Supporting Information

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SI Text

SI Materials and Methods. ***Cloning and purification of HA6.*** *Escherichia coli* codon optimized genes corresponding to the designed proteins (HA6 and HA6a) were synthesized and cloned into the bacterial expression vector pET-26b(+) between the *Nde*I and *Hind*III sites of the multiple cloning site. The purification of the proteins after expression in bacteria was done as indicated below. *E. coli* BL21(DE3) cells transformed with the plasmid were grown in Terrific Broth at 37 °C to an A_{600} of 0.8 before inducing the culture with 1 mM IPTG. The cells were grown for another 6 h before harvesting them by centrifugation at 3,500 g. The cells were resuspended in 20 mM Tris (pH 8.0) and lysed by sonication. The cell lysate was separated from the inclusion bodies by centrifugation at $18,500 \times g$. The inclusion bodies were washed in 0.05% Triton X-100, 20 mM Tris (pH 8.0) and subjected to centrifugation at $18,500 \times g$. The inclusion bodies (insoluble fraction) were then solubilized in 8 M GdnCl, 1 mM DTT, 20 mM Tris (pH 8.0). The protein was purified from clarified, solubilized inclusion bodies by binding and refolding on a Ni-NTA column (Amersham). The protein was eluted in 1 M GdnCl, 500 mM Imidazole, 20 mM Tris (pH 8.0). The eluted protein was desalted into deionized water on an (Amersham) Hitrap Desalting column and stored in aliquots at −80 °C.

Construction and purification of model HA2 peptide. The gene corresponding to the residues (75–98) of HA2 was cloned into the vector pET-21a(+)cytb5-tev (1) as a cytochrome b5 fusion. The mutations 63D and/or 73D were introduced into the plasmid by complementary primers as described in ref. 2. *E. coli* BL21 (DE3) cells transformed with the plasmid were grown in LB broth at 37 °C and induced with 0.4 mM IPTG at an A_{600} of 0.6. The cells were harvested 12 h after induction, resuspended in 25 mM phosphate buffer pH 6.0, and lysed by sonication. The fusion proteins were purified on a DEAE Sephacel column and cleaved using tobacco etch virus protease. The cleaved peptides were purified from the mixture using a semipreparative RP C-18 column (10 mm × 250 mm) on a Shimadzu HPLC system using a gradient of water and acetonitrile (going from 55% to 75% acetonitrile at 1%/ min using a flow rate of 5 mL/ min).

CD and fluorescence spectroscopy. All spectra were acquired at 298 K. The CD spectrum of the protein at a concentration of 5 μM was recorded in 1XPBS on a JASCO J-175 Spectropolarimeter. The spectra were recorded using a 0.1-cm path length cuvette by scanning from 250 nm to 195 nm at a rate of 50 nm/ min. The helix content of the molecule was determined as described in ref. 3. To study the effect of pH on the structure, the θ_{222} of the protein/peptide in 5 mM citrate glycine HEPES buffer was monitored as a function of pH.

The intrinsic fluorescence emission spectrum of the protein at a concentration of 2 μM was recorded using a 1-cm path length cuvette under native (20 mM Tris, pH 8.0) or denaturing conditions (6 M GdnCl, 20 mM Tris, pH 8.0) after excitation at 280 nm on a Fluoromax-3 Fluorimeter. Equilibrium unfolding studies were carried out on the molecule using the denaturant GdnCl. The unfolding of 2 μM HA6 in Tris buffer (pH 8.0) was monitored by measuring the intrinsic Trp fluorescence emission at 338 nm after exciting the molecule at 280 nm in the presence of increasing amounts of denaturant. To test for binding to ANS (1-anilino-8-napthalene-sulphonate), 1 μM of the protein was incubated in the presence of 100 μM ANS in 20 mM Tris (pH 8.0) for 30 min, and the fluorescence emission at 482 nm after excitation at 388 nm was monitored. A previously characterized molten globule of 1 μM Controller of Cell Division B protein (CcdB) at pH 4.0 (4) was also tested for ANS binding as a control.

Determination of free thiol content. The oxidation state of the protein was determined by reacting 5 μM of the protein with 500 μM DTNB (5,5′-dithio-bis(2-nitrobenzoic acid) in 4 M GdnCl, 50 mM Tris (pH 8.0) and monitoring the absorbance at 412 nm on a Varian Cary 100 Bio UV-Vis Spectrophotometer after 15 min of incubation. The free thiol content was calculated using an extinction coefficient of 13,700 M^{-1} for the TNB anion (5). The oxidation state of the proteins was also investigated using reverse phase HPLC. 50 μM of each of the proteins in 4 M GdnCl, 50 mM Tris (pH 8.0) either with or without 500 μM Tris(2-Carboxyethyl) phosphine was injected onto an analytical RP C5 (15 cm × 4.6 mm) column and eluted using a gradient of water and acetonitrile at a flow rate of 1 mL/ min (30% acetonitrile to 45% acetonitrile).

Mouse immunization and challenge. Female BALB/c mice, 10 per group, were vaccinated intramuscularly with different doses of the immunogen (HA6 or HA6a) at weeks 0 and 4. The naïve control group was not vaccinated, and a positive control group received a single dose of 0.1 LD90 live virus either intramuscularly or intranasally at week 0. Sera were collected two weeks after the second immunization, and all animals were challenged with either the homologous H3N2 strain (A/HK/68) or an H1N1 strain (A/PR/8/34) at a dose of 1LD90 a week later. The different proteins used for immunization and the dosage regimen used is shown in Table S2. The body mass of the mice was monitored for 20 days after the challenge by which time all the control mice had died.

Guinea pig immunizations. Female guinea pigs were immunized with 100 μg of HA6 intramuscularly three times in four-week intervals. Two weeks after the last immunization, sera were collected and used for the mAb 12D1 competition assay.

Flow cytometric analysis of antibody binding to the viral infected cells and ELISA of antibody binding to recombinant HA protein. Mardin Darby canine kidney (MDCK) cells were inoculated with either A/HK/68 or A/PR/8/34 virus at an moi of 1 and incubated overnight at 37 °C. The infected MDCK cells were harvested at 1×10^7 cells per mL in PBS plus 0.1% BSA and transferred to a 96-well plate with 100-μL cells per well. Test sera were diluted at 1:200 in PBSB and added to the plate at 100 μL per well and plates were incubated at 37 °C for 1 h. After washing with PBSB, cells were centrifuged at 500 × *g* and resuspended in 100 μL of 5 μg/mL FITC labeled goat anti-mouse immunoglobulin (BD Biosciences). After incubation for 30 min, cells were fixed with 1% formaldehyde in PBS. Samples were analyzed for antibody binding by flow cytometry using a Becton Dickinson FACScalibur. H3N2 recombinant HA protein A/Brisbane/16/2007 was obtained from Protein Sciences Corporation. Binding of immune sera to the proteins was carried out by ELISA as described below.

Antibody titers. Serum antibody titers were determined by ELISA. Ninety-six well plates were coated with 50 μL per well of a test antigen at a concentration of 4 μg per mL in PBS at 4 °C overnight. Plates were washed six times with PBS containing 0.05% Tween-20 (PBST) and blocked with 3% skim milk in PBST

(milk-PBST). Sera were diluted in a 4-fold series in milk-PBST and added in a volume of 100 μL per well. Plates were incubated for 2 h at room temperature followed by six washes with PBST. Fifty μL of predetermined dilution (1:5,000) of HRP-conjugated goat anti-mouse secondary antibodies (Invitrogen) in milk-PBST was added per well and incubated at room temperature for 1 h. Plates were washed six times followed by addition of 100 μL per well of substrate 3,3′,5,5′-tetramethylbenzidine (Virolabs, Inc.) and stopped after 3–5 min of development. The antibody titer was defined as the reciprocal of the highest dilution that gave an OD450-nm value above the mean plus 2 standard deviations of the conjugate control wells.

Binding of 12D1 to HA6 by ELISA. Each of HA6, peptide (57–98) HA2, recombinant A/Brisbane/10/07 HA, recombinant A/Wyoming/03 HA and Vaxigrip seasonal flu vaccine (for 2009–2010) were coated onto Nunc 96 well plates (250 ng per well). Recombinant HA proteins were obtained from Protein Science Corporation. The wells were blocked with 1% BSA in PBS (0.05% Tween 20) and different concentrations of mAb12D1 were added. After 2-h incubation at room temperature (RT), the plates were washed and 50 μL of 1:10,000 ALP conjugated anti-mouse Ab was added. After 2-h incubation at RT and washing, 50 μL ALP substrate was added. The absorbance at 405 nm was measured after 10 min using a Tecan microplate reader.

Competition binding of 12D1 to recombinant A/Brisbane/10/07 HA in the presence of anti-HA6 sera. Sector(tm) Imager 6000 96-well plates (Meso Scale Discovery) were coated with 10 ng of recombinant HA (A/Brisbane/10/07) per well as per manufacturer's instructions. The plates were blocked with 3% dry milk in PBS containing 0.05% Tween 20. MAb 12D1 at 10 ng/mL was mixed with serial dilutions of the test guinea pig serum. To each well, 25 μl of the mixture was added and the plates were incubated at room temperature for one hour. Plates were washed with PBST, added with 25 μL of Meso Scale Discovery Sulfo-tag labeled goat anti-mouse IgG at 1 μg/mL in PBST per well and incubated at room temperature for 1 h, which was followed with additional wash and addition of 125 μL of substrate (read buffer T, Meso Scale Discovery) per well. The plates were read with Sector(tm) Imager 6000. All samples were run in duplicate.

Sequence analysis. The HA protein sequences for vaccine strains (H1, H3, and B type) for all the flu seasons over the past 10 years (recommended by World Health Organization) were obtained from the Influenza Virus Resource at the National Center for Biotechnology Information (NCBI) (6). All nonidentical, full-length HA sequences of H1, H3, and B-type strains were also obtained from the NBCI resource. The HA sequences of each subtype were multiply aligned using ClustalX (7). The percent conservation of residues at each position was mapped onto the HA neutral pH structure (PDB ID code 1HGD) for the individual subtypes. Conserved, exposed regions of the molecule were identified by calculating the accessibilities of the residues and using an accessibility cutoff of >20% to identify exposed residues. The analysis was repeated for an HA6 version using a model of HA6 (derived from the structure of neutral pH HA).

Neutralization assay. The neutralizing antibody titers were measured against A/HK/68 in a microneutralization assay as described previously (8). Briefly, 10 μL of heat inactivated serially diluted test sera were taken in a microtiter plate, incubated with diluted virus, and incubated for 1 h. Then 1.5×10^4 MDCK cells were added per well and incubated overnight. The cells were then fixed and growth of the virus was measured by performing an ELISA with anti-Influenza A Nucleoprotein mouse monoclonal antibodies as the primary antibody and HRP-conjugated anti-mouse antibody as the secondary antibody. Five to ten minutes after adding the substrate o-phenylenediamine, the reaction was stopped and the absorbance at 490 nm was measured.

1. Mitra A, et al. (2005) High level expression of peptides and proteins using cytochrome b5 as a fusion host. *Protein Expr Purif* 41:84–97.
2. Bajaj K, Chakrabarti P, Varadarajan R (2005) Mutagenesis-based definitions and probes of residue burial in proteins. *Proc Natl Acad Sci USA*102:16221–16226.
3. Joyce JG, et al. (2002) Enhancement of alpha-helicity in the HIV-1 inhibitory peptide DP178 leads to an increased affinity for human monoclonal antibody 2F5 but does not elicit neutralizing responses in vitro. Implications for vaccine design. *J Biol Chem* 277:45811–45820.
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6. Bao Y, et al. (2008) The influenza virus resource at the National Center for Biotechnology Information. *J Virol* 82:596–601.
7. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG (1997) The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* 25:4876–4882.
8. Rowe T, et al. (1999) Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. *J Clin Microbiol* 37:937–943.

*M*GLFGAIAGFIENGWEGMIDGWYGFRHQNSEGTGQAADLKSTQAAIDQINGKLN

RVIEKTNEKDHQIEKEFSEDEGRIQDLEKYVEDTKIDLWSYNAELLVALENQHTI

DLTDSEMNKLFEKTRRQLRENAEEMGNGCFKIYHKCDNACIESIRNGTYDHDVY

RDEALNNRFQ*GSAGSAG*DNSTATLCLGHHAVPNGTLVKTITDDQIEVTNATELVQ

SS*GSAGSA*NDKPFQNTNKETTGATPKYVKQNTLKLATGMR*KLAAALEHHHHHH*

Fig. S1. Sequence of the designed construct HA6. Cloning into the vector pET-26b(+) resulted in a C-terminal hexa-His tag. The designed protein has residues (1–172) of HA2, a 7-amino acid linker (GSAGSAG), (7–46) of HA1 and a 6 amino acid linker (GSAGSA) followed by residues (290–321) of HA1. Mutations were incorporated into the construct to remove exposed hydrophobic patches and stabilize the neutral pH conformation. Mutated residues are underlined, and linkers and vector derived sequences are shown in italics.

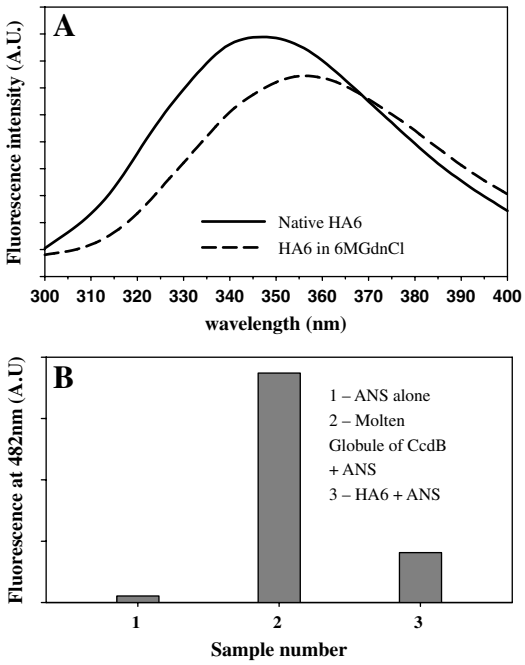

Fig. S2. Fluorescence studies of HA6. (*A*) Fluorescence emission spectra of 2 μM HA6 under native conditions (20 mM Tris pH 8.0, solid line) or denaturing conditions (6 M GdnCl, 20 mM Tris, pH 8.0, dashed line) at 25 °C. (*B*) Binding of ANS to 1 μM HA6 in a buffer containing 100 μM ANS. The protein does not bind ANS to a significant extent (sample 3) as compared to a control molten globule of CcdB (sample 2), indicating that the molecule is quite compact and does not have large, exposed hydrophobic patches.

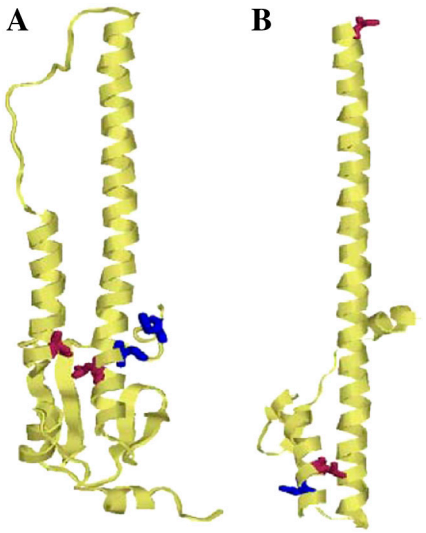

Fig. S3. Use of engineered disulfides to confirm that HA6 adopts the neutral pH conformation of HA. Monomers from trimeric HA2 at neutral pH (*A*) and low pH (*B*). Residues that were mutated to cysteines are shown in stick representation. In blue the (3F,116N) pair and in red the (40S, 118L) pair. The introduced cysteine pairs (3C, 116C) and (40C, 118C) can form disulfides only if the molecule is in the neutral pH conformation. The low pH structure does not have the coordinates of residue F3 (HA2), but this residue is expected to be in the top part of the figure, over 90 Å away from residue 116. Only a monomer of HA2 is shown for clarity.

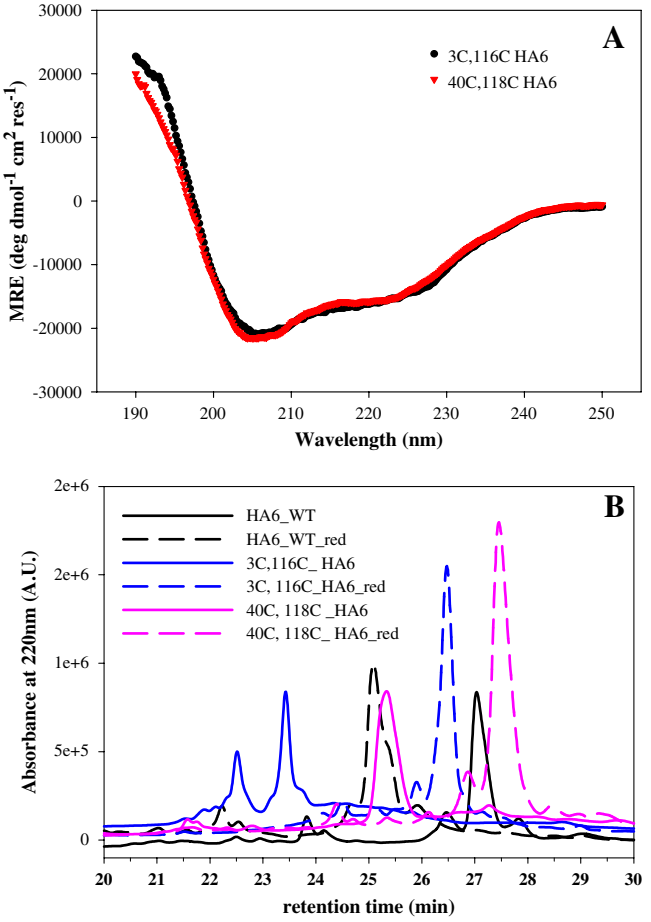

Fig. S4. Characterization of (3C, 116C) HA6 and (40C, 118C) HA6 proteins. (*A*) Far UV CD spectra of (3C, 116C) HA6 (black) and (40C, 118C) HA6 (red) in PBS, pH 7.4 at 25 °C. Both the mutant proteins are well folded and have CD spectra similar to WT HA6 (Fig. 3*A*). (*B*) Reverse Phase HPLC profiles of WT, (3C, 116C) and (40C, 118C) mutants. The differences in the retention times of the reduced proteins (dashed lines) in comparison with the native proteins (solid lines) and with each other indicate formation of the expected disulfide bonds. WT and (40C, 118C) HA6 show single peaks on RP-HPLC indicating that they are well folded with only a single combination of disulfides. (3C, 116C) HA6 shows one major and one minor conformer in the oxidized state.

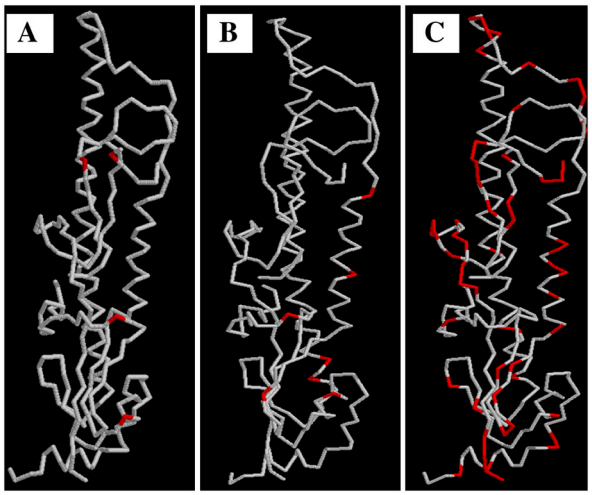

Fig. S5. Exposed, nonidentical residues of HA mapped on to the target HA6 structure. The sequences of HA proteins [for the vaccine strains of the past 10 years obtained from the Influenza Virus Resource at NCBI (6)] were multiply aligned using ClustalX (7) to identify the residues that are variable. The accessibilities of the residues in the target structure were calculated and the exposed, nonidentical regions of the molecule identified. The same was done by comparing the sequences of the H3N2 (A/HK/68) and H1N1 (A/PR/8/34) HAs. Exposed nonidentical residues in H1 vaccine strains (*A*), H3 vaccine strains (*B*), and between the A/HK/68 (H3N2) and A/PR/8/34 (H1N1) used in the mice challenge studies (*C*) are shown in red. The exposed regions in the HA6 version of H1 and H3 vaccine strains have an identity of 96% and 91%, respectively, whereas the identity is only 45% between the H1 (A/PR/8/34) and H3 (A/HK/68) in the exposed regions of the HA6 molecule.

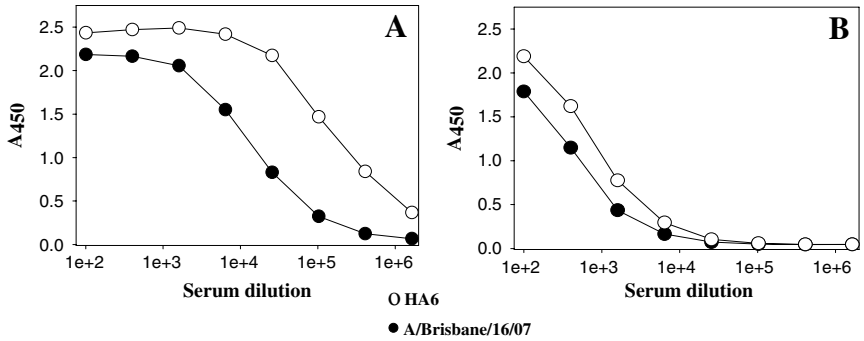

Fig. S6. Binding of the anti-HA6 sera to recombinant H3 HA proteins. Anti-HA6 sera from study group 3 in Table S2 (*A*) and convalescent sera from A/HK/68 infected mice (*B*) were tested for binding activities to recombinant H3 A/Brisbane/16/07 HA by ELISA assays. HA6 was included as a control. The data represent the mean OD450-nm values of 10 mouse sera from each respective group. Convalescent sera bind both HA6 and A/Brisbane/07 poorly, confirming that antibodies in these sera are largely directed to the globular head of HA. In contrast, anti-HA6 sera bind well to both HA6 and A/Brisbane/07. Anti-HA6 sera have an approximately 100-fold higher half-maximal binding titer for A/Brisbane/07 than convalescent sera.

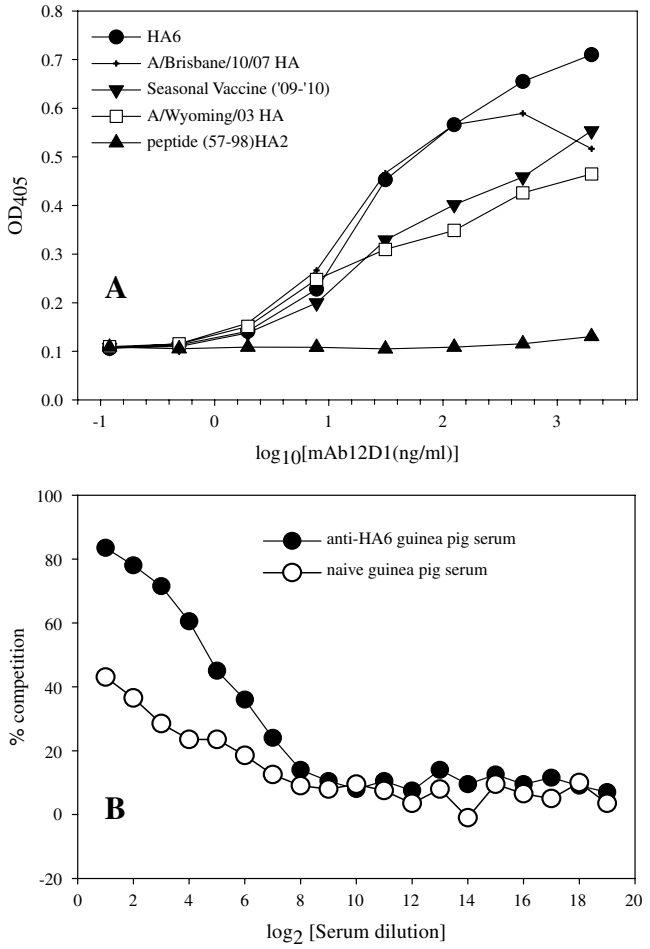

Fig. S7. Binding of mAb12D1 to immobilized HA6 and competition binding in the presence of anti-HA6 serum. (*A*) ELISA binding of mAb 12D1 to HA6, recombinant HA proteins (A/Brisbane/10/07, A/Wyoming/03), seasonal vaccine and peptide (57–98) HA2 (250 ng per well in each case). The antibody does not bind to a linear peptide (57–98) HA2 containing the putative epitope residues but binds well to HA6 and the other full length, soluble recombinant HA proteins. (*B*) Competition binding of 12D1 (10 ng/mL) to recombinant A/Brisbane/10/07 HA (10 ng per well) in the presence of various dilutions of anti-HA6 guinea pig serum and naïve guinea pig serum. Anti-HA6 serum competes with 12D1 indicating the presence of 12D1 like Abs in the serum. In all cases binding of 12D1 to immobilized protein was detected by an appropriately tagged anti-mouse IgG.

Table S1. Fraction of exposed residues that are highly conserved in different HA fragments

	Influenza A						Influenza B		
	H1			H3					
	HA1, %*	HA2, %*	HA6, %*	HA1, %*	HA2, %*	HA6, %*	HA1, %*	HA2, %*	HA6, %*
Vaccine strains[†]	86	97	96	80	91	91	82	99	98
All strains[‡]	71	92	90	64	88	88	81	97	97

Residues that are at least 95% conserved and have accessibilities greater than 20% (exposed) were identified by mapping the percent conservation of the residues onto the structure of HA (PDB ID code 1HGD)

*Fraction of exposed residues that are ≥95% conserved. The total number of exposed residues in HA1, HA2, and HA6 is 146, 74, and 105, respectively.

[†]Sequences of the vaccine strains of the past 10 years were obtained from the NCBI Influenza Virus resource (6).

[‡]All the nonidentical, full-length HA sequences available at the NCBI flu resource were used for the analysis. The average conservation (in the exposed regions of HA) for H1 and H3 strains is 67%, 90%, and 89% for HA1, HA2, and HA6, respectively.

Table S2. Dosage regimen used for mice challenge studies

Group	Vaccine antigen/adjuvant*	Log_{10} (Anti-HA6 antibody titres)[†]	% Survival after challenge[‡]
1	1 μg of HA6 WT/100 μg CpG7909	6.0	90
2	5 μg of HA6 WT/100 μg CpG7909	6.2	100
3	20 μg of HA6 WT/100 μg CpG7909[§]	6.2	90
4	1 μg of HA6 (3C, 116C mutant)/100 μg CpG7909	6.0	90
5	5 μg of HA6 (3C, 116C mutant)/100 μg CpG7909	6.2	100
6	20 μg of HA6 (3C,116C mutant)/100 μg CpG7909	6.2	100
7	1 μg of HA6 (40C, 118C mutant)/100 μg CpG7909	6.2	90
8	5 μg of HA6 (40C, 118C mutant)/100 μg CpG7909	6.2	100
9	20 μg of HA6 (40C, 118C mutant)/100 μg CpG7909	6.2	90
10	20 μg of HA6a (A/Phil/2/82)/100 μg CpG7909	6.1	80
11	20 μg HA6: Tg Fcer1g (FcRγ)[¶]/100 μg CpG7909	6.0	40
12	Single dose of HA6 WT (20 μg)	5.9	90
13	Naive (None)	ND[‖]	0
14	100 μg CpG7909	ND[‖]	0
15	A/HK/68 0.1 LD90, i.n. (week 0 only)	4.1	90
16	A/HK/68 0.1 LD90, i.m. (week 0 only)	ND[‖]	10
17	A/HK68 0.1 LD90, i.n.: Tg Fcer1g (FcRγ)[¶] (week 0 only)	4.6	100

Each group consisted of 10 BALB/c mice. The survival of the mice after viral challenge was monitored for 20 days by which time the naïve control mice died. All the immunogens tested [WT, (3C, 116C) and (40C, 118C) mutants of HA6 and HA6a] were highly immunogenic and conferred protection against (H3N2) viral challenge (A/HK/68). However, there was no protection against an H1N1 virus challenge (A/PR/8/34).

*The mice were vaccinated at week 0 and given a booster at Week 4.

[†]At week 6, bleeds were collected and the anti-HA6 antibody titers were determined by ELISA against WT HA6.

[‡]At week 7, the mice were challenged with 1LD90 of the homologous H3 virus (A/HK/68) and the survival was monitored up to 20 days.

[§]Similar immunogenicity and protection was seen when Merck Aluminium Adjuvant and ISCOMATRIX were used as adjuvants.

[¶]Mice in which the FcR-γ chain is knocked out.

[‖]ND, not determined; i.n., intranasal.