparable to that of WT. In particular, Y276F itself exhibited no fitness defect, which may facilitate its expansion in IAV populations. Consistent with this idea, Y276F was identified, albeit at low frequency, in human isolates in 2009 and 2010 (15). Moreover, Y276F did not affect sensitivity of the H275Y enzyme to oseltamivir, so H275Y/Y276F may confer resistance to oseltamivir treatment without diminishing viral fitness.

In a survey of H1N1 sequences in the Influenza Research Database (28), Y276F was observed in six of 20,433 full-length NA sequences obtained from human isolates since 1933. The dearth of Y276F in the database indicates that it has not attained high frequency, by selection or otherwise, during this period in circulating viral populations. In addition, we do not know of any observations of Y276F in laboratory selection experiments, suggesting that it has not arisen to high frequency in experimental evolution studies. Multiple factors could underlie the low frequency of Y276F, despite its apparent fitness benefit in A/WSN/33 in laboratory experiments. These include discrepancies between selection pressures *in vitro* versus *in vivo*, negative epistasis with other sites in proximity (as suggested by our results), or interference with other linked sites experiencing purifying selection (29, 30).

Importantly, as our biochemical analyses demonstrate that Y276F rescues H275Y (i.e., rendering it neutral rather than deleterious) in both A/WSN/33 and A/California/04/2009 in the absence of oseltamivir, the double mutant would be expected to be segregating only at low frequency under mutation-drift equilibrium in the absence of treatment, consistent with the database observation. Furthering this logic, as H275Y is strongly beneficial in the presence of oseltamivir regardless of the presence or absence of Y276F on the genetic background, there would similarly not be a particular expectation of observing the double mutant at high frequency in populations experiencing treatment. However, if H275Y were to be brought to high frequency by positive selection in response to oseltamivir treatment, and treatment were ceased, it would then experience purifying selection (as the mutant would be less fit than the WT in the absence of drug) and begin rapidly decreasing in frequency. If Y276F were to occur on the H275Y background in this rather specific temporal period, the double mutant would be expected to be governed by genetic drift rather than purifying selection and as such might maintain H275Y at appreciable frequency longer than expected. This scenario would render the population more susceptible to rapid resistance evolution should drug treatment resume.

In our experiments, Y276F compensated only for the fitness defect of H275Y, not for oseltamivir resistance mutations at residue 223 or 295. Intriguingly, Y276F did not significantly impact the experimental fitness or NA activity of other resistance mutations. If Y276F and resistant mutations at positions 223 and 295 acted independently, these resistance mutations would be more fit with Y276F; however, observations rather

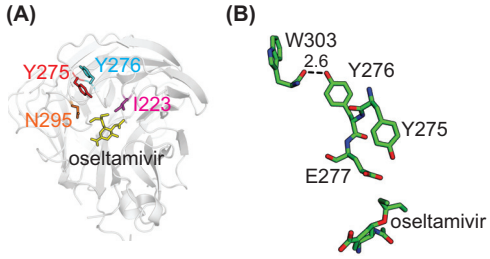

FIG 6 Position of Y276 relative to drug resistance mutations in NA. Residue 223 (magenta), 275 (red), 276 (cyan), and 295 (orange) are highlighted as sticks. The inhibitor oseltamivir is also shown as sticks and is colored yellow. Position 276 is close to 275 but distal from positions 223 and 295. (B) Residues 275 to 277, 303, and oseltamivir are highlighted as sticks and colored based on atom type (C, green; O, red; N, blue). The molecular images of NA in both panels were generated from PDB ID 3CL0 (20).

indicate negative epistasis among these mutations. We investigated the structural basis of the specific compensatory function of Y276F for H275Y. Among the positions with resistant mutations that we studied, residue 275 was in the closest structural proximity to residue 276 (Fig. 6). Inspecting the local conformation of residue 275 to 277 (Fig. 6B) reveals that the H275Y substitution sterically forces the glutamate at position 277 closer to oseltamivir, as we previously described (31), which alters the active site and disrupts drug binding. The WT tyrosine at position 276 forms a hydrogen bond with the main-chain carbonyl oxygen at position 303. The Y276F substitution generates space in the structure that may permit the repositioning of both residues 276 and 275. Thus, it appears that the Y276F substitution may locally reorganize the active site. Further experimental work could aid in understanding the physical interactions governing epistasis between Y276F and drug resistance mutations in NA.

In conclusion, we identified Y276F as a permissive mutation of H275Y that can restore NA expression level and enzymatic activity. The appearance of Y276F in circulating strains of IAV may increase the expected frequency of H275Y. The likely effect of this would be to move from mutation selection equilibrium to mutation drift equilibrium and thus facilitate oseltamivir resistance.

MATERIALS AND METHODS

Generation of single and double mutations by site-directed mutagenesis. Plasmids encoding the WT NA gene and the other seven gene segments of the H1N1 A/WSN/33 strain in the pHW2000 vector were kindly provided by R. Webster (St. Jude Children's Research Hospital, Memphis, TN). Mutations were introduced using site-directed mutagenesis and confirmed by Sanger sequencing. Plasmids for expressing NA in 293T cells were based on pJB992, kindly provided by J. Bloom (University of Washington, Seattle, WA). In this plasmid, a cytomegalovirus (CMV) promoter drives the expression of NA with a C-terminal V5 epitope, used for surface staining, followed by an internal ribosome entry site-green fluorescent protein (IRES-GFP), used for calculating transfection efficiency (32). The NA genes were cloned between EcoRI and NotI restriction sites, and mutations were introduced by site-directed mutagenesis.

Cell culture. 293T and MDCK cell lines were obtained from the American Type Culture Collection (Manassas, VA). The 293T cell line was maintained in Opti-MEM I reduced-serum medium (Gibco, Grand Island, NY) supplemented with 5% fetal bovine serum (HyClone, Logan, UT), 100 U/mL penicillin, and 100 μg/mL of streptomycin at 37°C with 5% carbon dioxide. The MDCK cell line was maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM sodium pyruvate, 1× nonessential amino acid, 100 U/mL penicillin, and 100 μg/mL of streptomycin at 37°C with 5% carbon dioxide. All cell culture reagents were from Corning (Manassas, VA) unless otherwise indicated.

Fitness analyses. The experimental fitness of virus stocks was assessed based on plaque size as previously described (15). Briefly, serial dilutions were performed on the viral samples, followed by 1 h of binding on confluent MDCK cells. Unbound virus was washed away with phosphate-buffered saline (PBS), and the cells were overlaid with 0.5% agar in Dulbecco's modified Eagle medium-nutrient mixture F-12 supplemented with penicillin-streptomycin, L-glutamine, bovine serum albumin, HEPES, sodium bicarbonate, and acetylated trypsin. After the agar solidified, the plates were incubated for 48 h. Cells were fixed and stained with anti-H1 primary antibody (MAB8261; MilliporeSigma) and visualized with a horseradish peroxidase-conjugated secondary antibody. All variants were analyzed in parallel to minimize experimental variability. For each variant, a well with clearly separated plaques was chosen for size analysis (Fig. 1A). The diameter of all individual plaques in the chosen well were measured (average of

24 plaques per well measured; range, 8 to 38). For fitness estimates, the diameter of each variant was normalized to the diameter of the parental WT virus. Full experimental replicates were performed in triplicate for each variant and WT. To estimate independent fitness effects for H275Y/Y276F, we multiplied the normalized plaque size of H275Y and Y276F and propagated errors.

To determine experimental viral growth, MDCK or A549 cells were seeded in 6-well plates and infected with virus variants at a multiplicity of infection (MOI) of 0.01 for MDCK cells or 0.1 for A549 cells. After inoculation, cells were washed and replenished with growth medium. Supernatant samples were collected at 1, 24, and 48 h postinfection and stored at −80°C. After thawing of supernatant samples, viral RNA was isolated in TRIzol LS following the manufacturer's instructions (Thermo Fisher). Viral RNA was quantified by RT-qPCR. TaqMan primers and probe sequences targeting IAV-M were as follows: FLUAM-7-F, CTTCTAACC GAGGTCGAAACGTA; FLUAM-161-R, GGTGACAGGATTGGTCTTGTCTTTA; and FLUAM-49-P6, TCAGGCCCCC TCAAAGCCGAG.

Quantification of IAV RNA was performed with the QuantiFast pathogen RT-PCR kit (Qiagen). *P* values were determined for the data at 24 h postinfection (Fig. 2A and C) using ordinary one-way analysis of variance (ANOVA) in GraphPad Prism 9.

Generation of recombinant influenza viruses. Viral mutants were recovered from plasmids as previously described (15). Briefly, equal numbers of 293T and MDCK cells were seeded in 6-well plates and transfected the next day with 1 μg of NA plasmid (WT or mutants) as well as 1 μg of plasmids carrying the other seven gene segments using TransIT-LT1 reagent (Mirus, Madison, WI). At 6 h posttransfection, cell growth medium was replaced with fresh Opti-MEM I reduced-serum medium. At 30 h posttransfection, tosylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-trypsin (Sigma-Aldrich, St. Louis, MO) was added to cell growth medium to a final concentration of 0.5 μg/mL. At 72 h posttransfection, supernatant containing viral particles was harvested and centrifuged at 1,200 rpm for 15 min. Supernatants were stored at −80°C. Plaque assays were performed to determine the titer (PFU/mL) of each sample as previously described (33). The radius of all well-separated plaques in each well was measured using a Nikon SMZ1500 microscope, and the mean of all measurements was calculated as an estimate of relative fitness.

NA enzymatic activity *in vitro*. Enzyme activity of NA was determined using fluorogenic 2′-(4-methylubelliferyl)-α-D-*N*-acetylneuraminic acid (MUNANA; Life Technologies, Carlsbad, CA) according to a previously published protocol (15). The relative fluorescence was normalized to the titer of the virus stock in order to estimate fluorescence activity per infectious virion. The data were then normalized to the WT. To estimate sensitivity to oseltamivir, MUNANA assays were performed in the presence of a range of oseltamivir concentrations to generate a dose-response curve following a previously published protocol (15). For each viral variant, the fluorescence signals in increasing concentrations of oseltamivir were normalized to the signal without drug. IC_{50}s were estimated by fitting normalized signal to a standard binding equation.

Surface enzymatic activity and expression of NA on 293T cells. Surface enzymatic activity and expression of WT, H275Y, Y276F, and H275Y/Y276F NA were estimated by transiently expressing A/WSN/33 and A/California/04/09 NA on the surface of 293T cells according to a previously published protocol with modifications (23, 34). Briefly, 1 μg plasmid harboring WT or mutant NA was transfected into 293T cells using TransIT-LT1. 293T cells were harvested 22 to 24 h posttransfection and resuspended in nonlysis buffer. Cells (2%) were subjected to MUNANA assays as described for recombinant viruses to estimate enzyme activity. The rest of the cells were used for flow cytometry to estimate the surface expression of NA after staining with anti-V5 antibody conjugated to allophycocyanin (APC; Abcam, Cambridge, MA). Transfection efficiency was estimated from flow cytometry as the fraction of GFP-positive cells. The enzyme activity was normalized to transfection efficiency. The surface expression of NA was estimated as the mean fluorescence signal of APC. Relative surface expression and enzyme activity were determined by normalization to WT.

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