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Influenza Viruses Expressing Chimeric Hemagglutinins: Globular Head and Stalk Domains Derived from Different Subtypes

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The influenza virus hemagglutinin molecule possesses a globular head domain that mediates receptor binding and a stalk domain at the membrane-proximal region. We generated functional influenza viruses expressing chimeric hemagglutinins encompassing a variety of globular head and stalk combinations, not only from different hemagglutinin subtypes but also from different hemagglutinin phylogenetic groups. These chimeric recombinant viruses possess growth properties similar to those of wild-type influenza viruses and can be used as reagents to measure domain-specific antibodies in virological and immunological assays.

Hemagglutinin (HA) is a homotrimeric molecule that is the major surface glycoprotein of influenza virus. Each monomer consists of two disulfide-linked glycosylated polypeptides, HA1 and HA2, that are generated by proteolytic cleavage (15). These polypeptides make up two structurally distinct domains: a globular head, composed of part of HA1, and a stalk structure, composed of portions of HA1 and all of HA2 (27). These domains are involved in two essential functions for the initiation of viral infection. The globular head domain contains a sialic acid binding pocket that mediates virus attachment to the host cell (18), whereas the fusion peptide, located in the HA2 region of the stalk domain, induces pH-triggered membrane fusion between the viral envelope and the endosomal membrane of the cell. These functions allow the virus to enter the host cell and release genetic material so that replication, transcription, and translation of the viral genome—and the subsequent production of progeny virions—can occur.

The globular head domain of the HA is also the major antigenic component on the surface of the virus. A large percentage of the antibodies generated after infection by influenza viruses are directed against specific antigenic sites located in the globular head domain of the HA (15). Earlier studies from our laboratory have shown that foreign B-cell epitopes, either from another HA subtype (10) or from an unrelated virus (9, 12), can be introduced into the antigenic sites of the head domain of the HA, and infectious influenza viruses can be generated. Vaccination with such chimeric viruses can induce an immune response against both parental viruses.

Previously, we had used a highly conserved disulfide bond (Cys52-Cys277 [H3 numbering]) that separates the stalk and head domains to construct headless HA immunogens (20). We then hypothesized that we could use the same disulfide bond as a demarcation point to generate influenza viruses expressing chimeric HAs (cHAs) that consist of globular head and stalk domains from different influenza virus strains. We were able to generate a virus that expressed a cHA composed of the head from an H9 virus and the stalk domain from the A/Puerto Rico/8/34 (PR8) virus (16). We now extend our studies to see if this technique is broadly applicable to different HA subtypes and to HAs of different phylogenetic groups.

We have been able to successfully rescue recombinant viruses containing HAs that have entire domains replaced by those from another HA subtype. We have generated recombinant viruses with the following HA combinations: the head of A/California/4/09 (H1, group 1) (Cal/09) or A/Viet Nam/1203/04 (H5, group 1) (VN/04) on the stalk of PR8 (H1, group 1) and the head of VN/04 (H5, group 1) or A/mallard/Alberta/24/01 (H7, group 2) (Alb/01) on the stalk of A/Perth/16/2009 (H3, group 2) (Perth/09). The recombinant viruses bearing different chimeric HAs replicate efficiently *in vitro*, indicating that the cHAs generated fold correctly and are functional.

MATERIALS AND METHODS

Cells and viruses. 293T and MDCK cells were obtained from the American Type Culture Collection (ATCC) and were maintained either in Dulbecco's minimal essential medium (DMEM) or in MEM (Gibco, Invitrogen) supplemented with 10% fetal calf serum (HyClone; Thermo Scientific) and penicillin-streptomycin (Gibco, Invitrogen). Wild-type A/Puerto Rico/8/1934 (PR8) and A/Perth/16/2009 (Perth/09) (kindly provided by Alexander Klimov, CDC) and the recombinant viruses were grown in 10-day-old specific-pathogen-free embryonated hen's eggs (Charles River) at 37°C for 2 days.

Construction of plasmids. Plasmids encoding the different chimeric hemagglutinins were constructed using a strategy similar to those described previously (7, 8, 13). Briefly, the different segments of chimeric HA were amplified by PCR with primers containing SapI sites, digested with SapI, and cloned via multisegmental ligation into the SapI sites of the ambisense expression vector pDZ, in which viral RNA (vRNA) transcription is controlled by the human RNA polymerase I promoter and the mouse RNA polymerase I terminator, while mRNA/cRNA transcription is controlled by the chicken beta-actin polymerase II promoter (17). We thank Daniel Perez (University of Maryland) for the kind gift of the H7 HA plasmid (GenBank accession number DQ017504). The plasmids encoding PR8 genes were used as described previously (8).

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Flow cytometric analysis. To assess levels of hemagglutinin protein expression at the cell surface, 293T cells were transfected with 1 μg of the appropriate plasmid by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions, or MDCK cells were infected with cHA-expressing recombinant viruses. At 48 h posttransfection, 293T cells were trypsinized and resuspended in phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) prior to staining with monoclonal antibody (MAb) 6F12 (5 μg/ml), a MAb generated in our laboratory that is broadly reactive to the stalk domain of group 1 HAs (data not shown), or with MAb 12D1 (5 μg/ml) against H3 HAs (25). At 12 h postinfection, MDCK cells were resuspended by trypsinization and were stained with MAb 12D1. Stained cells were analyzed on a Beckman Coulter Cytomics FC 500 flow cytometer, and the results were analyzed using FlowJo software.

Pseudoparticle generation and entry assay. The procedure for pseudoparticle production was adapted from previous studies (6, 23). Briefly, we cotransfected 293T cells with four plasmids encoding (i) a provirus containing the desired reporter (V1-*Gaussia* luciferase) (6), (ii) HIV Gag-Pol (6), (iii) chimeric hemagglutinin protein, and (iv) B/Yamagata/16/88 virus neuraminidase (NA). Supernatants were collected 72 h posttransfection and were subsequently filtered (pore size, 0.45 μm). The presence of pseudotype virus-like particles (VLPs) was evaluated through hemagglutination assays. Different VLP preparations were adjusted to the same 4 hemagglutination units prior to inoculation of MDCK cells. All of the assays using pseudoparticles described below were performed in the presence of 1 μg/ml Polybrene (Sigma) to increase the efficiency of transduction (23).

The entry assay was performed by transducing MDCK cells with pseudoparticles that expressed different chimeric hemagglutinins and contained the *Gaussia* luciferase reporter. Twenty-four hours posttransduction, cells were washed three times with fresh medium to remove any residual *Gaussia* luciferase protein present in the inoculum. Forty-eight hours posttransduction, luciferase assays were performed (6).

Rescue of recombinant chimeric influenza A viruses. Influenza A viruses were rescued from plasmid DNA as described previously (7, 8, 13). To generate the recombinant wild-type (rWT) PR8 virus, 293T cells were cotransfected with 1 μg of each of the eight pDZ PR8 rescue plasmids using Lipofectamine 2000 (Invitrogen). The wild-type HA plasmid was replaced with a plasmid encoding the desired chimeric HA in order to generate cHA-expressing recombinant viruses. At 6 h posttransfection, the medium was replaced with DMEM containing 0.3% bovine serum albumin (BSA), 10 mM HEPES, and 1.5 μg/ml TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone)-treated trypsin (Sigma). After 24 h posttransfection, 8-day-old embryonated chicken eggs were inoculated with the virus-containing supernatant. Allantoic fluid was harvested after 2 days of incubation at 37°C and was assayed for the presence of virus by hemagglutination of chicken red blood cells. The titers of virus stocks were determined by plaque assays on MDCK cells as described previously (7, 8).

Virus growth kinetics assay. To analyze the replication characteristics of recombinant viruses, 10-day-old embryonated chicken eggs were inoculated with 100 PFU of wild-type or cHA-expressing recombinant viruses. Allantoic fluid was harvested and was subsequently assayed for viral growth at 0, 9, 24, 48, and 72 h postinfection (hpi). The titers of virus present in allantoic fluid were determined by plaque assays on MDCK cells as referenced above.

Immunostaining of plaques. Plaques were visualized by immunostaining with MAb HT103 against the influenza A virus nucleoprotein (NP) by use of a previously described protocol (1, 19).

Western blotting and indirect immunofluorescence analysis. Confluent MDCK cells either were infected (multiplicity of infection [MOI], 2) with recombinant influenza viruses or were mock infected with PBS for 1 h at 37°C. At 12 hpi, cells were lysed in 1× SDS loading buffer as described previously (8, 29). The reduced cell lysates were analyzed by Western blot analysis using MAbs against influenza A virus NP (HT103) (14), the PR8 HA head domain (PY102), the Cal/09 HA head domain (29E3) (11), and the VN/04 HA head domain (MAb 8) (19), as well as 12D1, a pan-H3 antibody reactive against the HA stalk (25). In order to detect H7 head domains, polyclonal goat serum NR-3152 (raised against the A/FPV/Dutch/27 [H7] virus; BEI Resources) was used. An anti-glyceraldehyde-3-phosphate dehydrogenase (anti-GAPDH) antibody (Abcam) was used as the loading control. Proteins were visualized using an enhanced chemiluminescence protein detection system (Perkin-Elmer Life Sciences).

For immunofluorescence analysis, confluent monolayers of MDCK cells on 15-mm-diameter coverslips were infected with recombinant viruses at an MOI of 2. At 15 hpi, cells were fixed and permeabilized with methanol-acetone (ratio, 1:1) at −20°C for 20 min. After being blocked with 1% bovine serum albumin in PBS containing 0.1% Tween 20, cells were incubated for 1 h with the antibodies described above, as well as with MAb 6F12. After three washes with PBS containing 0.1% Tween 20, cells were incubated for 1 h with Alexa Fluor 594-conjugated anti-mouse IgG (Invitrogen) or Alexa Fluor 594-conjugated anti-goat IgG (Invitrogen). Following the final three washes, infected cells were analyzed by fluorescence microscopy with an Olympus IX70 microscope.

Plaque reduction assay. The plaque reduction assay was performed as described previously (25). Approximately 60 to 80 PFU of recombinant viruses expressing cHA made up of a Cal/09 or VN/04 globular head domain atop a PR8 stalk was incubated with or without different concentrations (100, 20, 4, 0.8, 0.16, and 0.032 μg/ml) of MAb KB2, a broadly neutralizing anti-HA stalk antibody generated in our laboratory (data not shown), for 60 min in a total volume of 240 μl at room temperature. A confluent layer of MDCK cells in 6-well plates was washed twice with PBS and was then incubated with the antibody-virus mixture for 40 min at 37°C. A TPCK-trypsin agar overlay either with no antibody or supplemented with the antibody at the concentrations described above was then added to each well after the inoculum had been aspirated off. Plates were incubated for 2 days at 37°C. Plaques were then visualized by immunostaining (1, 19) with anti-influenza A virus NP antibody HT103.

Pseudotype particle neutralization assay. The procedure for pseudotype particle production was the same as that described above and used the cHA construct comprising either a VN/04 (H5) or a Cal/09 (H1) head and a PR8 (H1) stalk with the influenza B/Yamagata/16/88 virus NA. Particles were then incubated with different concentrations of MAb KB2 at 5-fold dilutions from 100 to 0.032 μg/ml. Then these mixtures were added to MDCK cells. Transductions proceeded for 6 h before cells were washed and fresh medium was placed over cells. All transductions using pseudotype particles were performed in the presence of 1 μg/ml Polybrene (Sigma, St. Louis, MO) (23). Forty-eight hours posttransduction, luciferase assays were performed in order to assay the degree in which entry was blocked by MAb KB2.

Nucleotide sequence accession numbers. All constructed cHA genes used in this study have been deposited in the Influenza Research Database and can be accessed under GenBank accession numbers CY110921 to -24. The chimeric cH1/1, cH5/1, cH7/3, and cH5/3 viruses are listed as A/Puerto Rico/8-RGcH1-1/34, A/Puerto Rico/8-RGcH5-1/34, A/Perth/16-RGcH7-3/09, and A/Perth/16-RGcH5-3/09, respectively.

RESULTS

Generation of chimeric hemagglutinins. We generated an alignment of influenza A virus HA sequences of the H1, H3, H5, and H7 subtypes used in this study in order to see if the cysteine residues forming the Cys52-Cys277 disulfide bond were conserved. Because these cysteine residues are highly conserved across HA subtypes, for both group 1 and group 2 HAs, we hypothesized that we could use this bond as a delineating point between the head and stalk domains. By defining the sequence between Cys52 and Cys277 as the head region and the remainder of the molecule as the stalk, we rationalized that we could engineer constructs that

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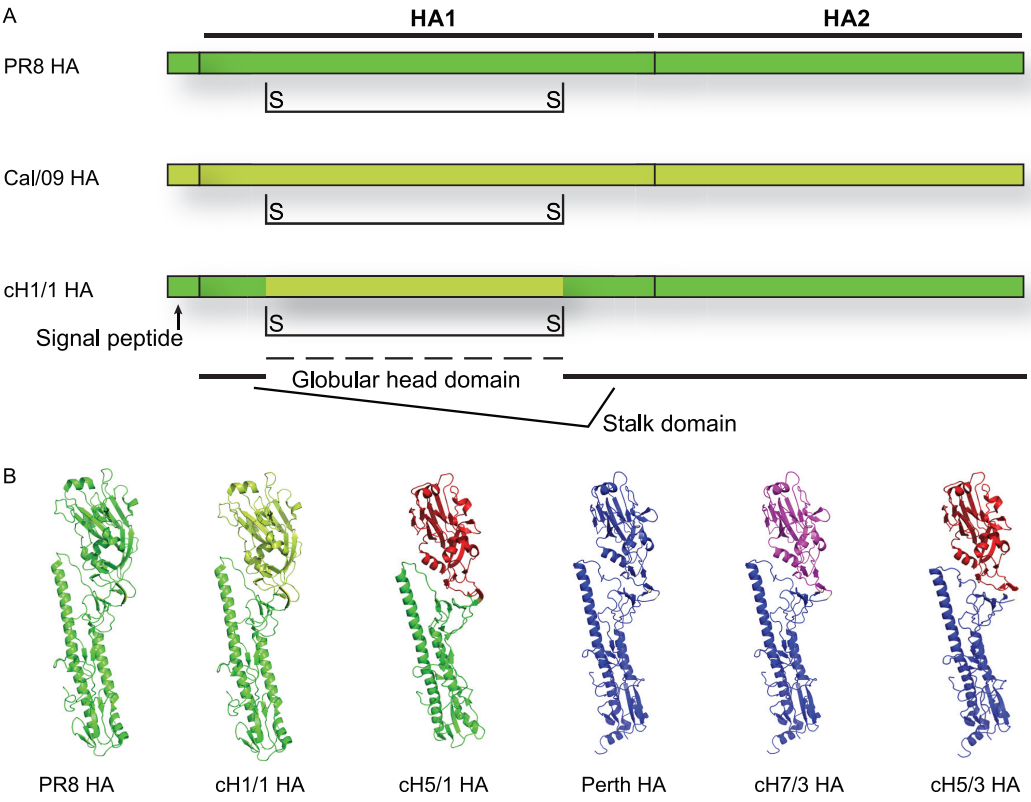

C

Percent amino acid identity in the globular head domain

	A/PR/8/34 (H1)	A/Cal/4/09 (H1)	A/VN/1203/04 (H5)	A/Perth/16/09 (H3)	A/mallard/Alb/24/01 (H7)
A/PR/8/34 (H1)		70	50	31	32
A/Cal/4/09 (H1)			46	31	27
A/VN/1203/04 (H5)				34	31
A/Perth/16/09 (H3)					30
A/mallard/Alb/24/01 (H7)					

Percent amino acid identity in the stalk domain

	A/PR/8/34 (H1)	A/Cal/4/09 (H1)	A/VN/1203/04 (H5)	A/Perth/16/09 (H3)	A/mallard/Alb/24/01 (H7)
A/PR/8/34 (H1)		90	77	47	47
A/Cal/4/09 (H1)			78	50	48
A/VN/1203/04 (H5)				46	49
A/Perth/16/09 (H3)					61
A/mallard/Alb/24/01 (H7)					

Percent amino acid identity of the full length hemagglutinin

	A/PR/8/34 (H1)	A/Cal/4/09 (H1)	A/VN/1203/04 (H5)	A/Perth/16/09 (H3)	A/mallard/Alb/24/01 (H7)
A/PR/8/34 (H1)		82	65	40	42
A/Cal/4/09 (H1)			65	42	40
A/VN/1203/04 (H5)				41	42
A/Perth/16/09 (H3)					49
A/mallard/Alb/24/01 (H7)					

FIG 1 Schematic of chimeric hemagglutinins and sequence comparisons. (A) Diagrams of wild-type and cH1/1 viruses. The chimeric HA was constructed by swapping the globular head domain located between Cys52 and Cys277 of PR8 (H1) HA (dark green) with that of the A/California/4/09 (H1) HA (light green). The resulting chimeric HA has the stalk region of the A/PR8/34 (H1) HA with the globular head domain of the A/California/4/09 (H1) HA and is designated cH1/1. (B) Theoretical schematics of the folded structures of the different wild-type and chimeric HAs (from left to right, wild-type PR8 HA, the chimeric cH1/1 HA, the chimeric cH5/1 HA, the wild-type Perth HA, the chimeric cH7/3 HA, and the chimeric cH5/3 HA). The different HAs are color coded as follows: dark green, PR8 HA; light green, Cal/09 HA; red, VN H5 HA; blue, Perth/09 HA; cyan, H7 HA. The following full-length HA structures were downloaded from the Protein Database (PDB): PR8 HA (PDB identification code [ID] 1RU7) and Perth/09 HA (represented by A/duck/Ukraine/63 [H3N8]) HA (PDB ID 1MQN). Final images were generated with PyMol (Delano Scientific). In order to represent the cHAs, only the colors of the HA head domains from the wild-type structures were changed. (C) Tables comparing amino acid identities between the H1, H3, H5, and H7 HAs used in this study. The percentages of amino acid identity were calculated using ClustalW (excluding the signal peptide) and were compared for full-length HA, the globular head domain, and the stalk domain. Shaded cells indicate 100% identity.

encode novel head and stalk combinations from a variety of HA subtypes (Fig. 1A and B).

The degree of amino acid identity that exists between the stalk regions of hemagglutinin subtypes further encouraged us to think that the swapping of head domains might be possible. Higher percentages of amino acid identity were seen in the stalk domains across all subtypes than in the head domains (Fig. 1C).

All 16 subtypes of influenza virus HA are classified into two

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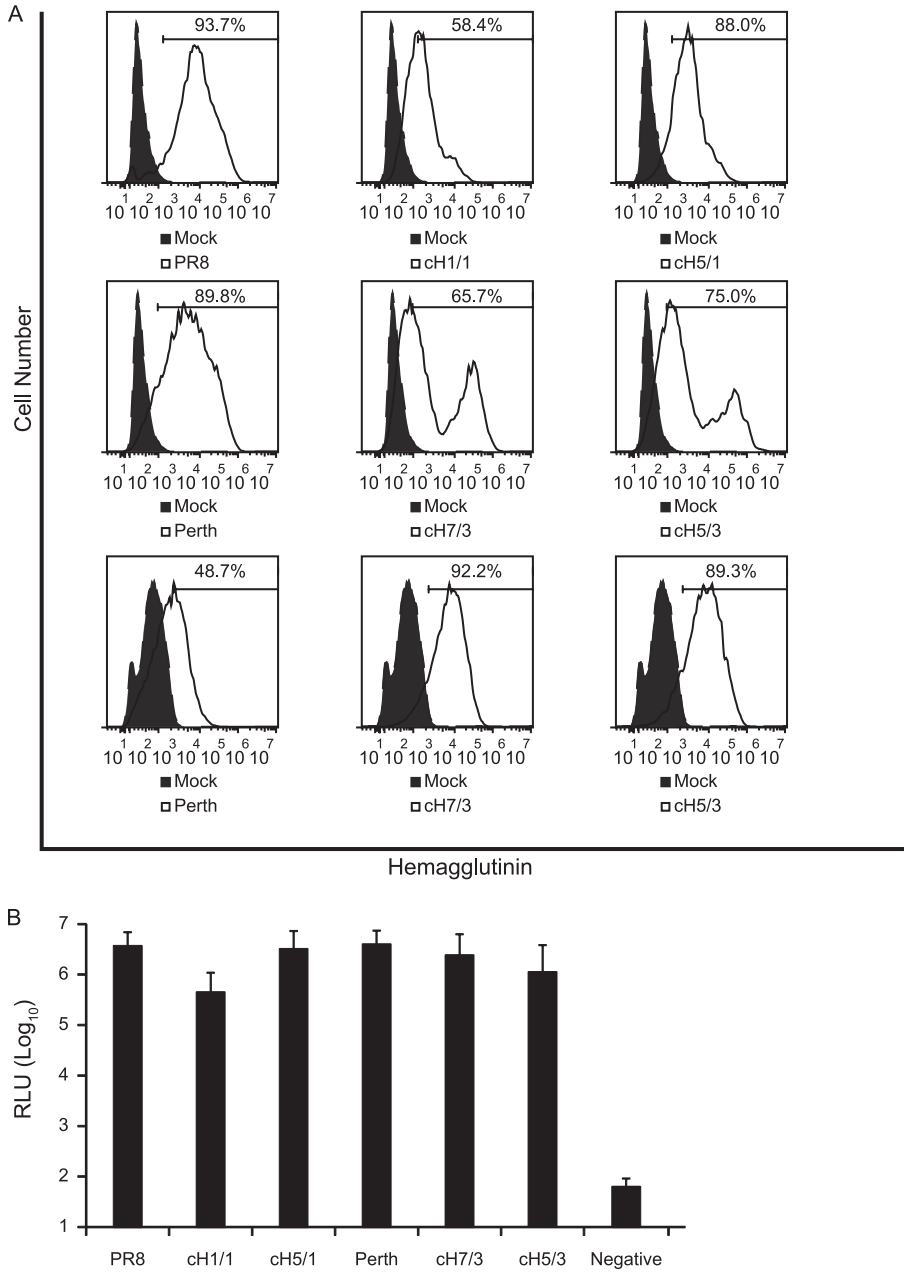

FIG 2 Surface expression and functional analysis of chimeric HA constructs. (A) The surface expression of chimeric HA constructs was evaluated in transiently transfected or infected cells. At 48 h posttransfection, 293T cells were trypsinized, and the expression of chimeric HA proteins on the cell surface was analyzed by flow cytometry. (Top) Mock-transfected 293T cells (shaded histograms) are compared to cells transfected with either PR8, cH1/1, or cH5/1 (open histograms). (Middle) Mock-transfected 293T cells (shaded histograms) are compared to cells transfected with either Perth/09, cH7/3, or cH5/3 (open histograms). (Bottom) MDCK cells were infected with recombinant viruses expressing either Perth/09, cH7/3, or cH5/3. At 12 h postinfection, the expression of the different HAs on the cell surface was analyzed using flow cytometry. (B) Luciferase-encoding pseudoparticles expressing chimeric HAs were used to transduce MDCK cells. The relative light units (RLU) generated in the luciferase assay indicate that pseudoparticles expressing chimeric HAs are able to enter cells.

phylogenetic groups (15). Because we saw higher percentages of amino acid identity within the stalk regions of a particular group (Fig. 1C), and because we had success in generating one cHA virus that contained head and stalk domains from group 1 viruses (16), we attempted to make intragroup cHAs. For group 1, we generated two chimeric hemagglutinin constructs that encode either the pandemic H1 Cal/09 or VN/04 globular head domain with the stalk region from PR8 HA (H1) (cH1/1 and cH5/1, respectively) (Fig. 1B). We applied a similar strategy to generate a chimeric HA that expressed head and stalk domains from different group 2 influenza strains: the head from Alb/01 HA (H7, group 2) and the stalk region from Perth/09 HA (H3, group 2) (cH7/3) (Fig. 1B). Finally, we evaluated whether we could swap the head and stalk domains to make an intergroup chimeric HA containing the head domain of VN/05 HA (H5, group 1) atop a Perth/09 HA (H3, group 2) stalk (cH5/3) (Fig. 1B).

Following the construction of these plasmids, we tested whether the different chimeric HA constructs could be expressed

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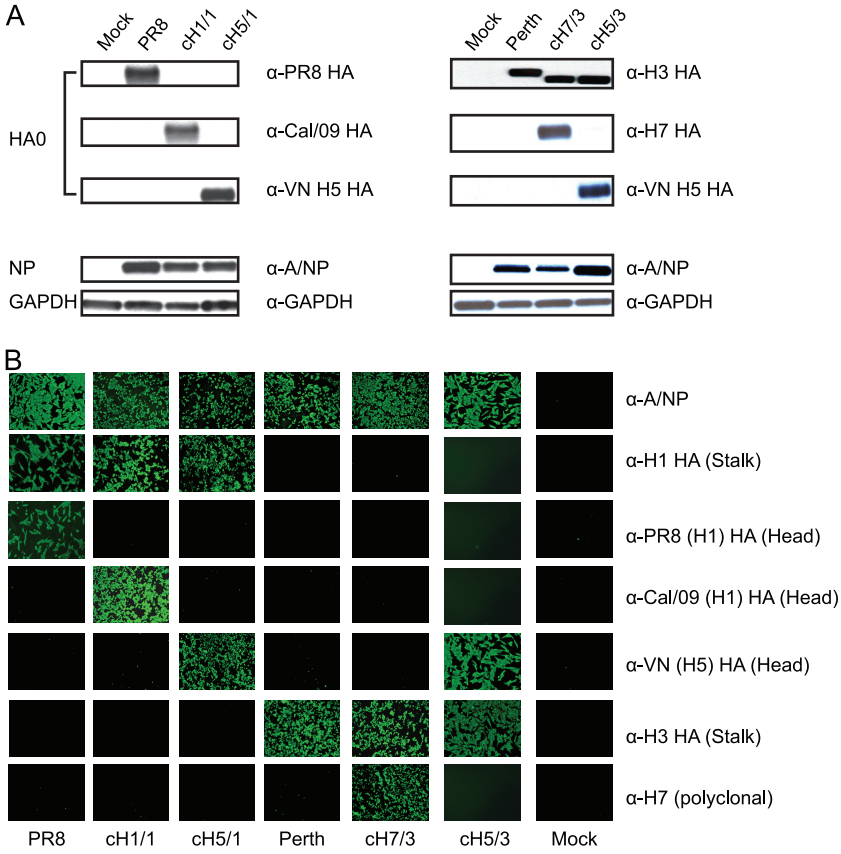

FIG 3 Generation of recombinant viruses bearing chimeric hemagglutinins. (A) Western blot analysis of cells infected with recombinant cHA-expressing viruses. Extracts from MDCK cells that were either mock infected or infected with the indicated viruses at an MOI of 2 were prepared and were probed with the following antibodies at 16 hpi: anti-A/PR8 HA (H1) (PY102), anti-A/Cal/09 HA (H1) (29E3), anti-A/VN HA (H5) (MAb 8), anti-H3 HA (12D1), anti-H7 HA (NR-3152), and anti-A/NP (HT103). Anti-GAPDH was used as a loading control. (B) Immunofluorescence analysis of MDCK cells infected with recombinant viruses using anti-A/NP (HT103), anti-A/H1 HA (6F12), anti-A/PR8 HA (PY102), anti-A/Cal/09 HA (29E3), anti-A/VN HA (MAb 8), anti-H3 HA (12D1), and anti-A/H7 (NR-3152) antibodies.

and transported to the cell surface like wild-type HAs. Fluorescence-activated cell sorter (FACS) analysis of transiently transfected 293T cells was performed following surface staining with H1 and H3 stalk domain-specific antibodies. Using this method, we were able to detect the expression of all four chimeric constructs on the cell surface (Fig. 2A). However, compared to that for the wild-type PR8 HA, less surface protein expression was detected for the cH1/1 construct, which could be attributed to the inherent character associated with the head domain of the Cal/09 HA or to a lower transfection efficiency for this chimeric DNA construct. In addition, it is noteworthy that the cell surface expression patterns of the cH7/3 and cH5/3 constructs were different. This "double-peak" expression pattern was observed only under transfection conditions and was reproducible. It was not detected upon infection with either cH7/3- or cH5/3-expressing recombinant viruses (Fig. 2A). Therefore, these data indicate that the cHAs can be transported through the Golgi complex to the cell surface.

Next, we examined the entry characteristics of the different cHAs through transduction of MDCK cells with retroviral pseudotype particles that contained a luciferase reporter construct and expressed the cHA and wild-type B/Yamagata/16/88 virus NA on the particle surface. The entry efficiency mediated by the cHA proteins was detected by the luciferase readout. Comparable levels of pseudotype particle-mediated luciferase expression were observed for cH5/1, cH7/3, and cH5/3 chimeric HAs and the corresponding wild-type proteins (Fig. 2B). Particles encoding the cH1/1 HA expressed lower luciferase levels than did the other HA constructs, which could be due either to the lower expression of cH1/1 in the producer cell line (leading to fewer HA trimers per particle) or to the less efficient entry properties of the cH1/1 HA. It is also possible that when one is normalizing the pseudotype particles to 4 hemagglutinin units, the actual number of pseudotype particles may differ due to differences in binding to red blood cells.

Generation of recombinant influenza viruses bearing chimeric hemagglutinins. Because we had determined that our cHA constructs were efficiently expressed and transported to the cell surface, we wanted to ascertain whether a recombinant influenza virus that encodes a cHA could be rescued. Using previously published protocols (7, 8), we succeeded in generating viruses containing the different cHAs. The resulting viruses were plaque purified and were amplified in 10-day-old embryonated eggs, and the chimeric segments were analyzed by reverse transcription-PCR (RT-PCR) and sequenced. In all cases, the virus was found to have the expected chimeric HA segment and no other HA segment (data not shown).

We further confirmed the presence of the cHAs in rescued viruses by Western blotting and indirect immunofluorescence of

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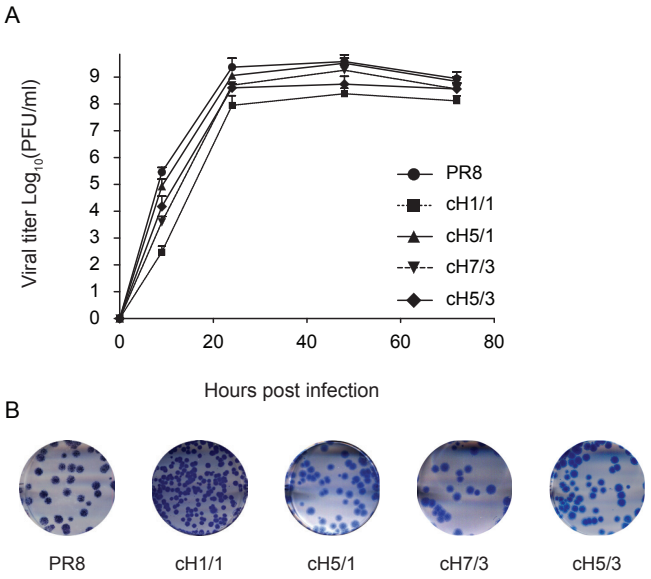

FIG 4 Growth kinetics and plaque phenotypes of wild-type and recombinant viruses. (A) Ten-day-old embryonated chicken eggs were infected with 100 PFU per egg of wild-type or recombinant virus, and viral growth was monitored for 72 h postinfection. Data points are averages and standard deviations for experimental replicates. (B) The plaque phenotypes of recombinant viruses were assessed by plaque assays. MDCK cells were infected with either a wild-type or a recombinant virus. Cells were fixed 48 h postinfection and were immunostained to reveal plaque phenotypes by using the antibody against A/NP (HT103).

infected cells (Fig. 3A and B). MDCK cells were infected with the rWT PR8, wild-type Perth/09, cH1/1, cH5/1, cH7/3, and cH5/3 viruses (Fig. 3A and B). cH1/1 and cH5/1 chimeric HA proteins were detected in the corresponding samples using antibodies reactive against the head domain of Cal/09 (H1) HA (29E3) (11) or VN/04 (H5) HA (MAb 8) (19), respectively (Fig. 3A). Using 12D1, a pan-H3 anti-stalk MAb (25), we could observe comparable expression levels among the cH7/3, cH5/3, and wild-type Perth HAs. The wild-type Perth HA showed slower migration on the gel, likely due to a higher number of glycosylation sites in the globular head domain. We confirmed that the correct HA head domain was expressed atop an H3 stalk using an anti-H7 polyclonal (NR-3152) or anti-H5 monoclonal (MAb 8) antibody on cH7/3 or cH5/3 infection samples, respectively. Positive bands were detected in both cases.

For the immunofluorescence study, the infection conditions were similar to those used for Western blot analysis. Infected cells were stained with corresponding antibodies as used in Fig. 3A. All infected cells showed the expected expression of chimeric and wild-type HAs, as well as of influenza A virus NP (Fig. 3B).

Replication characteristics of recombinant viruses. The growth properties of wild-type and recombinant viruses were assessed in 10-day-old embryonated chicken eggs at 37°C (Fig. 4A). The rWT PR8 virus was included for comparison of the growth kinetics of the recombinant viruses expressing chimeric HAs. The cH5/1 and cH5/3 viruses displayed replication kinetics comparable to that of the rWT PR8 virus. The cH7/3 virus grew to peak titers similar to those of rWT PR8 at 48 hpi (1×10^9 PFU/ml), although the cH7/3 viral titer was 2 log units lower than that of the rWT PR8 virus at 9 hpi. The cH1/1 virus was attenuated compared to the rWT PR8 virus, as shown by reduced viral titers at all time points. Nonetheless, the cH1/1 virus reached a respectable peak titer of approximately 10^8 PFU/ml. The Perth/09 wild-type virus grows to comparable peak titers in embryonated eggs (data not shown).

The plaque phenotype of each of the chimeric viruses was also evaluated in MDCK cells. All viruses formed comparably sized plaques, as shown in Fig. 4B. These data, taken together, confirm that the chimeric HA constructs fold correctly and are biologically functional.

Stalk-specific antibodies can neutralize cHA-expressing viruses and pseudoparticles. Finally, we wanted to test whether stalk-specific antibodies were able to neutralize our newly generated recombinant viruses expressing cHAs. We performed plaque reduction assays either without antibodies or in the presence of MAb KB2, an HA stalk-specific antibody with broad group 1 reactivity generated in our laboratory. We were able to show that MAb KB2 neutralizes all cHA-expressing viruses with similar efficiencies and in a dose-dependent manner. At 100 μg/ml, MAb KB2 was able to neutralize the cH1/1 and cH5/1 viruses with 100% efficiency, and it retained some neutralizing activity at concentrations as low as 4 μg/ml (Fig. 5A).

We confirmed these results by using a pseudotype particle inhibition assay with MAb KB2. Pseudotype particles expressing cH1/1 or cH5/1 and influenza B virus NA were added to MDCK cells either in the presence of MAb KB2 or without an antibody. Forty-eight hours posttransduction, the supernatant was collected, and luciferase activity was analyzed. As expected, MAb KB2 blocked the entry of cH1/1 and cH5/1 pseudotype particles in a dose-dependent manner at concentrations above 4 μg/ml. While lower concentrations of MAb KB2 than those effective in the plaque reduction assay were sufficient to inhibit the entry of pseudotype particles, this result was expected due to the assumed lower incorporation of HA trimers on the surfaces of pseudotype particles (3). This phenomenon of different neutralizing potencies of MAbs in assays that involve whole virus versus pseudotype particles has been recognized in other studies (3, 21).

DISCUSSION

Using the conserved disulfide bond Cys52-Cys277, which defines the border between the head and stalk domains, we developed a strategy to generate influenza viruses with chimeric HA proteins that express different HA globular head and stalk domain combinations. The constructs included combinations of heads and stalks within group 1 (cH1/1 and cH5/1) and group 2 (cH7/3) or between the two HA groups (cH5/3). All constructs were expressed on the cell surface and retained fusion activity. The generation of recombinant viruses bearing the cHAs further proved that the HAs folded correctly and retained biological functions. Furthermore, we have demonstrated that cHA-expressing recombinant viruses and pseudoparticles can be neutralized using monoclonal antibodies with known reactivity to HA stalk domains.

While we were able to detect surface expression and fusion capability for all cHAs, it is interesting that the surface expression level of cH1/1 HA was approximately 2-fold lower than that of the wild-type PR8 HA (Fig. 2A); fusion activity was also less efficient (Fig. 2B). Despite these minor deficiencies, we were able to generate recombinant viruses with the cH1/1 HA, which showed a peak titer only 1 log unit lower than that of wild-type PR8 (Fig. 4A). It is known that wild-type Cal/09 virus grows poorly in eggs; an

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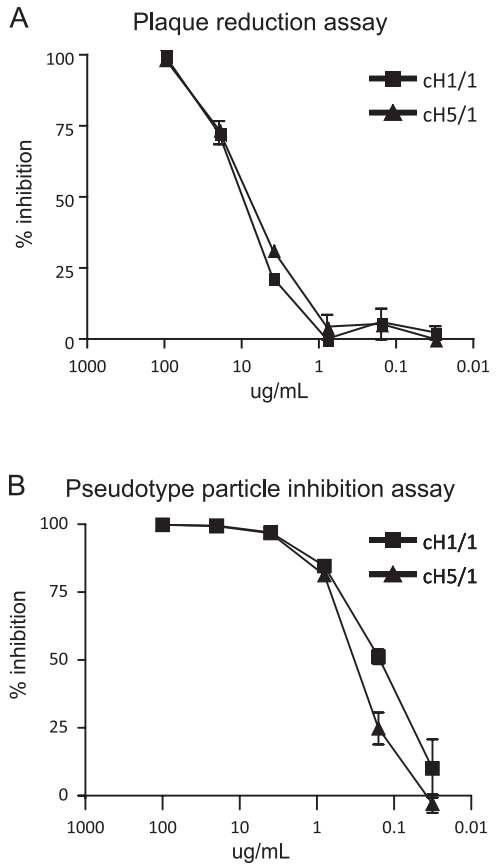

FIG 5 A stalk-specific monoclonal antibody neutralizes cHA-expressing viruses and pseudotype particles. The ability of a MAb (KB2) to neutralize cHA-expressing viruses or pseudotype particles was assessed by a plaque reduction assay or a pseudotype particle inhibition assay. MDCK cells were infected or transduced with cHA-expressing viruses or pseudotype particles either in the presence of the indicated amounts (in micrograms per milliliter) of the MAb or without an antibody. Plaque formation or luciferase activity was used as a readout to determine the degree of inhibition by the MAb. (A) MAb KB2 neutralizes the replication of the cH1/1 (squares) and cH5/1 (triangles) viruses in a dose-dependent manner, with 100% inhibition at concentrations above 100 μg/ml. Data points are averages and standard deviations for experimental replicates. (B) MAb KB2 also inhibits the entry of cH1/1 and cH5/1 pseudotype particles in a dose-dependent manner, with complete inhibition above 4 μg/ml. Data points are averages and standard deviations for experimental replicates. The pseudotype inhibition assays were processed independently.

early report implicates two amino acid substitutions with this phenotype (K119E and A186D) (2). Therefore, the attenuation of the cH1/1 virus is likely due to properties associated with the Cal/09 HA globular head domain and not to the design of the chimeric HA.

The successful generation of recombinant viruses expressing the chimeric cH5/3 HA (with the head [H5] domain from group 1 and the stalk [H3] from group 2) impressively demonstrates that a cHA can be generated using combinations of heads and stalks within or between the two defined HA phylogenetic groups. Although we were able to rescue these recombinant viruses, it is possible that certain head and stalk combinations could not be rescued. An inherent difference of compatibility might exist between the heads and stalks of different HA subtypes (or viral variants).

Previously, it has been shown that antigenic epitopes, from influenza A virus and other viruses, can be introduced into the globular head domain of the viral HA (9, 10, 12). In addition, Wang et al. have demonstrated that recombinant viruses can be generated that express an HA possessing the HA1 portion of Cal/09 and the HA2 region from A/South Dakota/6/07 (SD/07). Both Cal/09 and SD/07 are H1 viruses of the group 1 type and thus have a high degree of similarity in the HA2 region. Specifically, only 18 amino acids are different in the swapped HA2 domain (26). Conversely, we have been able to swap out regions that share low amino acid sequence identity (Fig. 1C), rescuing infectious influenza viruses that contain cHAs with head and stalk domains from viruses classified in different phylogenetic groups.

Although most antibodies elicited by the HA are strain specific and are directed against the globular head domain, several groups have reported various broadly neutralizing antibodies that bind to epitopes on the influenza virus HA stalk domain (4, 5, 22, 24, 28). These cHAs could be used as reagents for the study and quantitation of the binding activities of such stalk-specific antibodies. In addition, chimeric HA constructs may be useful in inducing stalk-specific (or head-specific) immune responses.

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