Supporting Information

Chen *et al.* 10.1073/pnas.0806901105

SI Materials and Methods

Functional Characterization of CHA5 with Immunoblot and Hemadsorption. Human 293T cells were transfected with pCHA5 or pVAX for 48 h. The transfected cells were subjected to immunoblotting analysis with a polyclonal anti-HA serum (provided by Y.-T. Wu, Academia Sinica, Taiwan). For analysis of hemadsorption, transfected HEK293 cells were treated with 10 mU neuraminidase at 37°C for 60 min followed by a 30-min incubation with 3% chicken red blood cells (cRBC). After several washes with 0.85% NaCl solution, the cells were examined with phase-contrast microscopy by using an IX71 inverted microscope with a 40X objective (Olympus).

Determination of HA-Specific Antibodies by Enzyme-Linked Immunosorbent Assay (ELISA). HA-specific antibodies in heat-inactivated serum were monitored by direct ELISA. The CHAedc-Fc-coated plates were incubated with mouse serum in twofold serial dilutions for 1 h. HA-specific IgG was monitored by using alkaline phosphatase (AP)-conjugated anti-mouse antibodies and the AMPAK kit (DAKO). The endpoint antibody titer was defined as the highest dilution of serum to produce an absorbance 2.5 times higher than the optical absorbance (OD) produced by the preimmune serum. The background endpoint antibody titer was assigned as <1:50.

Enzyme-Linked Immunospot (ELISpot) Assay for IFN-gamma (IFN-γ). The ELISpot assay was carried out by using the mouse IFN-γ development module manufactured by R&D Systems. Briefly, 96-well Millipore MultiScreen-IP sterile plates were coated with anti-IFN-γ monoclonal antibodies overnight. After the killing of the pCHA5- and pVAX-immunized mice, splenocytes were collected and cultured at 0.5×10^6 per well for 18 h at 37°C with a $CD8^+$ peptide (IYSTVASSL) from HA (1) for restimulation. The cells were then washed and incubated with biotinylated anti-mouse IFN-γ-specific antibody. The plates were washed again before the addition of streptavidin-AP conjugate. ELISpots were developed with one-step BCIP/NPT reagent and counted with an Immune Spot Reader (Cellular Technology Ltd.). Two to four wells were counted per group. Data were recorded as the mean spot-forming cells (SFC) per 10^6 cells for each group from repeating experiments (+ SD). Negative control wells were stimulated with the same amount of either an irrelevant peptide (HIV-1 9mer) (2) or no peptide, always yielding fewer than 10 spots per sample.

Hemagglutination Inhibition (HI) Activity of Antiserum. Both the whole-virus and the cell-based methods were used to evaluate the HI activity of immune sera. The HI assay using the reassortant viruses was performed following a standard procedure (3). Briefly, the antiserum in twofold dilutions was incubated with the indicated virus at a 4HA unit. The mixture was then added to an equal volume of 0.5% cRBC suspension and incubated in a V-shaped microtiter plate for 30 min at room temperature. Alternatively, HA-transfected HEK293 cells were treated with neuraminidase and then incubated with 3% cRBC in the presence or absence of pCHA5 antisera. After several washes with 0.85% NaCl solution, the bound cRBC were lysed and the released hemoglobin was quantitated based on O.D. at 540 nm.

Production of HA-Pseudotyped Virus and Determination of Neutralization Activity of Antiserum. Influenza HA-pseudotyped viruses expressing a luciferase reporter were produced to evaluate neutralization activity of immune sera. Briefly, human 293T cells were cotransfected with three plasmids: pNA, pCHA5, or other HA constructs, and pNL4–3.Luc.R-E- (AIDS Research and Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health) (4). The vesicular stomatitis virus-G (VSV-G) protein served as a control to evaluate entry efficiency as well as the specificity of the antiserum. After overnight incubation, the transfected cells were washed with PBS and incubated with fresh culture medium for another 24 h. The supernatant was then harvested, clarified through 0.45-μm syringe filters, and stored at −80°C until use. The infectivity of the HA-pseudotyped viruses for MDCK cells was titered by the Reed and Muench method (5). For the neutralization assay, 50 $TCID_{50}$ of HA-pseudotyped viruses was mixed with various dilutions of the antiserum at 37°C for 30 min. The mixtures were then added to MDCK cells (final volume 200 μl) in each well of a 96-well plate. After 4 h of incubation at 37°C, the plates were washed and replenished with fresh medium. After 48 h, the cells were lysed, and luciferase activity was determined by the Luciferase Assay System (Promega). The relative luminescence values determined in the wells containing cells and HA-pseudotyped virus were defined as 0% neutralization; the values in the cells-only wells were defined as 100% neutralization. The IC_{50}, the reciprocal of the antiserum dilution at which virus entry is inhibited by 50%, was calculated after curve-fitting with the Prism program (GraphPad).

1. Hoelscher M, *et al.* (2006) Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. *Lancet* 367:475–481.
2. Huang Y, *et al.* (2008) Enhancement of HIV DNA vaccine immunogenicity by the NKT cell ligand, alpha-galactosylceramide. *Vaccine* 26:1807–1816.
3. World Health Organization (2007) Recommendations and laboratory procedures for detection of avian influenza A(H5N1) virus in specimens from suspected human cases. Available at http://www.who.int/csr/disease/avian_influenza/guidelines/RecAllabtestsAug07.pdf. Accessed August 2007.
4. He J, *et al.* (1995) Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. *J Virol* 69:6705–6711.
5. Reed LJ, Muench H (1938) A simple method of estimating fifty percent endpoints. *Amer J Hygiene* 27:493–497.

Fig. S1. Expression and assessment of function of CHA5. Human 293T cells were transfected with pVAX or pCHA5 for 48 h to monitor the expression of CHA5. (*A*) After transfection, the cells were collected and processed for immunoblotting analysis with an anti-HA polyclonal antibody. The arrow marks the estimated size of full-length HA and HA1. (*B*) Transfected cells were incubated with cRBC and then examined for hemadsorption by phase-contrast microscopy (40X). The magnifications indicate that cRBC were attached to the surface of the 293T cells transfected with pCHA5.