Supporting Information

Mallajosyula et al. 10.1073/pnas.1402766111

(1) H1HA10 (H1N1 A/Puerto Rico/8/34)

DTVDTVLEKNVTVTHSVNLLEDSH**GSA**NSSLPYQN**T**HP**T**T**N**GESPKYVRSAKLRMVTG
LRN**GSAGSA**TQNAINGITNKVNTVIEKMNIQ***D***TA**T**GKEFNK***D***EKRMENLNKKVDDGFL
DIWTYNAELLVLLENERTLD**A**HDS

(2) H1HA10-IZ (H1N1 A/Puerto Rico/8/34)

DTVDTVLEKNVTVTHSVNLLEDSH**GSA**NSSLPYQN**T**HP**T**T**N**GESPKYVRSAKLRMVTG
LRN**GSAGSA**TQNAINGITNKVNTVIEKMNIQ***D***TA**T**GKEFNK***D***EKRMENLNKKVDDGFL
DIWTYNAELLVLLENERTLD**A**HDSQGT**GG***IKKEIEAIKKEQEAIKKKIEAIEKEIEA*

(3) H1HA10-Foldon (H1N1 A/Puerto Rico/8/34)

DTVDTVLEKNVTVTHSVNLLEDSH**GSA**NSSLPYQN**T**HP**T**T**N**GESPKYVRSAKLRMVTG
LRN**GSAGSA**TQNAINGITNKVNTVIEKMNIQ***D***TA**T**GKEFNK***D***EKRMENLNKKVDDGFL
DIWTYNAELLVLLENERTLD**A**HDSQGT**GG***GYIPEAPRDGQAYVRKDGEWVLLSTFL*

(4) NCH1HA10-Foldon (H1N1 A/New Caledonia/20/99)

DTVDTVLEKNVTVTHSVNLLEDSH**GSA**NSSLPFQN**T**HP**T**T**N**GESPKYVRSAKLRMVTG
LRN**GSAGSA**TQNAINGITNKVNSVIEKMNTQ***D***TAVGKEFNK***D***ERRMENLNKKVDDGFL
DIWTYNAELLVLLENERTLD**A**HDSQGT**GG***GYIPEAPRDGQAYVRKDGEWVLLSTFL*

(5) pH1HA10-Foldon (H1N1 A/California/04/2009)

DTVDTVLEKNVTVTHSVNLLEDKH**GSA**NTSLPFQN**T**HP**T**T**N**GKSPKYVKSTKLRLATG
LRN**GSAGSA**TQNAIDEITNKVNSVIEKMNTQ***D***TAVGKEFNH***D***EKRIENLNKKVDDGFL
DIWTYNAELLVLLENERTLD**A**HDSQGT**GG***GYIPEAPRDGQAYVRKDGEWVLLSTFL*

(6) H5HA10-Foldon (H5N1 A/Viet Nam/1203/2004)

EQVDTIMEKNVTVTHAQDILEKTH**GSA**NSSMPFHN**T**HP**N**T**T**GESPKYVKSNRLVLATG
LRN**GSAGSA**TQKAIDGVTNKVNSIIDKMNTQFEA***D***GREFNN***D***ERRIENLNKKMEDGFL
DVWTYNAELLVLMENERTLD**A**HDSQGT**GG***GYIPEAPRDGQAYVRKDGEWVLLSTFL*

Fig. S1. Sequence of the designed "headless" hemagglutinin (HA) stem constructs. The various constructs were designed from full-length HA sequences obtained from the National Center for Biotechnology Information Influenza Virus Database [H1N1 A/Puerto Rico/8/1934 (ABD77675.1), H1N1 A/New Caledonia/20/1999 (ACF41878.1), H1N1 A/California/04/2009 (ACS45035.1), and H5N1 A/Viet Nam/1203/2004 (ABW90125.1)]. Our preliminary construct H1HA10 included residue fragments 18_1–41_1 (HA1), 290_1–323_1 (HA1), and 41_2–113_2 (HA2). Mutations (bold and underlined) chosen by ROSETTA DESIGN (1) were incorporated to remove exposed hydrophobic patches. Selected residues in the B loop were mutated to Asp (bold and italics) to destabilize the low-pH conformation of HA. Cys306_1 was mutated to Ser to prevent intermolecular disulfide bonds. The HA fragments were connected by soluble, flexible linkers (bold). Derivatives of H1HA10 were made with C-terminal trimerization motifs: isoleucine zipper (IZ) or Foldon (italics). Constructs similar to H1HA10-Foldon were made from heterologous strains as indicated.

1. Kuhlman B, et al. (2003) Design of a novel globular protein fold with atomic-level accuracy. *Science* 302(5649):1364–1368.

Fig. S2. Strategy to extend our HA stem-based immunogen design to other strains. A pair-wise sequence alignment with H1HA10 (our preliminary construct) can guide immunogen design. Hydrophobic residues mutated (solid-line boxes) in H1HA10 to mask the newly exposed hydrophobic patches are identical/similar within a subtype. Mutations destabilizing the low-pH conformation of HA (dashed-line boxes) have been previously discussed (1). Cys306$_1$ is mutated to Ser (shaded boxes) to prevent intermolecular disulfide bond formation. The start of the HA2 fragment (41$_2$–113$_2$) is indicated with an arrow. An asterisk indicates a fully conserved residue. A colon or period indicates a conservative substitution between groups of strongly or weakly similar properties, respectively.

1. Bommakanti G, et al. (2012) Design of Escherichia coli-expressed stalk domain immunogens of H1N1 hemagglutinin that protect mice from lethal challenge. *J Virol* 86(24):13434–13444.

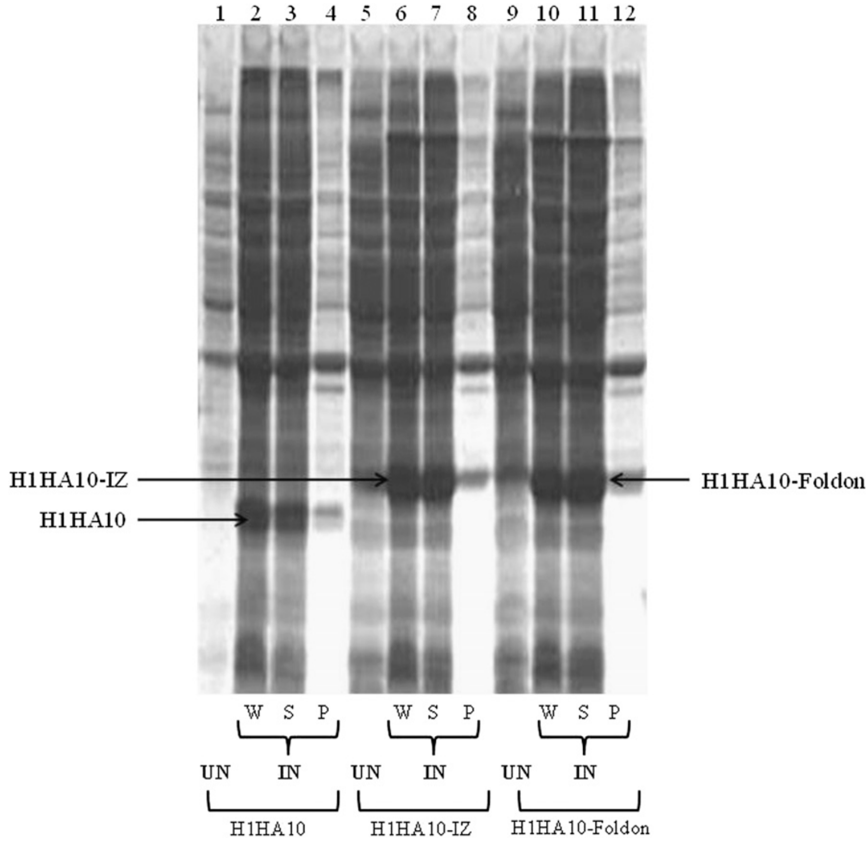

Fig. S3. The designed HA stem immunogens were purified from the soluble fraction of the cell culture lysate. The expression of all of the designed HA stem immunogens was monitored using a similar protocol. Briefly, a small-scale culture (6 mL) of *Escherichia coli* BL21(DE3) cells transformed with a plasmid encoding the construct was grown at 37 °C until OD_{600} of ∼0.6 was reached. An aliquot (1 mL) of the uninduced (UN) culture was removed for later analysis. The remaining culture was induced with 1 mM isopropyl-β-thiogalactopyranoside and grown at 20 °C for ∼6–8 h. One microliter of the induced (IN) whole cell culture (W) was removed and the rest of the culture lysed by sonication on ice. After sonication, the supernatant (S) and pellet (P) fractions were separated by centrifugation at 13,000 × *g* for 10 min at 4 °C. The UN and IN (W, S, and P) fractions for the various constructs were analyzed by SDS/PAGE. Lanes: 1–4, H1HA10; 5–8, H1HA10-IZ; and lanes 9–12, H1HA10-Foldon. As indicated (arrows), all of the designed HA stem immunogens are present in the soluble fraction of the cell culture lysate.

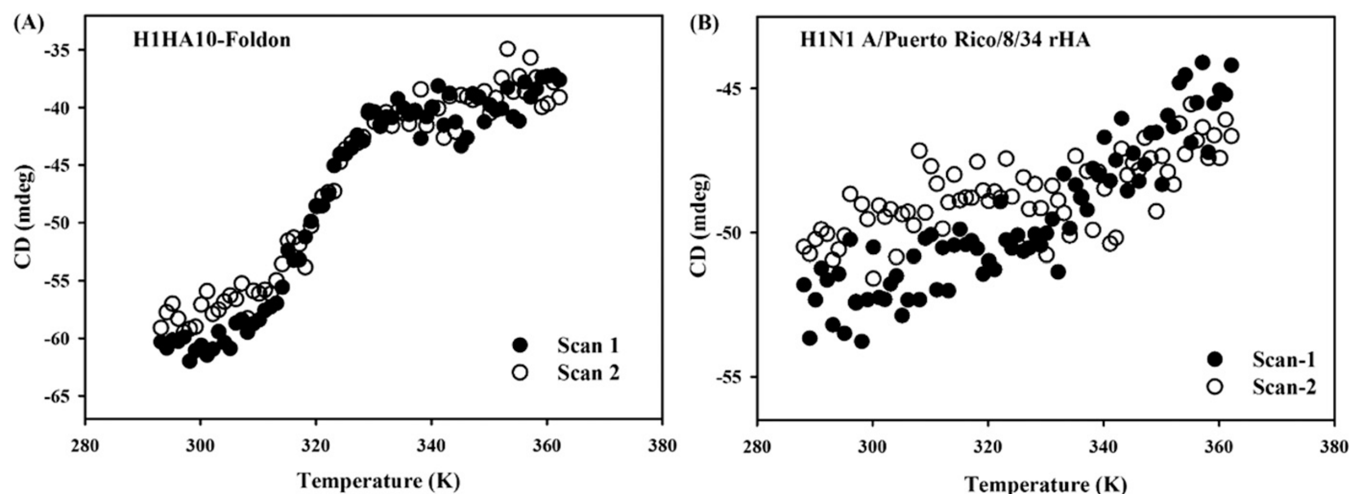

Fig. S4. Thermal stability of HA stem fragments. (*A*) Thermal melt of H1HA10-Foldon (~15 μM) was monitored by circular dichroism (CD) at 208 nm. H1HA10-Foldon showed a reversible and cooperative unfolding with an apparent transition midpoint of 323 K (50 °C). Consecutive scans of H1HA10-Foldon overlapped well with each other indicating reversible thermal unfolding. (*B*) Thermal melt of full-length H1N1 A/Puerto Rico/8/34 recombinant HA (rHA) (~12.5 μM) was monitored at 215 nm. The protein showed a broad transition and the repeat scan showed little or no change in CD signal indicating irreversible thermal unfolding. All protein concentrations are in monomer units.

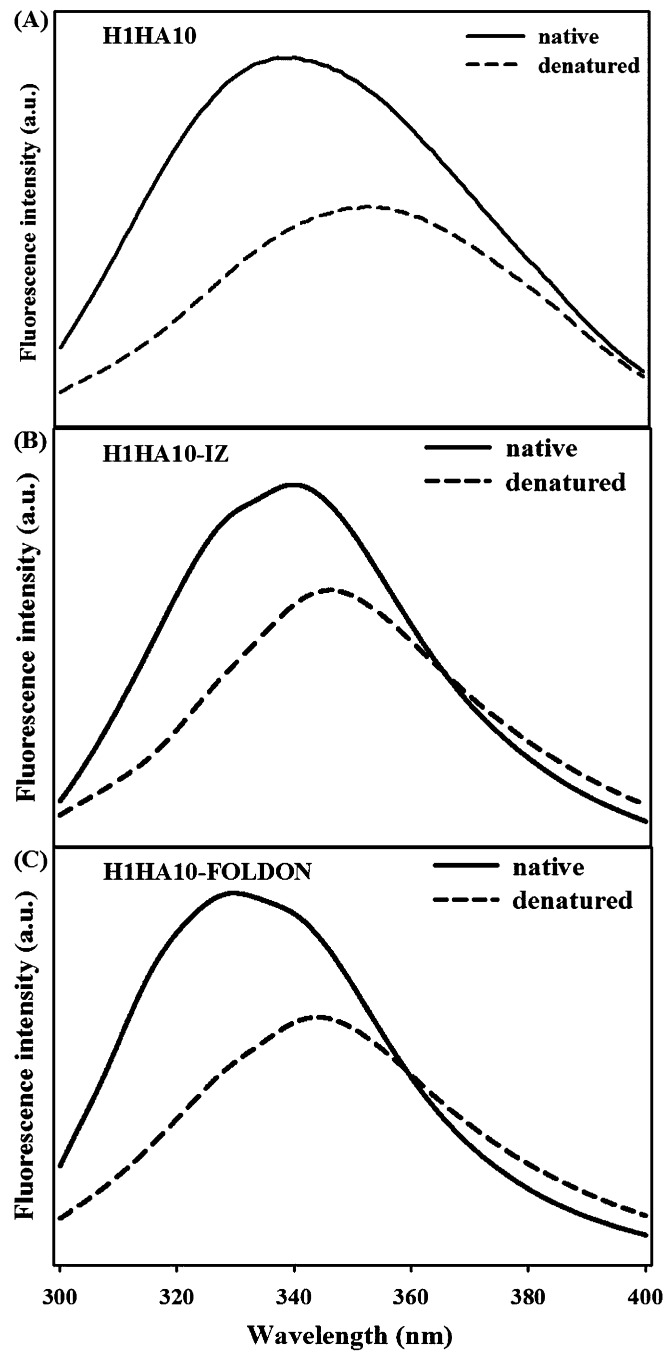

Fig. S5. Fluorescence emission spectra of HA stem immunogens. The samples were excited at 280 nm, and emission was monitored from 300 to 400 nm. The measurements were carried out under native (PBS, pH 7.4) or denaturing conditions [7 M guanidine hydrochloride (GdnCl) in PBS, pH 7.4] as indicated. (*A*) H1HA10, (*B*) H1HA10-IZ, and (*C*) H1HA10-Foldon. All proteins showed a considerable red-shift in the emission maxima upon denaturation with GdnCl, suggesting that they are folded under native conditions.

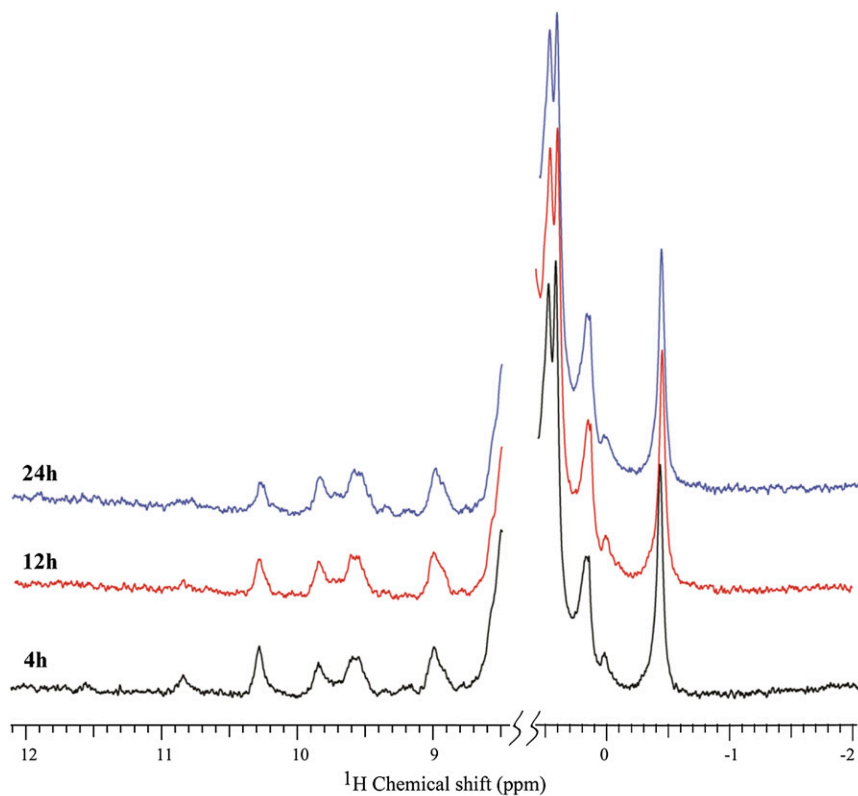

Fig. S6. Hydrogen exchange studies of H1HA10-Foldon. Overlay of the 1D ^{1}H NMR spectra at 4, 12, and 24 h is shown. The spectra were recorded at 25 °C (pH 7.4, 80% D_2O:20% H_2O). The slow exchange of the amide protons in the downfield (9–11 ppm) region is indicative of a compact, well-folded molecule.

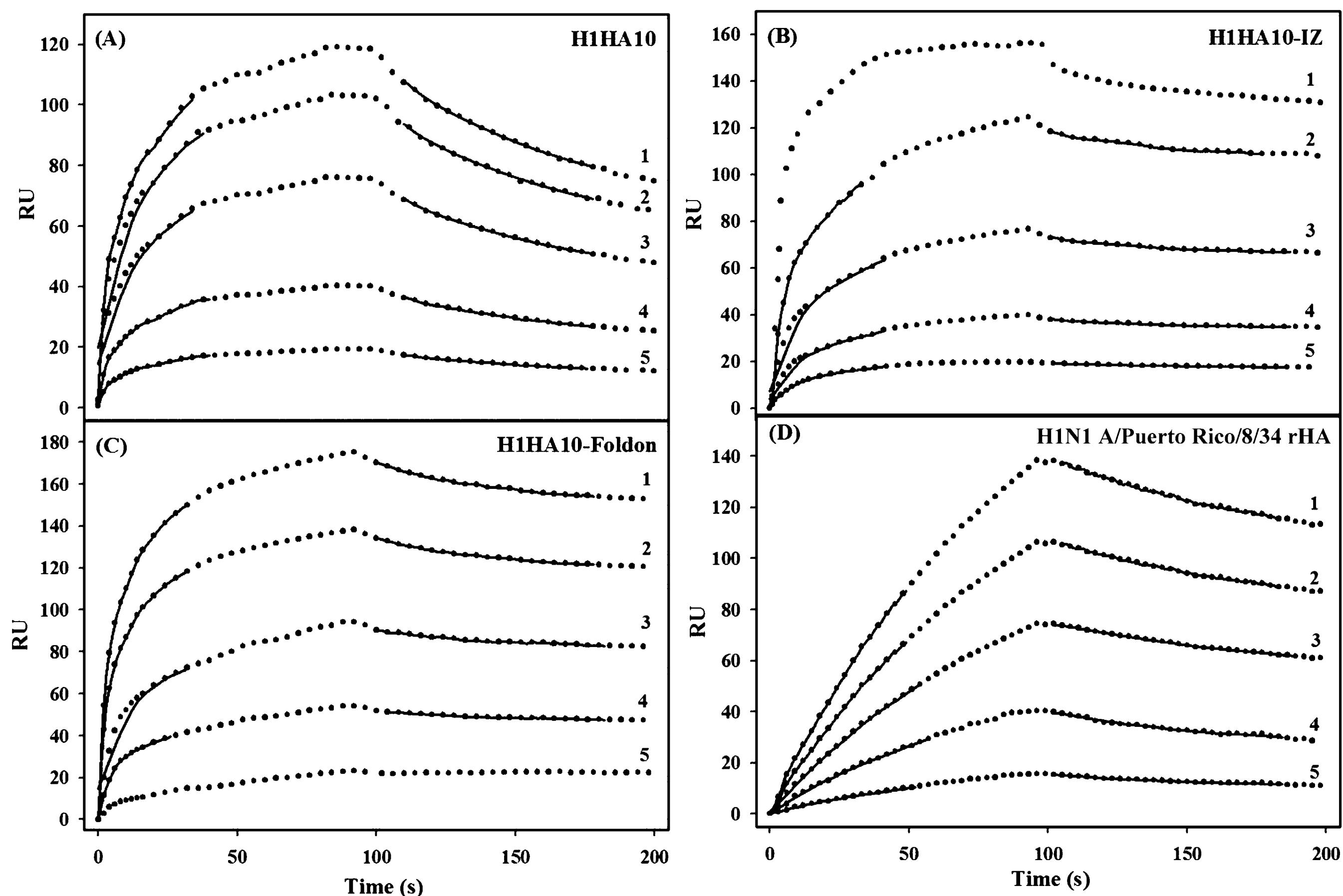

Fig. S7. HA stem immunogens bind the conformation specific stem-directed bnAb CR6261. The overlays of binding kinetics at different concentrations of the HA stem immunogens, studied with Biacore are shown. Five hundred response units (RU) of IgG-CR6261 were immobilized on the surface of a CM5 chip. Binding of various proteins to IgG-CR6261 was determined over a concentration range to obtain the kinetic parameters listed in Table 1. (*A*) H1HA10 (traces 1–5: 15, 10, 5, 2.5, and 1 μM). (*B* and *C*) H1HA10-IZ and H1HA10-Foldon (traces 1–5: 7.5, 5, 2.5, 0.75, and 0.2 μM). (*D*) H1N1 A/Puerto Rico/8/34 rHA (traces 1–5: 0.1, 0.08, 0.06, 0.04, and 0.02 μM). At high concentrations (7.5 μM), H1HA10-IZ shows biphasic binding to the immobilized ligand probably because of its heterogenous oligomeric states in solution (trace 1 could not be fitted to a 1:1 Langmuir interaction model). H1HA10-Foldon binds IgG-CR6261 with high affinity (52.4 ± 1.8 nM). The dissociation phase for H1HA10-Foldon binding (0.2 μM) could not be fitted because there was no measurable change in RU. The binding of H1HA10-Foldon to IgG-CR6261 is about sixfold weaker than full-length H1N1 A/Puerto Rico/8/34 rHA (8.9 ± 0.3 nM). Our design H1HA10 has ∼80% of the antibody footprint, which possibly explains the marginally weaker affinity of the designed stem immunogens to IgG-CR6261 compared with the full-length rHA. Low-density ligand immobilization enabled us to globally fit the data to a 1:1 Langmuir interaction model using BIA EVALUATION 3.1 software as described previously (1). The data points are shown as solid circles, whereas the fits are solid lines.

1. Bhattacharyya S, et al. (2013) Design of an *Escherichia coli* expressed HIV-1 gp120 fragment immunogen that binds to b12 and induces broad and potent neutralizing antibodies. *J Biol Chem* 288(14):9815–9825.

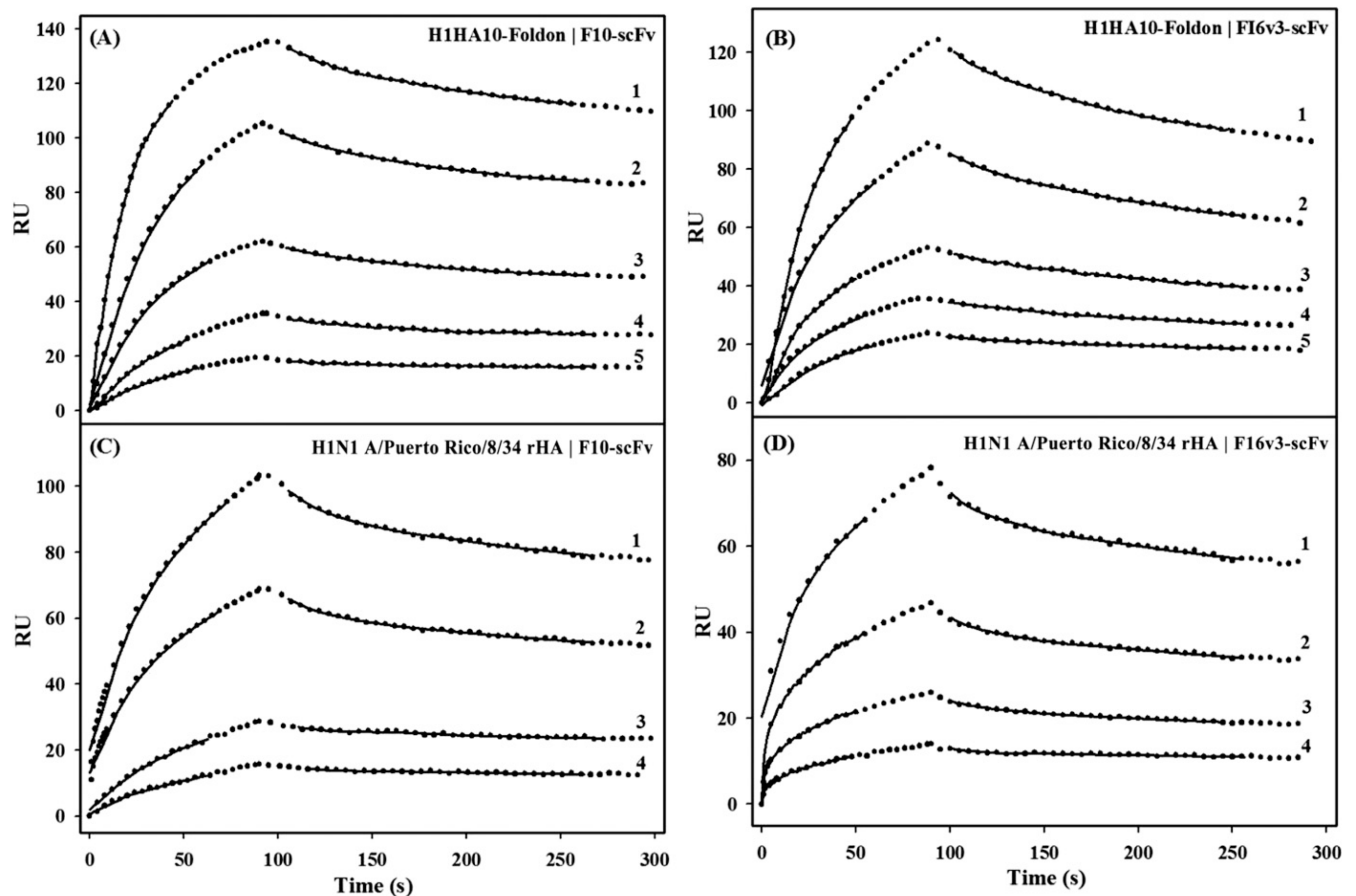

Fig. S8. H1HA10-Foldon binds conformation-specific, stem-directed broadly neutralizing antibodies, F10 and FI6v3, with high affinity. The overlays of binding kinetics of H1HA10-Foldon and H1N1 A/Puerto Rico/8/34 rHA, studied with Biacore are shown. Seven hundred fifty RU of purified F10–single-chain variable fragment (scFv) or FI6v3-scFv were immobilized on the surface of a CM5 chip. Analyte binding was determined over a concentration range to obtain the kinetic parameters (Table 1). (*A* and *B*) H1HA10-Foldon (traces 1–5: 500, 250, 150, 75, and 50 nM). (*C* and *D*) H1N1 A/Puerto Rico/8/34 rHA (traces 1–4: 150, 75, 25, and 10 nM). H1HA10-Foldon binds both F10-scFv (K_D 9.8 ± 2.1 nM) and FI6v3-scFv (K_D 12.1 ± 3.4nM) with high affinity. The kinetic parameters were obtained by globally fitting the data to a 1:1 Langmuir interaction model using BIA EVALUATION 3.1 software as described previously (1). The data points are shown as solid circles, whereas the fits are solid lines.

1. Bhattacharyya S, et al. (2013) Design of an *Escherichia coli* expressed HIV-1 gp120 fragment immunogen that binds to b12 and induces broad and potent neutralizing antibodies. *J Biol Chem* 288(14):9815–9825.

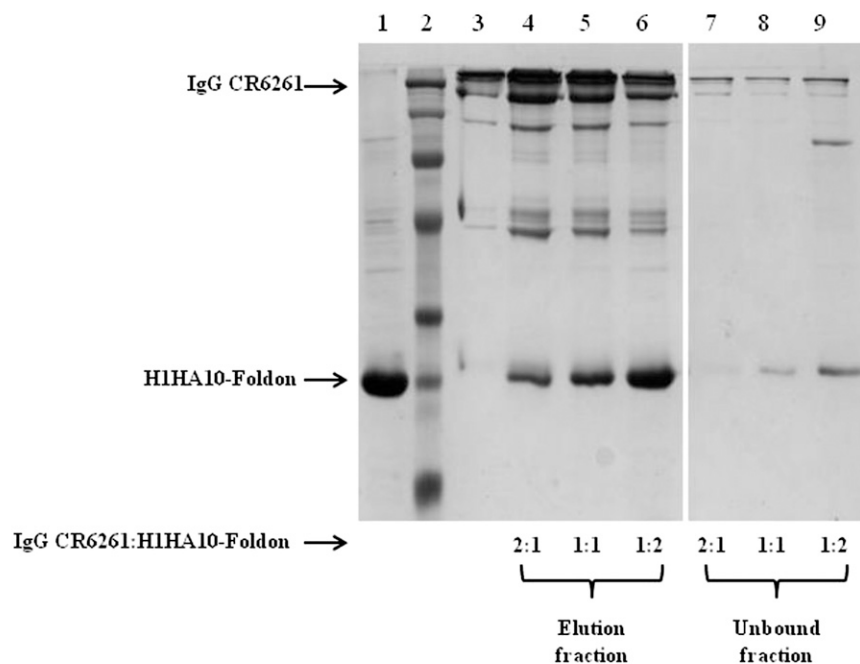

Fig. S9. H1HA10-Foldon forms a stable complex with the stem-directed bnAb CR6261. H1HA10-Foldon and IgG-CR6261 were mixed together at different molar ratios as indicated and incubated for 2 h at 4 °C. Protein G beads specific for the IgG could pull down the antibody–antigen (Ab–Agn) complex. The Protein G-bound protein was eluted (elution fraction) with 100 mM glycine·HCl (pH 3). The absence of H1HA10-Foldon in the unbound fraction indicates the formation of a stable Ab–Agn complex. Lanes: 1, H1HA10-Foldon, lane 2: prestained broad range marker (Biorad), lane 3: IgG CR6261, lanes 4–6: elution fraction; and 7–9, unbound fraction. All of the samples (without reducing agent) were analyzed on a denaturing SDS/PAGE.

Table S1. Residue conservation across influenza subtypes

Strain	Percent identity with full-length H1N1 A/Puerto Rico/8/34 HA	Percent identity with designed H1HA10 (H1N1 A/Puerto Rico/8/34)
H1N1 A/New Caledonia/20/1999	88	96
H1N1 A/California/04/2009	81	89
H5N1 A/Viet Nam/1203/2004	64	70

The residue conservation within our designed HA stem immunogen is greater than in full-length HA.

Table S2. HA-stem immunogens (designed from H1N1 A/Puerto Rico/8/1934) induce broadly cross-reactive HA-specific antibodies

	Log_{10}, serum dilution*			
Influenza strain	H1HA10	H1HA10-IZ	H1HA10-Foldon	A/Puerto Rico/8/34 virus†
H1N1 A/Puerto Rico/8/1934	6.14	6.14	≥6.21	4.62
H1N1 A/California/04/2009	5.57	5.59	≥6.21	2.81
H1N1 A/Brisbane/59/2007	6.01	5.17	≥6.21	3.74
H5N1 A/Viet Nam/1194/2004	5.07	5.40	≥6.21	2.75
H3N2 A/Aichi/2/1968	3.81	3.85	4.61	NB
H3N2 A/Brisbane/10/07	3.61	3.99	4.84	NB

Antibody titer against full-length rHA was determined by ELISA. NB, no detectable binding.

*Sera were collected from individual mice of each group 14 d postsecondary immunization of the indicated HA stem immunogens and pooled.

†Mice were intranasally immunized with a sublethal dose ($0.1LD_{90}$) of A/Puerto Rico/8/34 virus.