

Influenza Virus Vaccine Based on the Conserved Hemagglutinin Stalk Domain

John Steel,^a Anice C. Lowen,^a Taia T. Wang,^a Mark Yondola,^a Qinshan Gao,^a Kester Haye,^a Adolfo García-Sastre,^{a,b,c} and Peter Palese^{a,b}

Department of Microbiology,^a Department of Medicine, Division of Infectious Diseases,^b and Emerging Pathogens Institute,^c Mount Sinai School of Medicine, New York, New York, USA

John Steel and Anice C. Lowen contributed equally to this article.

ABSTRACT Although highly effective in the general population when well matched to circulating influenza virus strains, current influenza vaccines are limited in their utility due to the narrow breadth of protection they provide. The strain specificity of vaccines presently in use mirrors the exquisite specificity of the neutralizing antibodies that they induce, that is, antibodies which bind to the highly variable globular head domain of hemagglutinin (HA). Herein, we describe the construction of a novel immunogen comprising the conserved influenza HA stalk domain and lacking the globular head. Vaccination of mice with this headless HA construct elicited immune sera with broader reactivity than those obtained from mice immunized with a full-length HA. Furthermore, the headless HA vaccine provided full protection against death and partial protection against disease following lethal viral challenge. Our results suggest that the response induced by headless HA vaccines is sufficiently potent to warrant their further development toward a universal influenza virus vaccine.

IMPORTANCE Current influenza vaccines are effective against only a narrow range of influenza virus strains. It is for this reason that new vaccines must be generated and administered each year. We now report progress toward the goal of an influenza virus vaccine which would protect against multiple strains. Our approach is based on presentation to the host immune system of a region of the influenza virus—called a “headless hemagglutinin” (headless HA)—which is similar among a multitude of diverse strains. We show that vaccination of mice with a headless HA confers protection to these animals against a lethal influenza virus challenge, thereby demonstrating the viability of the approach. Through further development and testing, we predict that a single immunization with a headless HA vaccine will offer effective protection through several influenza epidemics.

Received 28 January 2010 Accepted 29 January 2010 Published 18 May 2010

Citation Steel, J., A. C. Lowen, T. T. Wang, M. Yondola, Q. Gao, et al. 2010. An influenza virus vaccine based on the conserved hemagglutinin stalk domain. *mBio* 1(1):e00018-10. doi:10.1128/mBio.00018-10.

Editor Terence Dermody, Vanderbilt University Medical Center

Copyright © 2010 Steel et al. This is an open-access article distributed under the terms of the Creative Commons Attribution-Noncommercial Share-Alike 3.0 Unported License, which permits unrestricted noncommercial use, distribution, and reproduction in any medium, provided the original author and source are credited.

Address correspondence to Peter Palese, peter.palese@mssm.edu.

Influenza in humans is a respiratory disease that ranges in severity from subclinical infection to primary viral pneumonia, which can result in death. The clinical effects of infection vary with the virulence of the influenza strain and the exposure history, age, and immune status of the host. The cumulative morbidity and mortality caused by seasonal influenza are substantial due to the relatively high attack rate: in the United States, influenza viruses infect an estimated 10% to 15% of the population (1, 2) and are associated with approximately 30,000 deaths each year (3, 4). In addition to causing annual epidemics, influenza viruses are the cause of infrequent pandemics. Due to the lack of preformed immunity against the major viral antigen (hemagglutinin [HA]), pandemic influenza can affect more than 50% of the population in a single year and often causes more severe disease than epidemic influenza. A stark example is the pandemic of 1918, in which an estimated 50 to 100 million people were killed (5).

The most effective way to protect against influenza virus infection is through vaccination; however, current vaccination approaches rely on achieving a good match between circulating strains and the isolates included in the vaccine. Such a match is

often difficult to attain due to a combination of factors. First, influenza viruses are constantly undergoing change: every 3 to 5 years the predominant strains of influenza A viruses are replaced by variants that have undergone sufficient antigenic drift to evade existing antibody responses (6). Isolates to be included in vaccine preparations must therefore be selected each year based on the intensive surveillance efforts of the WHO collaborating centers. Second, to allow sufficient time for vaccine manufacture and distribution, strains must be selected approximately 9 months prior to the initiation of the influenza season. Unfortunately, the predictions of the vaccine strain selection committee can be inaccurate, resulting in a substantial drop in the efficacy of vaccination (7, 8).

Herein, we describe a novel influenza vaccine construct which is based on a conserved region of the HA protein. The rationale for our approach follows (or builds on) from an earlier effort reported in 1983 (9), which in turn was prompted by our realization that the HA2 subunit of the influenza virus hemagglutinin is relatively well conserved (10). We reasoned that antisera elicited against the HA2 protein should be cross-reactive between strains and poten-

tially cross-protective. However, during natural infection or vaccination with conventional influenza vaccines, HA2 is thought to be masked by the membrane-distal portion of HA, a bulky and highly immunogenic globular head domain. With the aim of producing a vaccine which would elicit cross-reactive anti-HA2 antibodies, we therefore derived a method of chemically treating purified influenza virions in order to remove the HA1 subunit. While immunization of rabbits or mice with subviral particles produced in this way yielded a strong humoral response against HA2, this response was not found to be protective against influenza viral challenge (9). In the context of the H2 subtype, a truncated hemagglutinin molecule lacking a significant portion of the globular head has been shown to offer protection to mice upon subsequent challenge with influenza virus. In animals vaccinated with the truncated HA, 7 out of 10 mice were protected from death, while in mock vaccinated animals only 1 out of 10 mice survived infection (28). Since that time, we and others have identified monoclonal antibodies (MAbs) which bind the HA stalk domain and are both broadly cross-reactive and neutralizing (11–14). In light of our earlier efforts to raise immune sera against HA2, a key feature of these antibodies is that they map to epitopes which comprise membrane-proximal portions of both the HA1 and HA2 subunits. Using modern molecular techniques, we have now identified a way of expressing a modified HA molecule which lacks the globular head domain and maintains the integrity of the stalk region, including both the HA1 and HA2 portions. Vaccination of mice with such a headless HA immunogen elicited antisera that were cross-reactive against multiple subtypes of hemagglutinin and furthermore was found to provide protection against lethal influenza virus challenge. Our data suggest that, through optimization of antigen delivery and immunogenicity, a headless HA molecule could form the basis for a broadly protective influenza virus vaccine.

RESULTS

Design and construction of headless HA constructs. We sought to generate an immunogen consisting of the complete HA2 polypeptide and the regions of HA1 contributing to the stalk region but lacking the globular head domain of HA1. With this aim in mind, we noted the existence of a conserved disulfide bond linking cysteines 52 and 277 (H3 numbering) of HA1. The loop flanked by these two cysteines comprises the bulk of the globular head domain, while the N-terminal 51 and the C-terminal 52 amino acids of HA1 extend downward from the cysteine bridge and contribute to the stalk region. Due to the proximity of cysteines 52 and 277 in the three-dimensional structure of HA (Fig. 1) (15), we predicted that replacement of the intervening loop with a short linker peptide would not disrupt the folding of the remainder of the molecule. Based on this principle, we designed a panel of headless HA constructs (Fig. 1). First, sequences encoding linker peptides of two glycines (2G), four glycines (4G), or a proline and a glycine (PG) were inserted into the open reading frames of the A/Puerto Rico/8/1934 (H1N1) (PR8) and the A/Hong Kong/1968 (H3N2) (HK68) hemagglutinins in place of the respective nucleotide sequences encoding amino acids 53 to 276. These three linker peptides were selected to have a range of flexibilities, with 4G predicted to be the most flexible and PG the most rigid. To test whether insertion of a linker in the absence of a disulfide bond at this position would yield a more stable product, we designed three additional constructs in the context of the PR8 HA: sequences

encoding one, two, or three glycines were inserted in place of the sequences encoding amino acids 52 to 277 (that is, both the cysteines and the connecting loop were replaced). Based on the hypothesis that glycosylation may improve trafficking through the Golgi complex, we also tested the insertion of a glycosylation site (Asn-Ala-Ser) in place of amino acids 52 to 277 in both the PR8 and HK68 backgrounds. Finally, a series of three constructs was made in each of the PR8 and HK68 HAs in which existing wild-type amino acids were directly linked: amino acids 51 to 278, 51 to 279, or 50 to 280. Constructs were made in the context of an H1 (representative of group 1) and an H3 (representative of group 2) HA, since the activity of neutralizing antibodies targeting the stalk region appears to be limited to HA subtypes within the same major phylogenetic group (11–14).

Expression of headless HA constructs in transfected cell cultures. As a preliminary test of protein integrity and stability, levels of the headless HA constructs expressed in transiently transfected cells were assessed by Western blotting. As shown in Fig. 2A, headless HA constructs based on the PR8 HA protein were expressed to levels comparable to the corresponding full-length protein at 24 h posttransfection. Within the panel of constructs tested, those which retained Cys 52 and Cys 277 and carried the linker peptide 2G, 4G, or PG exhibited the highest steady-state levels. In the context of the HK68 HA (Fig. 2B), similar results were seen: all HK68 headless HAs tested were detected using an antibody specific to the stalk domain, and those carrying the 2G, 4G, or PG linker between Cys 52 and Cys 277 were the most abundant. For both HK68 and PR8, the least abundant constructs were those with the direct linkage between amino acids 50 and 280 and those with the inserted glycosylation site (Fig. 2).

To test whether the headless HA constructs were also being transported to the cell surface, fluorescence-activated cell sorter (FACS) analysis of transiently transfected 293T cells was performed following surface staining with HA2-specific antibodies. Only the 2G, 4G, and PG constructs, which showed high levels of protein expression by Western blotting, were tested in this assay. As shown in Fig. 3, the three PR8-based constructs and the three HK68-based constructs were detected, indicating that transport through the Golgi complex to the cell surface was not disrupted by removal of the globular head domain. No marked differences among the three linker bridges were noted in either the Western blotting or FACS-based assays. The constructs carrying the 4G linker bridge were selected for further characterization.

Incorporation of headless HA into VLPs. As a further test of the functionality of the headless HA molecules, we assessed their ability to bud from the cell surface to produce virus-like particles (VLPs). While transient expression of the headless HA constructs alone in 293T cells was not found to result in VLP production (data not shown), cotransfection with an HIV Gag-based construct did lead to the production of headless HA-containing particles. Specifically, when either the PR8 or HK68 4G headless HA construct was coexpressed in 293T cells with a Gag-EGFP (enhanced green fluorescent protein) fusion protein, particles capable of sedimenting through a 30% sucrose cushion and containing headless HA proteins were released into the cell culture medium (Fig. 4). Similar results were obtained in the presence (Fig. 4) or absence (data not shown) of exogenous trypsin. Unlike the full-length HA protein, and as expected based on the lack of a globular head domain, the release of headless HA-containing particles was not found to be dependent on the presence of neuraminidase

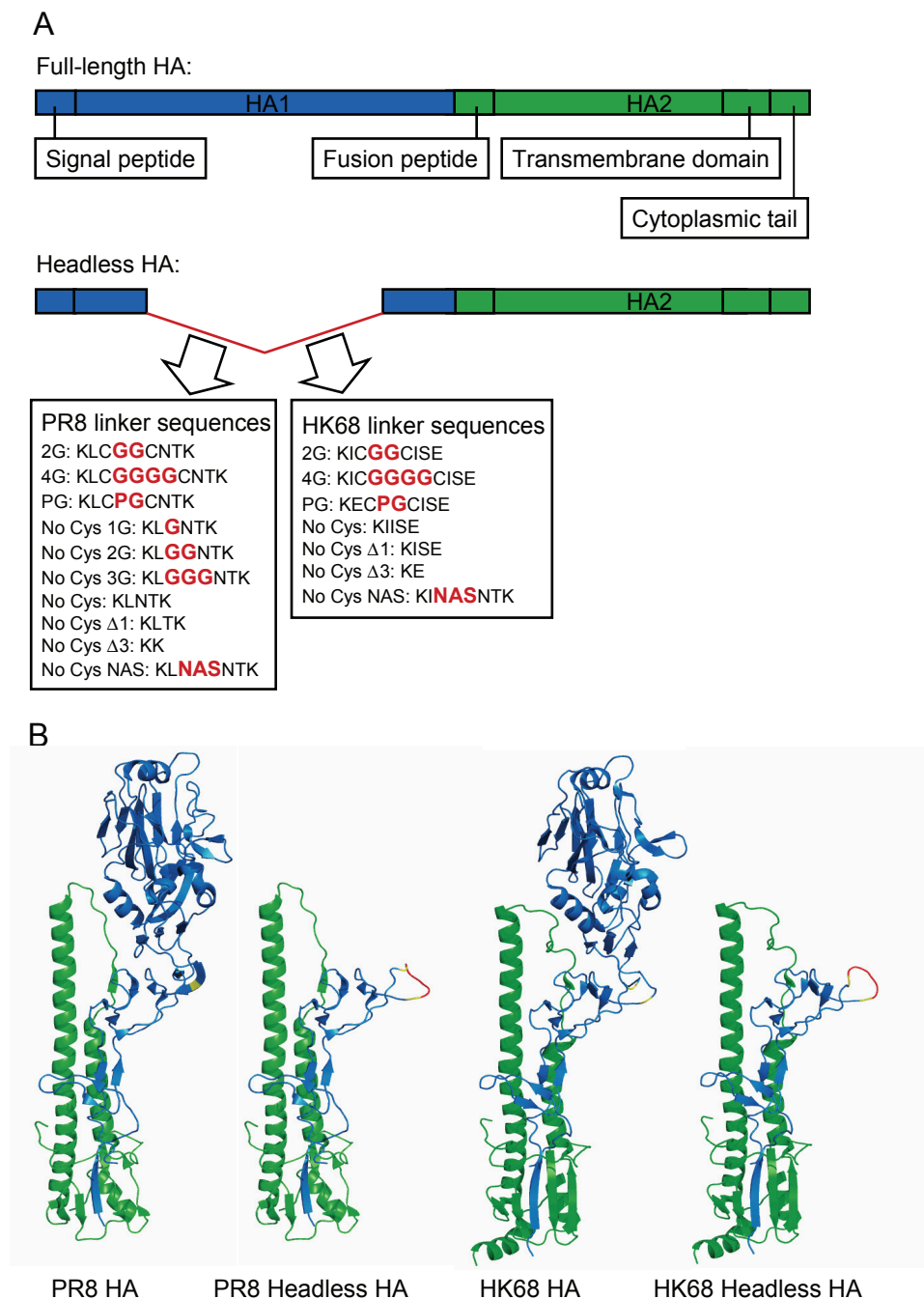


FIG 1 Schematic of headless HA constructs. (A) Schematic of the linear structure of the full-length influenza virus HA protein (top) and a generalized headless HA protein (bottom). Linker peptides tested in the context of the PR8 and HK68 HA sequences are shown. Inserted amino acids are shown in bold red type, while amino acids present in the native HA sequence are in black. (B) Schematic of the folded structures of the full-length and headless HAs of PR8 virus (left panel) and HK68 virus (right panel). In both cases, headless HAs carrying the 4G linker bridge are depicted. The HA1 subunit is blue, and the HA2 subunit is green. Cysteines 52 and 277 are shown in yellow, and 4G linker sequences are shown in red. The full-length HA structures were downloaded from the Protein Database (PDB): PR8 HA (PDB ID 1RVX) and HK68 HA (PDB ID 1MQN). Schematics of headless HAs were generated using the full-length HA coordinates as a starting point, and 4G loops were manually docked into the headless HA carbon to close the discontinuous alpha-carbon amino acid chain. Final images were generated with PyMol (Delano Scientific).

(NA) activity. The neuraminidase activity required for VLPs to carry full-length HA proteins was provided by the inclusion of an expression vector encoding the cognate NA protein in the respective transfections. The presence of NA in the PR8 full-length HA VLPs is visible in Fig. 4 due to the use of a polyclonal antiserum.

Vaccination with the PR8 headless HA provides protection against homologous challenge in mice. The potential of vaccination with a headless HA construct to induce a protective immune response was evaluated in the mouse model. The following three-dose vaccine regimen was used: mice received plasmid DNA on

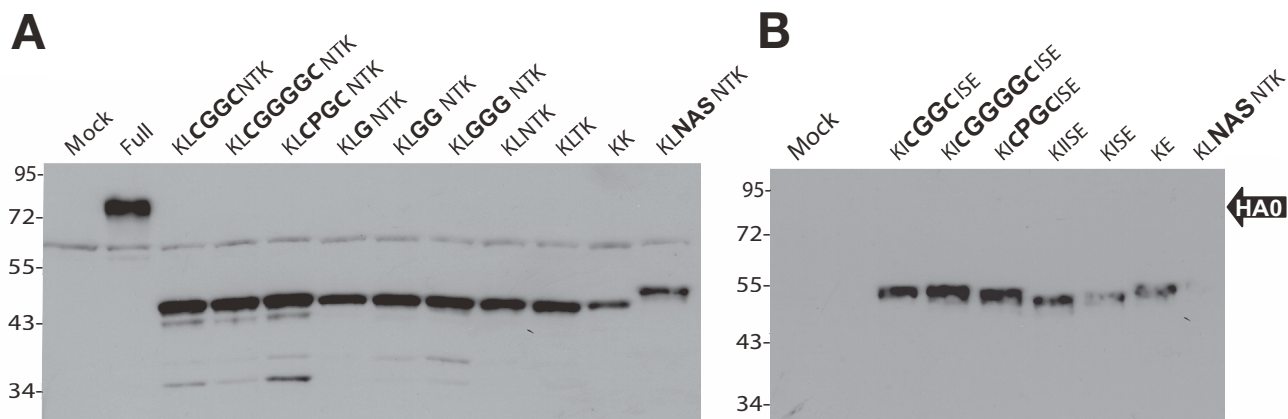


FIG 2 Expression of headless HA constructs in transiently transfected cells. Headless HA constructs were expressed in 293T cells by plasmid transfection in the absence of exogenous trypsin. At 24 h posttransfection, whole-cell lysates were prepared and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blotting. HA proteins were detected using the polyclonal antiserum 3951 (for PR8) or the MAb 12D1 (for HK68). Molecular mass markers in kilodaltons are shown to the left of each blot, and transfected constructs are identified above the appropriate lane. Mock, untransfected cells; full, full-length HA protein. For the headless HA constructs, the amino acid sequence bridging the N- and C-terminal strands of HA1 is shown. Boldface letters indicate inserted amino acids, while lightface letters represent residues present in the wild-type HA. In the region of the Cys 52 to Cys 277 disulfide bond, the wild-type sequences are as follows: for PR8, $K_{50}L_{51}C_{52} \dots C_{277}N_{278}T_{279}K_{280}$; for HK68, $K_{50}I_{51}C_{52} \dots C_{277}I_{278}S_{279}E_{280}$. (A) PR8-based constructs; (B) HK68-based constructs. In panel B, the molecular weight of the full-length HK68 HA0 protein is indicated by an arrow.

days 0 and 21, and VLP preparations were delivered with Freund's adjuvant on day 56. Each DNA vaccine comprised pGag-EGFP alone or in combination with a protein expression vector encoding the PR8 full-length HA, the PR8 4G headless HA, or the HK68

4G headless HA and was administered intramuscularly with electroporation. For a final boost, VLP preparations with an HA content of 150 ng (or an equivalent amount of Gag-only VLP) were combined with Freund's complete adjuvant and administered in-

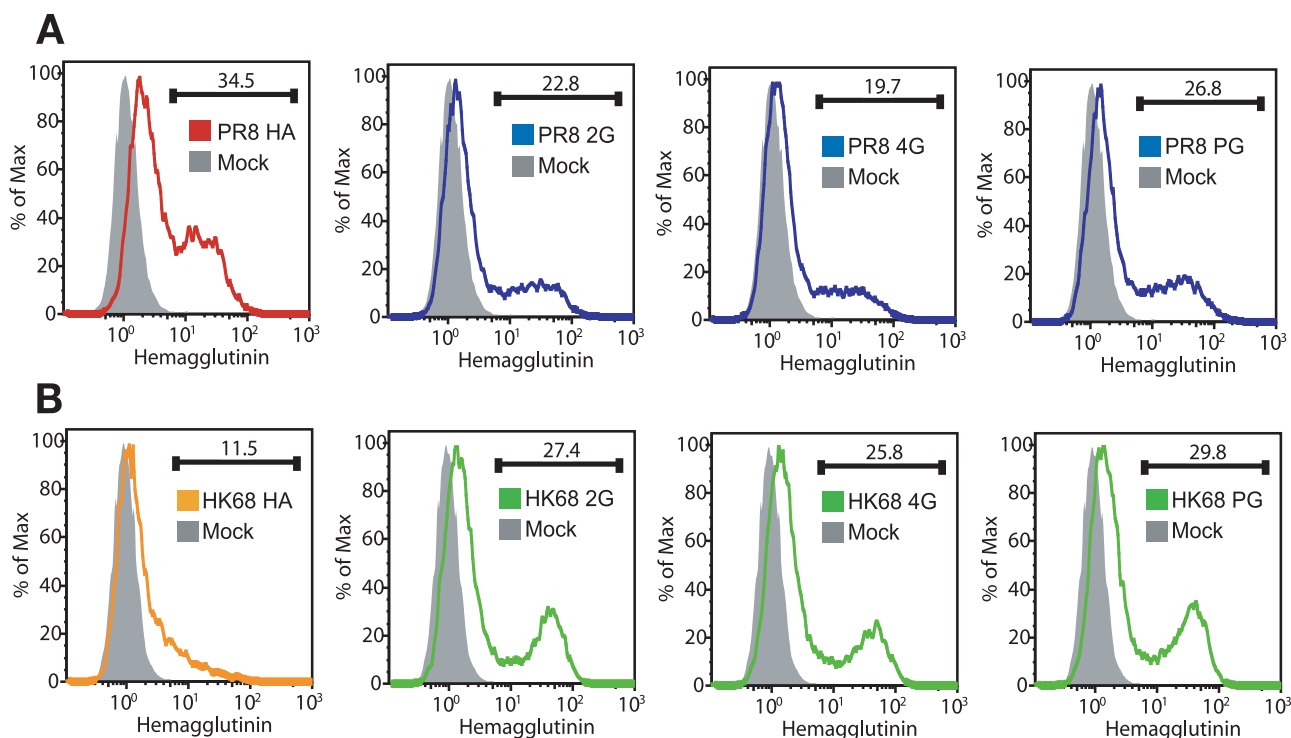


FIG 3 Detection of headless HA proteins on the surfaces of transfected cells. Full-length and headless HA constructs were expressed in 293T cells by plasmid transfection. At 24 h posttransfection, cells were trypsinized and HA proteins on the cell surface were stained using polyclonal antiserum 3951 (for PR8) or MAb 12D1 (for HK68) before analysis by flow cytometry. (A) Mock-transfected cells stained with immune serum 3951 (gray) are compared to cells transfected with pDZ PR8 HA (red) or cells transfected with pCAGGS PR8 2G, 4G, or PG headless HA constructs (blue). (B) Mock-transfected cells stained with MAb 12D1 (gray) are compared to cells transfected with pCAGGS HK68 HA (orange) or cells transfected with pCAGGS HK68 2G, 4G, or PG headless HA constructs (green).

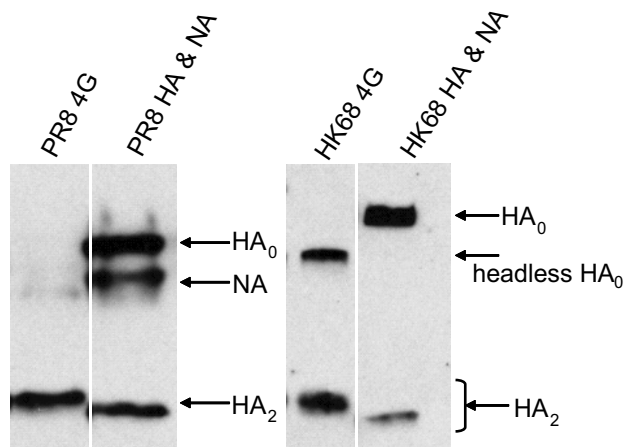


FIG 4 Incorporation of headless HA proteins into VLPs. The HA content of VLPs generated by cotransfection of HA constructs with pGag-EGFP was assessed by Western blotting. PR8-based VLPs were probed with polyclonal antiserum 3951, and HK68-based proteins were detected with MAb 12D1. Bands are identified to the right of each blot. Note that VLPs were produced in the presence of exogenous trypsin, resulting in the cleavage of HA₀ to produce HA₁ (not visualized here) and HA₂. Each lane shows VLPs harvested from the equivalent of one 10-cm dish.

traperitoneally to each mouse. The VLPs used for this purpose were produced in the absence of trypsin and comprised Gag-EGFP alone or in combination with full-length PR8 HA and PR8 NA, the PR8 4G headless HA, or the HK68 4G headless HA. On day 77, mice were challenged intranasally with PR8 virus and then monitored daily for morbidity and mortality for 10 days. In the Gag-only-vaccinated group, three out of four mice lost >25% of their initial body weight and were therefore scored as dead, and the fourth animal was seen to lose 15% of its initial body weight. In contrast, all mice vaccinated with the PR8 4G headless HA survived and experienced a maximum average weight loss of only 6% (Fig. 5).

Vaccination of mice with the PR8 headless HA elicits cross-reactive antisera. The reactivity of serum collected from vaccinated mice against influenza virus HA proteins was assessed by hemagglutination inhibition (HAI) assay, neutralization assay, and enzyme-linked immunosorbent assay (ELISA). As expected based on the absence of a globular head domain in the vaccine constructs, the pooled sera from mice immunized with Gag alone, the HK68 4G headless HA, or the PR8 4G headless HA did not show HAI activity against PR8 virus before challenge. In contrast, prechallenge sera obtained from mice that received the PR8 full-length-HA vaccine, as well as all postchallenge sera, were strongly reactive against PR8 virus in the HAI assay (Table 1). In an MDCK cell-based neutralization assay, pooled serum collected from mice immunized with PR8 full-length HA exhibited potent neutralizing activity, whereas that collected from PR8 4G-vaccinated mice showed marginally higher activity than the control sera derived from Gag-only- or HK68 4G-vaccinated mice (Table 1). Thus, the presence of neutralizing antibodies in the sera of mice immunized with PR8 headless HA was not conclusively demonstrated.

By ELISA, prechallenge sera were tested against a panel of HA substrates in order to evaluate the breadth of reactivity (Fig. 6). Against concentrated PR8 virion (Fig. 6A), Gag-only and HK68 4G antisera showed only a low level of background activity at the

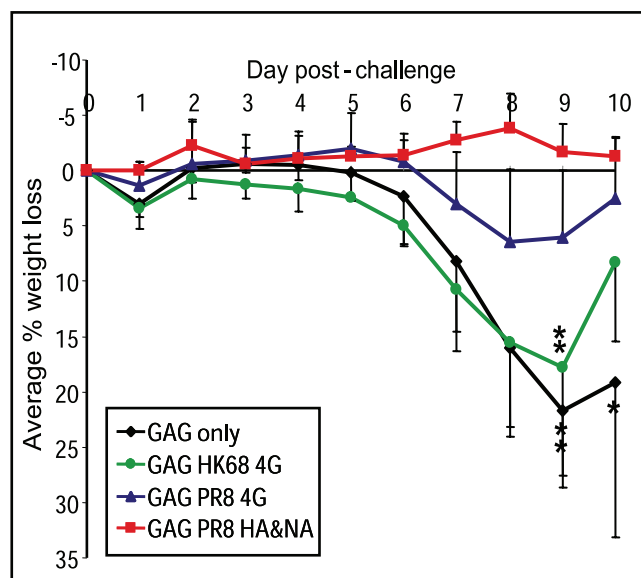


FIG 5 Vaccination of mice with headless HA constructs provides protection from death. The average body weight loss in each group of vaccinated mice following challenge with PR8 virus is shown. Error bars represent standard deviations. A star indicates the death of a mouse.

lowest dilution (1:50), while sera from the animals vaccinated with PR8 full-length HA gave a positive signal at a 1:6,250 dilution. Antisera against the PR8 4G headless HA were less potent than those against the full-length HA but reacted positively at a 1:50 dilution. When tested against recombinant HA proteins derived from a recent seasonal H1N1 influenza virus (A/New Caledonia/20/1999) (Fig. 6B) and a 2009 pandemic H1N1 influenza virus (A/California/04/2009) (Fig. 6C), Gag-only and HK68 4G antisera were negative, while sera from mice that received the PR8 full-length HA were either negative or showed a low level of reactivity. On these heterologous H1 substrates, the PR8 4G antisera showed the highest levels of reactivity, with the sera from two of the five mice in particular demonstrating high titers. Similar results were seen with recombinant HA proteins of the H2 and H5 subtypes: against the A/Singapore/1/1957 (H2N2) and A/Viet Nam/1203/2004 (H5N1) HAs, sera derived from PR8 4G-vaccinated mice showed moderate to high activity, while sera from the remaining groups (including those receiving the full-length HA) were largely negative (Fig. 6D and E). Finally, against the H3 subtype HA of A/Hong Kong/1/1968 (H3N2), only the HK68 4G antisera produced a positive signal (Fig. 6F). Thus, overall, sera obtained from mice vaccinated with the headless PR8 HA showed greater activity against heterologous strains than did sera from animals vaccinated with PR8 full-length HA. While serum titers of PR8 4G-vaccinated mice appeared to be higher against the heterologous HA proteins than against the homologous PR8 virus, a direct comparison should not be made due to the differing substrates used (purified HA versus whole virus). Within the PR8 4G group, the sera from two mice in particular consistently showed relatively high titers by ELISA. These serologic findings correlated with the protection data in that these same two mice were fully protected from disease while their three remaining counterparts each exhibited some weight loss after challenge (data not shown).

TABLE 1 HAI and neutralizing activity in immune sera of vaccinated mice

Vaccine	HAI ^a		Neutralization postvaccination ^b
	Postvaccination	Postchallenge	
Gag only		8	1:20
HK68 4G headless HA plus Gag	1	8	1:20
PR8 4G headless HA plus Gag	1	8	1:40
PR8 full-length HA plus Gag	≥128	≥128	1:20,480

^a The fold increase in titer over Gag-only prechallenge serum is shown.

^b The highest dilution of serum which achieved at least 50% neutralization is shown.

DISCUSSION

We describe a novel influenza vaccine construct which lacks the highly immunogenic globular head of the HA protein and is thereby designed to present the conserved HA stalk region to immune cells. We show that this construct can be stably expressed in mammalian cells and is targeted to the cell surface in a manner similar to that of full-length HA proteins. Our observations to date indicate that headless HA proteins cannot bud independently to form VLPs. Provision of the HIV Gag protein did, however, allow incorporation of headless HA molecules into VLPs, a finding which we have exploited to produce VLP-based vaccines. Immunization of mice with a PR8-based headless HA construct in plasmid DNA and VLP formats provided full protection against death and partial protection against weight loss following a lethal homologous challenge.

While vaccination with the PR8 full-length HA offered a higher degree of protection than vaccination with a PR8 headless HA, this observation was not unexpected. The inclusion of the PR8 NA protein in VLPs administered to mice vaccinated with PR8 full-length HA may have contributed to the protection seen: antibodies against NA are likely to have been elicited and would have been active upon challenge with the wild-type PR8 virus. Perhaps more importantly, however, the globular head domain of HA is well recognized to include the major protective antigens of influenza viruses (16). This region of the virus is highly immunogenic and therefore elicits high serum antibody titers. As a result of their ability to block attachment to host cell receptors, these antibodies are furthermore often potently neutralizing. If a sufficiently potent humoral response against the HA stem can be attained, however, protection extending to multiple strains would be expected due to the high sequence conservation of this region. Thus, an immunogenic vaccine which presents the HA stalk domain such that it is accessible to B cells is an important step toward the development of a broadly protective influenza vaccine. Our results herein with homologous viral challenge indicate that a headless HA vaccine is sufficiently immunogenic to induce protective immunity.

The 16 hemagglutinin subtypes of influenza A viruses fall into two major phylogenetic groupings, identified as group 1 (H1, H2, H5, H6, H8, H9, H11, H12, H13, and H16) and group 2 (H3, H4, H7, H10, H14, and H15). As underlined by the ability of MAbs described by Kashyap et al. (12) and MAbs C179, F10, and CR6261 (11, 13, 14) to cross-react with group 1, but not group 2, HAs, the sequence and structure of the HA stalk domain differ significantly between the two groups. For example, the PR8 headless HA shares only 49% identity with the HK68 headless HA but 95%, 77%, and 75% identity, respectively, with the corresponding regions of the drifted H1 A/New Caledonia/20/1999 HA, the H2 A/Singapore/1/1957 HA, and the H5 A/Viet Nam/1203/2004 HA. Consistent with

this lack of conservation between H1 and H3 HAs in the targeted region, vaccination of mice with an HK68 headless HA construct did not elicit antisera that were cross-reactive with group 1 HAs or provide protection against PR8 challenge. Whether immunization with the HK68 construct will offer protection against challenge with H3 and possibly other group 2 subtype influenza viruses remains to be determined. It is encouraging to note, however, that characterization of the 12D1 MAb, which has cross-neutralizing activity against H3 HAs representing 40 years of antigenic drift, has mapped its binding site to the stalk domain (17). Thus, epitopes which elicit broadly reactive, neutralizing antibodies are present not only in group 1, but also in group 2, HA stalk domains.

The protection seen following vaccination with the PR8 headless HA raises the question of why a vaccine comprising influenza virions stripped of HA1 did not provide a similar level of protection (9). We believe the key difference between the two approaches is in the conformation of the HA. In our earlier attempts, acid and dithiothreitol (DTT) treatments were used to remove HA1 from the viral surface; thus, the remaining HA2 portion of the molecule was in the extended, low-pH conformation (16). Since it is expressed under neutral pH conditions and such that the terminal strands of HA1 remain covalently linked to HA2, we expect that the headless HAs described herein remain in the prefusion conformation as depicted in Fig. 1B. Antibodies which bind to the stalk and prevent infection are more likely to be elicited by a molecule in the prefusion form.

Among the five mice vaccinated with PR8 4G headless HA, two were seen to develop relatively high cross-reactive antibody titers and were fully protected from disease, while the remaining three showed serum titers only marginally above background and some weight loss upon challenge. These results suggest that the vaccine dose used (particularly, we think, for the VLP boost at 150 ng HA per mouse) may have been at a borderline level required for full protection, such that the outcome was subject to stochastic effects. To address this problem, approaches are now being explored to increase yields of headless HA protein, either in the context of VLPs or as recombinant protein.

Earlier reports have indicated that antibodies targeting the HA stalk domain are able to neutralize virus infectivity by blocking fusion (11, 13, 14). The protection against challenge observed in vaccinated mice suggests that such fusion-inhibiting antibodies may have been elicited by the PR8 headless HA vaccine; conclusive evidence for the presence of such antibodies by *in vitro* neutralization has proven to be technically challenging, however, due to the high limit of detection of the assay. Serologic analysis by ELISA did, however, reveal that the PR8 4G headless HA vaccine, but not the PR8 full-length HA vaccine, induced antibodies which are cross-reactive among group 1 HA subtypes. This finding supports

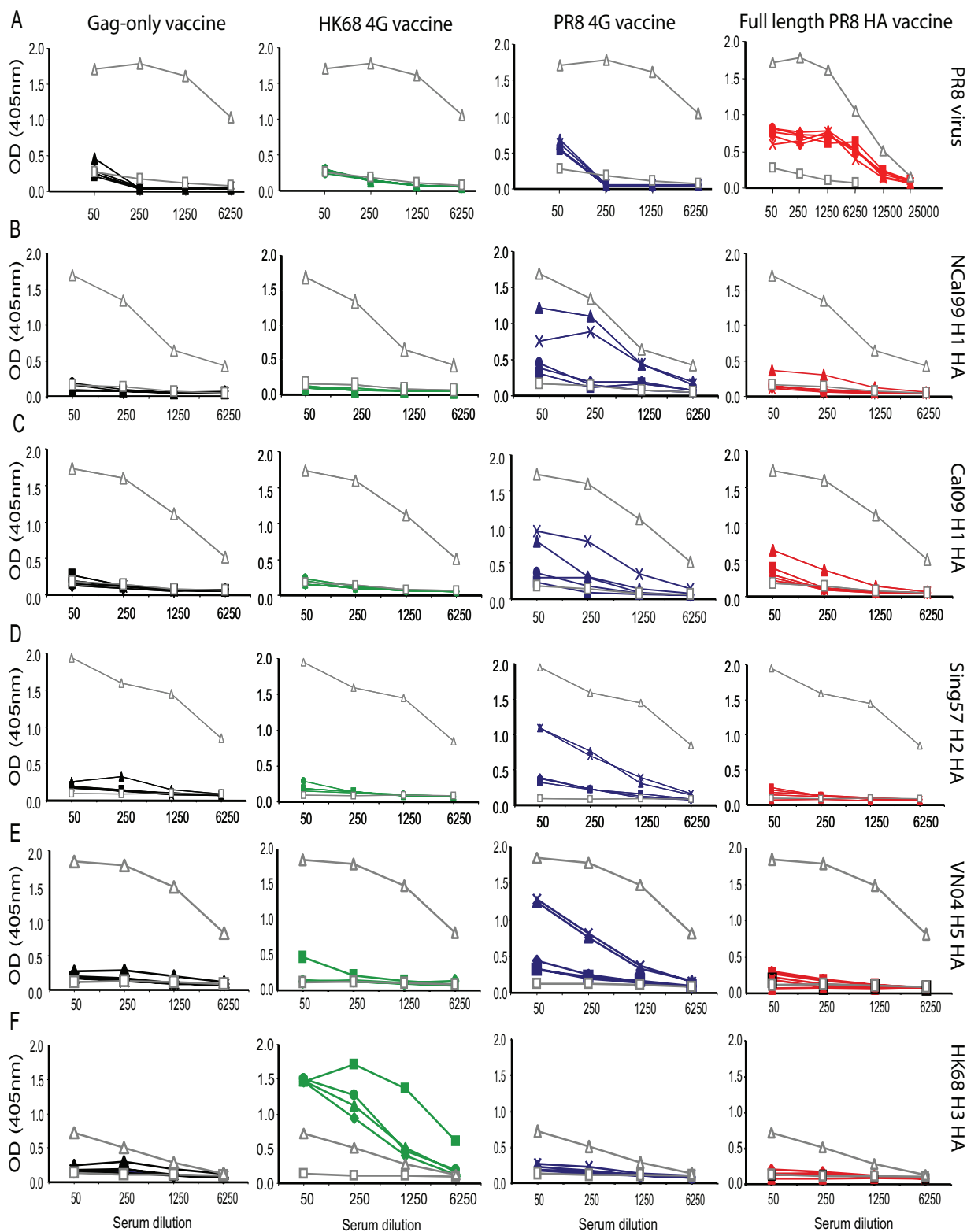


FIG 6 Antisera from mice vaccinated with the PR8 4G headless HA show broad cross-reactivity by ELISA. The vaccine groups from which sera are derived are identified at the top of each column, and the ELISA substrate used is indicated to the right of each row. The colors used are consistent throughout the figure, with sera from Gag-only-vaccinated mice indicated in black, HK68 4G-vaccinated mice indicated in green, PR8 4G-vaccinated mice indicated in blue, and full-length-PR8-HA-vaccinated mice indicated in red. Each mouse is represented by a unique symbol that is the same in each panel. A rabbit antiserum raised against whole PR8 virus is shown in gray with open triangles, and a serum sample taken from a naive mouse is shown in gray with open squares. The reactivities of mouse sera to whole PR8 virus (A), purified recombinant A/New Caledonia/20/1999 HA protein (B), purified recombinant A/California/04/2009 HA (C), purified recombinant A/Singapore/1/1957 HA (D), purified recombinant A/Viet Nam/ 1203/2004 HA (E), and purified recombinant A/Hong Kong/ 1/1968 HA (F) are shown.

our hypothesis that the globular head domain of an intact HA molecule inhibits recognition of the stem region by immune cells, either through steric shielding or due to the immune dominance of the membrane-distal portion of the protein. These data furthermore suggest that vaccination with a headless HA may lead to protection against divergent influenza strains.

MATERIALS AND METHODS

Plasmids. pGag-EGFP was generously provided by Carol Carter, Stony Brook University (18). The pCAGGS expression plasmid was kindly provided by J. Miyazaki, Osaka University (19). The plasmids pDZ PR8 HA and pDZ PR8 NA were constructed as described previously (20). For the construction of pCAGGS HK68 HA and pCAGGS HK68 NA, viral genes were reverse transcribed (Transcriptor reverse transcriptase; Roche) from purified virion RNA, amplified (PFU Turbo; Stratagene), and cloned into the vector pPOL1 (21) by following the recombinational protocol described by Wang et al. (22). Protein coding regions were then amplified with primers carrying the appropriate restriction enzyme sites and subcloned into the multiple-cloning site of pCAGGS between the NotI and NheI sites. Headless HA constructs were generated by either excise or fusion PCR methods. Excise PCR was performed on the pPOL1 PR8 HA or pPOL1 HK68 HA plasmids. The resulting PCR products were circularized by ligation, and the open reading frame of the headless HA was then subcloned into pCAGGS at the NotI and NheI sites. Fusion PCR was performed on pDZ PR8 HA or pCAGGS HK68 HA plasmid templates, and products were inserted into pCAGGS at the NotI and NruI sites. Primer sequences are available on request.

Antibodies. MAb 12D1 was generated by sequential intramuscular immunization of BALB/c mice with plasmid DNAs encoding the HK68 HA, the A/Alabama/1/1981 (H3N2) HA, and the A/Beijing/46/1992 (H3N2) HA, followed by a boost with whole A/Wyoming/03/2003 (H3N2) virus. This MAb binds multiple H3 HA proteins and maps to the HA2 subunit (17). Since a similar monoclonal antibody was not readily available for detection of the PR8 headless HAs, a rabbit polyclonal serum, 3951, was used. The polyclonal serum 3951 was raised against PR8 virus from which the HA1 subunit had been removed by treatment with acid and DTT (9) and therefore detects all viral structural proteins with the exception of the HA1 subunit of HA.

Cells and viruses. 293T cells were obtained from the ATCC and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; Clontech). Madin-Darby canine kidney cells were maintained in minimal essential medium supplemented with 10% FBS (Clontech), 100 U/ml of penicillin, and 100 μ g/ml of streptomycin.

Influenza A/Puerto Rico/8/1934 (H1N1) virus was obtained by reverse genetics as previously described (23) using plasmids bearing the eight genes defined by accession numbers AF389115 to AF189122 (A/Puerto Rico/8/34/Mount Sinai) in the NCBI database. The virus was amplified in 10- to 11-day-old embryonated chickens eggs and titrated by plaque assay.

Western blotting. To assess the expression levels of HA-based proteins, 293T cells were transfected with 2 μ g of the appropriate plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 24 h posttransfection, cells were lysed in 2 \times protein loading buffer (125 mM Tris-HCl [pH 6.8], 4% sodium dodecyl sulfate, 20% glycerol, 10% β -mercaptoethanol, and 0.1% bromophenol blue). Lysates were heated at 100°C for 5 min and then separated on a 10% sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane (Whatman, Inc.). To detect HA-based proteins in VLP preparations, pelleted VLPs were suspended in phosphate-buffered saline (PBS), lysed through 1:1 mixing with 2 \times protein loading buffer, boiled for 5 min, separated on a 10% sodium dodecyl sulfate-polyacrylamide gel, and transferred to a nitrocellulose membrane. Membranes were then probed with MAb 12D1 or 3951 antiserum at a 1:2,000 dilution.

Flow cytometric analysis. To assess levels of HA-based proteins at the cell surface, 293T cells were transfected with 1 μ g of the appropriate plas-

mid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. At 24 h posttransfection, cells were trypsinized and resuspended in PBS containing 2% FBS prior to staining them with 3951 antiserum at a 1/250 dilution or with MAb 12D1 at a 1/200 dilution. Stained cells were enumerated on a Beckman Coulter Cytomics FC 500 flow cytometer, and the results were analyzed using FlowJo software.

Generation of VLPs. For the production of VLPs, 6×10^6 293T cells were seeded into a 10-cm dish in 8 ml of DMEM with 10% FBS. While still in suspension, cells were transfected with Lipofectamine 2000 (Invitrogen) combined with the desired plasmid DNAs at a 4:1 ratio and as described in the manufacturer's instructions. The amounts of plasmid DNA used were as follows: for Gag-only VLPs, 7.5 μ g pGag-EGFP was transfected; for Gag plus PR8 4G VLPs, 4.5 μ g of pGag-EGFP plus 4.5 μ g of pCAGGS PR8 4G was used; for Gag plus HK68 4G VLPs, 4.5 μ g of pGag-EGFP plus 4.5 μ g of pCAGGS HK68 4G was used; and for Gag plus PR8 HA plus PR8 NA VLPs, 3 μ g of pGag-EGFP was combined with 3 μ g each of pDZ PR8 HA and pDZ PR8 NA. At 6 h posttransfection, medium was changed to fresh DMEM containing 10% FBS or to Opti-MEM (Gibco) supplemented with 3% bovine serum albumin (Sigma) and 10 μ g/ml TPCK (N-p-tosyl-L-phenylalanine chloromethyl ketone)-treated trypsin (Sigma). VLPs were harvested at 28 h posttransfection by layering clarified cell culture supernatant over a cushion of 30% sucrose in NTE buffer (1 M sodium chloride, 0.1 M Tris, 0.01 M EDTA, pH 7.4) and centrifuging for 2 h at 4°C and 25,000 rpm in an SW28 rotor (Beckman). Pellets were resuspended in PBS, and HA protein content was assessed by Western blotting in parallel with performing serial dilutions of a known amount of gradient-purified PR8 or HK68 virus (prepared as described in reference 24). VLPs shown in Fig. 4 were produced in the presence of exogenous trypsin, while those used to boost mice were produced without the addition of trypsin.

Mouse vaccine challenge experiment. Female, 6- to 8-week-old, C57BL/6 mice (Charles River Laboratories) were initially vaccinated by intramuscular administration of plasmid DNA, followed immediately by the application of electrical stimulation (TriGrid delivery system; Ichor Medical Systems) (25, 26). The spacing of the TriGrid electrode array is 2.5 mm, and the electrical field is applied at an amplitude of 250 V/cm of electrode spacing for six pulses for a total duration of 40 ms applied over a 400-ms interval. Each DNA vaccination comprised 37.5 μ g of pGag-EGFP alone or in combination with 75 μ g of pDZ PR8 HA, pCAGGS PR8 4G, or pCAGGS HK68 4G. Three weeks later, a DNA boost was performed by following the same procedure. Five weeks after the second dose of DNA was administered, the mice were boosted a second time with VLPs. For the HA-containing VLPs, HA content was normalized such that each mouse received approximately 150 ng HA protein. Prior to intraperitoneal administration, VLP suspensions were combined in a 1:1 ratio and emulsified with complete Freund's adjuvant (Pierce). Mice were challenged 3 weeks after the VLP boost via intranasal inoculation of two 50% mouse lethal doses (MLD₅₀) of PR8 virus in a total volume of 50 μ l PBS. Body weight was monitored daily, and mice losing more than 25% of their initial weight were sacrificed and scored as dead.

Serological assays. Sera were collected from mice immediately before challenge and at 21 days postchallenge. To remove nonspecific inhibitors of hemagglutination, trypsin-heat-periodate treatment was performed as described previously (27). For hemagglutination inhibition (HAI) assays, all sera from each vaccination group were pooled; for ELISAs, sera collected from individual mice were evaluated separately; and for neutralization assays, the two serum samples in each group exhibiting the highest titers by ELISA were pooled. HAI assays were carried out according to standard methods as described previously (27). For neutralization assays, 2-fold serial dilutions of treated sera were mixed with 10 50% tissue culture infective doses (TCID₅₀) of PR8 virus and incubated for 45 min at 37°C. After being washed with PBS, MDCK cells were inoculated with the virus-antibody mixtures. Following a 45-min incubation, serum-free medium supplemented with 1 μ g/ml TPCK-treated trypsin and 3% bovine serum albumin was added to each well. Cells were incubated at 37°C and,

at 72 h postinfection, supernatants were collected and tested for virus growth by hemagglutination assay. For ELISAs, 96-well plates (Costar) were coated with 0.25 μg per well of PR8 virus or with 0.1 μg per well of purified recombinant HA protein in PBS. PR8 virus for this purpose was prepared from allantoic fluid by concentration through a 30% sucrose cushion as previously described (27). The recombinant HA proteins of the A/California/04/2009, A/Viet Nam/1203/2004, and A/Singapore/1/1957 viruses were obtained from BEI Resources; the A/Hong Kong/1/1968 HA was the generous gift of Ian Wilson; and the A/New Caledonia/20/99 HA was purchased from Feldan-Bio. Fivefold serial dilutions of antisera were incubated on the plates, and after extensive washing, bound antibody was detected with an alkaline phosphatase-linked anti-mouse IgG antibody (Caltag) and *p*-nitrophenyl phosphate (PNPP) substrate (Sigma). In each assay, a rabbit immune serum raised against whole PR8 virus was included as a positive control, and serum obtained from a naive C57BL/6 mouse was included as a negative control.

ACKNOWLEDGMENTS

We are grateful to David Ho for the loan of and technical assistance with the TriGrid delivery system (Ichor Medical Systems) and to Carol Carter for providing the pGag-EGFP plasmid. We thank Estanislao Nistal-Villan for preparing the three-dimensional structures shown in Fig. 1, Silke Stertz for assistance with mouse vaccinations, Judith Alamares for help with preliminary experiments, and Lily Ngai and Carly Levine for excellent technical support.

This research was supported by the National Institutes of Health (grant 1 RC1 AI086061-01 to P.P. and grant U01 AI070469 to A.G.-S.) and a Northeast Biodefense Center career development fellowship (U54 AI057158) to J.S. A.C.L. is a Parker B. Francis Fellow in Pulmonary Research.

We wish to disclose that Mount Sinai School of Medicine has submitted patent applications on influenza virus vaccines based on conserved regions of the HA. U.S. Patent Application No. 61/319,137 and PCT application US2010, 29202 were filed March 30, 2010.

REFERENCES

1. Fox, J. P., M. K. Cooney, C. E. Hall, and H. M. Foy. 1982. Influenza virus infections in Seattle families, 1975-1979. II. Pattern of infection in invaded households and relation of age and prior antibody to occurrence of infection and related illness. *Am. J. Epidemiol.* 116:228-242.
2. Glezen, W. P., and R. B. Couch. 1978. Interpandemic influenza in the Houston area, 1974-1976. *N. Engl. J. Med.* 298:587-592.
3. Belshe, R. B. 2007. Translational research on vaccines: influenza as an example. *Clin. Pharmacol. Ther.* 82:745-749.
4. Thompson, W. W., D. K. Shay, E. Weintraub, L. Brammer, N. Cox, L. J. Anderson, and K. Fukuda. 2003. Mortality associated with influenza and respiratory syncytial virus in the United States. *JAMA* 289:179-186.
5. Johnson, N. P., and J. Mueller. 2002. Updating the accounts: global mortality of the 1918-1920 "Spanish" influenza pandemic. *Bull. Hist. Med.* 76:105-115.
6. Wright, P. F., G. Neumann, and Y. Kawaoka. 2006. Orthomyxoviruses. In D. M. Knipe, and P. M. Howley (ed.), *Fields virology*, 5th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, PA.
7. Bridges, C. B., W. W. Thompson, M. I. Meltzer, G. R. Reeve, W. J. Talamonti, N. J. Cox, H. A. Lilac, H. Hall, A. Klimov, and K. Fukuda. 2000. Effectiveness and cost-benefit of influenza vaccination of healthy working adults: a randomized controlled trial. *JAMA* 284:1655-1663.
8. de Jong, J. C., W. E. Beyer, A. M. Palache, G. F. Rimmelzwaan, and A. D. Osterhaus. 2000. Mismatch between the 1997/1998 influenza vaccine and the major epidemic A(H3N2) virus strain as the cause of an inadequate vaccine-induced antibody response to this strain in the elderly. *J. Med. Virol.* 61:94-99.
9. Graves, P. N., J. L. Schulman, J. F. Young, and P. Palese. 1983. Preparation of influenza virus subviral particles lacking the HA1 subunit of hemagglutinin: unmasking of cross-reactive HA2 determinants. *Virology* 126:106-116.
10. Krystal, M., R. M. Elliott, E. W. Benz, Jr., J. F. Young, and P. Palese. 1982. Evolution of influenza A and B viruses: conservation of structural features in the hemagglutinin genes. *Proc. Natl. Acad. Sci. U. S. A.* 79:4800-4804.
11. Ekiert, D. C., G. Bhabha, M. A. Elsliger, R. H. Friesen, M. Jongeneelen, M. Throsby, J. Goudsmit, and I. A. Wilson. 2009. Antibody recognition of a highly conserved influenza virus epitope. *Science* 324:246-251.
12. Kashyap, A. K., J. Steel, A. F. Oner, M. A. Dillon, R. E. Swale, K. M. Wall, K. J. Perry, A. Faynboym, M. Ilhan, M. Horowitz, L. Horowitz, P. Palese, R. R. Bhatt, and R. A. Lerner. 2008. Combinatorial antibody libraries from survivors of the Turkish H5N1 avian influenza outbreak reveal virus neutralization strategies. *Proc. Natl. Acad. Sci. U. S. A.* 105:5986-5991.
13. Okuno, Y., Y. Isegawa, F. Sasao, and S. Ueda. 1993. A common neutralizing epitope conserved between the hemagglutinins of influenza A virus H1 and H2 strains. *J. Virol.* 67:2552-2558.
14. Sui, J., W. C. Hwang, S. Perez, G. Wei, D. Aird, L. M. Chen, E. Santelli, B. Stec, G. Cadwell, M. Ali, H. Wan, A. Murakami, A. Yammanuru, T. Han, N. J. Cox, L. A. Bankston, R. O. Donis, R. C. Liddington, and W. A. Marasco. 2009. Structural and functional bases for broad-spectrum neutralization of avian and human influenza A viruses. *Nat. Struct. Mol. Biol.* 16:265-273.
15. Stevens, J., A. L. Corper, C. F. Basler, J. K. Taubenberger, P. Palese, and I. A. Wilson. 2004. Structure of the uncleaved human H1 hemagglutinin from the extinct 1918 influenza virus. *Science* 303:1866-1870.
16. Palese, P., and M. L. Shaw. 2006. Orthomyxoviridae: the viruses and their replication. In D. M. Knipe and P. M. Howley (ed.), *Fields virology*, 5th ed., vol. 1. Lippincott Williams & Wilkins, Philadelphia, PA.
17. Wang, T. T., G. S. Tan, R. Hai, N. Pica, E. Petersen, T. Moran, and P. Palese. 2010. Broadly protective monoclonal antibodies against H3 influenza viruses following sequential immunization with different hemagglutinins. *PLoS Pathog.* 6:e100796.
18. Hermida-Matsumoto, L., and M. D. Resh. 2000. Localization of human immunodeficiency virus type 1 Gag and Env at the plasma membrane by confocal imaging. *J. Virol.* 74:8670-8679.
19. Miyazaki, J., S. Takaki, K. Araki, F. Tashiro, A. Tominaga, K. Takatsu, and K. Yamamura. 1989. Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5. *Gene* 79:269-277.
20. Quinlivan, M., D. Zamarin, A. Garcia-Sastre, A. Cullinane, T. Chambers, and P. Palese. 2005. Attenuation of equine influenza viruses through truncations of the NS1 protein. *J. Virol.* 79:8431-8439.
21. Fodor, E., L. Devenish, O. G. Engelhardt, P. Palese, G. G. Brownlee, and A. Garcia-Sastre. 1999. Rescue of influenza A virus from recombinant DNA. *J. Virol.* 73:9679-9682.
22. Wang, S., Q. Liu, J. Pu, Y. Li, L. Keleta, Y. W. Hu, J. Liu, and E. G. Brown. 2008. Simplified recombinational approach for influenza A virus reverse genetics. *J. Virol. Methods* 151:74-78.
23. Steel, J., S. V. Burmakina, C. Thomas, E. Spackman, A. Garcia-Sastre, D. E. Swayne, and P. Palese. 2008. A combination in-ovo vaccine for avian influenza virus and Newcastle disease virus. *Vaccine* 26:522-531.
24. Palese, P., and J. L. Schulman. 1976. Differences in RNA patterns of influenza A viruses. *J. Virol.* 17:876-884.
25. Luxembourg, A., C. F. Evans, and D. Hannaman. 2007. Electroporation-based DNA immunisation: translation to the clinic. *Expert Opin. Biol. Ther.* 7:1647-1664.
26. Luxembourg, A., D. Hannaman, E. Nolan, B. Ellefsen, G. Nakamura, L. Chau, O. Tellez, S. Little, and R. Bernard. 2008. Potentiation of an anthrax DNA vaccine with electroporation. *Vaccine* 26:5216-5222.
27. Lowen, A. C., J. Steel, S. Mubareka, E. Carnero, A. Garcia-Sastre, and P. Palese. 2009. Blocking interhost transmission of influenza virus by vaccination in the guinea pig model. *J. Virol.* 83:2803-2818.
28. Sagawa, H., A. Ohshima, I. Kato, Y. Okuno, and Y. Isegawa. 1996. The immunological activity of a deletion mutant of influenza virus haemagglutinin lacking the globular head. *J. Gen. Virol.* 77:1483-1487.