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Hemagglutinin stalk domain from H5N1 strain as a potentially universal antigen

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Influenza A virus infections are the major public health concern and cause significant morbidity and mortality each year worldwide. Vaccination is the main strategy of influenza epidemic prevention. However, seasonal vaccines induce strain-specific immunity and must be reformulated annually based on prediction of the strains that will circulate in the next season. Thus, it is essential to develop vaccines that would induce broad and persistent immunity to influenza viruses. Hemagglutinin is the major surface antigen of the influenza virus. Recent studies revealed the importance of HA stalk-specific antibodies in neutralization of different influenza virus strains. Therefore, it is important to design an immunogen that would focus the immune response on the HA stalk domain in order to elicit neutralizing antibodies. In the present study, we report characterization of a conserved truncated protein, potentially a universal influenza virus antigen from the H5N1 Highly Pathogenic Avian Influenza A virus strain. Our results indicate that exposure of the HA stalk domain containing conserved epitopes results in cross reactivity with different antibodies (against group 1 and 2 HAs). Additionally, we conclude that HA stalk domain contains not only conformational epitopes recognized by universal FI6 antibody, but also linear epitopes recognized by other antibodies.

Key words: H5N1, universal influenza antigen, hemagglutinin stalk domain, universal influenza antibodies, FI6

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INTRODUCTION

The influenza virus belongs to the Orthomyxoviridae family and is one of the life-threatening pathogens in the world. There are three types of influenza virus identified as A, B and C, while subtype A is predominantly a serious public health problem causing acute infections in humans, characterized by high mortality rate. According to the World Health Organization (WHO), each year influenza virus infects up to 15% of the population (approximately 1 billion cases). Epidemiological data show a possibility of emergence of new pandemic strains.

Outbreak of the human pandemic influenza A (pH1N1) has caused the intensification of the scientific research and clinical trials so as to develop pandemic influenza vaccines and therapies. Blood samples were collected from donors exposed to pH1N1 and plasma cells were isolated with the aim of screening for secretion of an antibody that would bind to diverse hemagglutinins. A human monoclonal antibody FI6 was found to recognize all subtypes of HAs. FI6 cross-reacts with members of both HAs groups via the attachment to the conserved epitope in hemagglutinin (Corti *et al.*, 2011).

Hemagglutinin (HA) is the major surface glycoprotein of influenza virus. It is a homotrimeric protein molecule, where each monomer consists of two subunits linked by disulfide bonds: HA1 subunit, which forms a globular head and HA2 subunit, which forms a stalk domain (Fig. 1)(Kang *et al.*, 2011). Hemagglutinin plays a crucial role in the viral entry. The globular head domain contains a receptor-binding site for sialic acid that enables attachment of the virus to the host cell, while fusion peptide located in the stalk domain is responsible for the pH-induced fusion of the viral envelope with the endosome membrane (Bouvier & Palese, 2008).

HA proteins of influenza A viruses are divided into at least 17 subtypes and are categorized into two major phylogenetic groups based on distinct structures in the HA2 stalk domain: group 1 (subtypes H1, H2, H5, H6, H8, H9, H11, H12, H13, H16 and H17) and group 2 (subtypes H3, H4, H7, H10, H14, H15) (Gamblin & Skehel, 2010; Krammer *et al.*, 2012a).

Current seasonal vaccines predominantly induce anti-HA antibodies that recognize antigenic sites in the globular head domain and act by blocking its receptor-binding activity (Margine *et al.*, 2013a). However, HA head is highly variable among diverse virus strains which is related to frequent amino-acid changes in this region which allows efficient immune evasion. This leads to the lack of protection against viruses that are different from the strain used for vaccine formulation (Margine *et al.*, 2013b).

Recent studies aiming to characterize conserved epitopes of influenza virus revealed that specific regions in the HA stalk domain are highly preserved both in the structure and the sequence among various subtypes of viruses. It has been discovered that neutralizing antibodies to the HA stalk domain can be found after influenza infection (Wrammert *et al.*, 2011) and vaccination (Corti *et al.*, 2010). These anti-stalk antibodies demonstrate cross-reactivity between HAs of many strains from group 1 as well as from group 2 and it has been shown

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Abbreviations: HA, hemagglutinin; PAb, polyclonal antibody; MAb, monoclonal antibody; HPAI, highly pathogenic avian influenza; pH1N1, pandemic strain H1N1; rHA, recombinant hemagglutinin; WT AcNPV, *Autographa californica nuclear polyhedrosis virus* wild type strain.

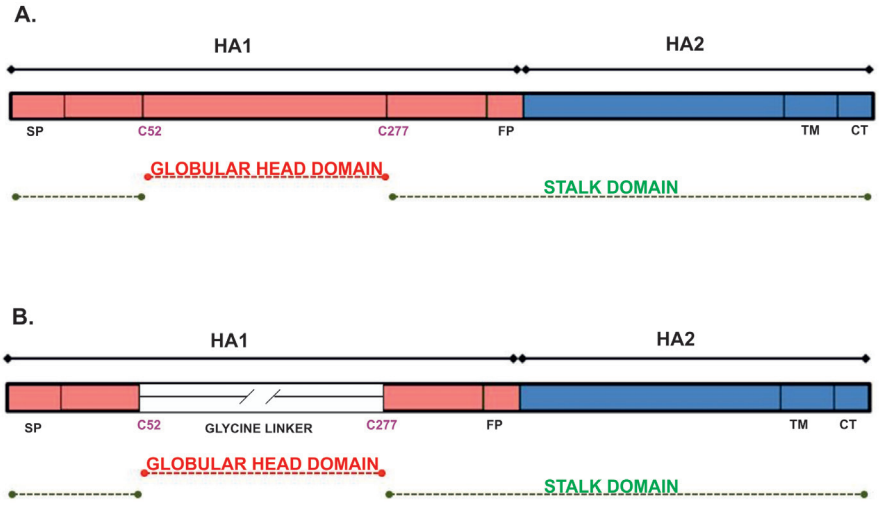

Figure 1. Schematic description of the full length and "headless" hemagglutinin from H5N1 strain.
(**A**) Full length HA (H5) containing globular head and stalk domain. Globular head region is mainly located between cysteines 52 and 277; stalk domain consists of N- and C-terminal parts of HA1 subunit and all of HA2 subunit; SP — signal peptide, FP — fusion peptide, TM — transmembrane domain, CT — cytoplasmic tail (**B**) HA stalk domain construct from the avian influenza virus H5N1 ("headless" HA) lacks the region located between cysteines 52 and 277 on the HA1 subunit and possesses instead a four glycine peptide linker.

that they act by preventing the fusion step of viral entry. It was also confirmed that anti-stalk antibodies elicited by infection with the 2009 pH1N1 have contributed to the disappearance of normally circulating H1N1 influenza virus strains in the following season (Pica *et al.,* 2012; Sangster *et al*., 2013).

Antigenically conserved HA stalk domain is thus a promising candidate for preparation of a broadly protective universal influenza vaccine; a potential immunogen that would focus the immune response on the conserved epitopes and would elicit anti-stalk neutralizing antibodies, and may probably give higher protection against different strains of the virus.

In this study we present the design, expression in a baculovirus system and characterization of a conserved truncated protein, HA stalk from the H5N1 HPAI strain, which might potentially be an universal influenza virus antigen. Such a "headless" HA lacks the region located between cysteines 52 and 277 on the HA1 subunit, and instead it posseses a short linker peptide (Fig. 1). Experiments were conducted to determine, whether the antibodies raised against various Influenza A virus strains can bind to HA stalk domain. In addition, we wanted to explore, whether immunization of rabbits with full length H5N1 HA elicits HA stalk-specific antibodies.

MATERIALS AND METHODS

Virus cDNA synthesis and PCR amplification. The full length HA gene was obtained according to the methods described by Gromadzka *et al*., 2008. The H5N1 HA stalk domain was designed based on current knowledge of its structure (Steel *et al*., 2010). Gene coding for the headless hemaglutinin from H5N1 Polish strain was synthesized and cloned into p GEM T-easy vector (accesion number FM163448).

Plasmid construction and sequencing. Products of amplification (full HA and truncated HA) were cloned into pGEM-T easy vector according to the manufacturer's manual (Promega). Plasmids were propagated in *Escherichia coli* cells and purified on an affinity column (A&A Biotechnology). DNA sequencing was performed to confirm the correct sequence using universal primers for T7 and Sp6 promoter in pGEM-T easy vector.

Generation of recombinant baculoviruses. Amplified DNA of full-length HA was resolved on 1% agarose gel, purified (Gel-out, A&A Biotechnology) and ligated to the pFastBac1 (Invitrogen) donor vector using the BamHI and SpeI restriction enzyme sites. The truncated form of HA gene was cloned into pFastBac1 using the EcoRI and NotI restriction enzyme sites. The resulting recombinant transfer vectors (pFastBac1-HA, pFastBac1-HA stalk), which contained full-length HA and truncated form of HA gene respectively, were introduced into the target bacmids by site-specific recombination in *E. coli* (DH10Bac) as described in the Bac-to-Bac® protocol (Invitrogen).

Cell culture. *Spodoptera frugiperda Sf9* insect cells (Invitrogen) were cultured in monolayers at 27°C using HyQ SFX medium (Thermo Scientific). Transfection and recovery of recombinant baculovirus from *Sf9* cultures was performed according to the Bac-to-Bac® protocol and virus infectivity titer was determined using *Sf9*-Easy Titer (*Sf9*-ET) cell line. The *Sf9*-ET cells possess plasmid DNA containing the enhanced green fluorescent protein (eGFP) gene under the control of a baculovirus polyhedrin promoter. When used in the titration assay, the *Sf*-9ET cells turn green when they are infected with the baculovirus as a result of the activation of the polyhedrin promoter/eGFP complex by baculovirus gene products expressed during the infection. (Hopkins & Esposito, 2009). Recombinant baculoviruses containing both forms of HA gene were confirmed by a PCR reaction with specific primers based on a universal sequence in the bacmid genome according to the manual instructions.

Production and purification of hemagglutinin recombinant proteins. *Sf9* cells were grown as monolayers at 27°C in a 12-well culture plate using HyQ SFX medium (10^6 cells/ml). Cultures were infected with the recombinant baculovirus with a multiplicity of infection of 1 and cells were collected from the culture medium 48h postinfection. Harvested cells and supernatant were separated by centrifugation in a microcentrifuge at 13 300 rpm for 5 min. Both the supernatant and the cell

Table 1. Description of different antibodies against group 1 hemagglutinins used in this study.

Group 1. Hemagglutinins				
	Strain	Immunogen		Antibody description
Human	H1N1	HA	Purified human-cell derived recombinant H1N1 A/California/04/2009 and A/California/07/2009 HA extracellular domain. Clone ID 9G1G8	Mouse Monoclonal Antibody Sino Biological Inc. cat. no 11048-MM08
	H1N1	HA	Purified influenza virus type A strain H1N1	Mouse Monoclonal Antibody Santa Cruz Biotechnology cat. No sc-52025
	H1N1	HA	Recombinant HA protein eluted from PVDF membrane	Monospecific Rabbit Polyclonal Antibody
Avian/Human	H5N1	HA0	Recombinant HA protein eluted from PVDF membrane	Monospecific Rabbit Polyclonal Antibodies
	H5N1	HA1	116–256 aa rHA peptide	Monospecific Rabbit Polyclonal Antibodies
	H5N1	HA1/HA2	Recombinant HA protein eluted from PVDF membrane	Monospecific Rabbit Polyclonal Antibodies
	H5N1	H5N1 — reference	Whole virus H5N1: A/Ck/Scot/59	Polyclonal Chicken Antibodies - Animal Health and Veterinary Laboratories Agency (VLA) cat. no RAA7002
	H5N1	HA	Purified human-cell derived recombinant influenza A virus A/Anhui/1/2005/H5N1 HA extracellular domain. Clone ID 9F2E3F3	Mouse Monoclonal Antibodies. Sino Biological Inc. cat. no 11048-MM01
	H5N1	HA	Purified human-cell derived recombinant influenza A virus A/Anhui/1/2005/H5N1 HA extracellular domain. Clone ID 1C5B1A10	Mouse Monoclonal Antibodies. Sino Biological Inc. cat. no 11048-MM06
	H5N1	HA	Purified human-cell derived recombinant influenza A virus A/Anhui/1/2005/H5N1 HA extracellular domain. Clone ID 14B1E2G6	Mouse Monoclonal Antibodies. Sino Biological Inc. cat. no 11048-MM10
Avian	H5N2	H5N2 — reference	Whole virus H5N2:A/Ost/Den/72420/96	Chicken Polyclonal Antibody cat. no RAA7003 1:500
	H5Nx	H5Nx	Whole virus — H5 avian influenza	Chicken Polyclonal Antibody National Veterinary Research Institute PIWet

lysates were examined for recombinant proteins (HA full and HA stalk) by SDS-PAGE and Western blotting.

Time course of recombinant H5N1 HA stalk protein production in Sf9 cells. *Sf9* cells were grown as monolayers at 27°C in 12-well culture plate using HyQ SFX medium (10^6 cells/ml). Cultures were infected with the recombinant baculoviruses at the multiplicity of infection of 1 and cells were collected from the culture medium at different times post infection (6 h, 24 h, 48 h, 72 h, 96 h). Supernatants (medium) were separated from the cell pellet by centrifugation at 13300 rpm for 5 minutes. Proteins from the medium were precipitated using 10% trichloroacetic acid (TCA). In parallel, cell pellets were treated with lysis buffer on ice (100 mM Tris pH 7.4, 100 mM NaCl and 1% Nonidet P-40) in order to separate cell membranes from the cytoplasm. Resulting samples were boiled for 10 minutes at 100°C in a loading buffer (Life technologies) prior to SDS-PAGE on 10% gels.

Purification of recombinant HA protein from polyvinylidene difluoride (PVDF) membranes. Cultures were infected with the recombinant baculoviruses at the multiplicity of infection of 1 and cells were collected from the culture medium 72 hours post infection. Supernatants (medium) were separated from the cell pellet by centrifugation at 8500 rpm for 10 minutes. Cell pellets were lysed for 10 min. on ice with a lysis buffer (100 mM Tris pH 7.5, 1% Triton X-100, 150 mM NaCl) in order to separate cell membranes from the cytoplasm. Membrane fraction samples were separated by 8% SDS-PAGE followed by transferring the samples onto PVDF membranes. Electrotransfer was performed in a Tris-glycine transfer buffer at 25V overnight. As a control Western Blotting was performed before the blotted proteins were visualized using Ponceau S. HA protein was eluted from PVDF membrane with elution buffer containing 50 mM Tris pH 9.5, 2% SDS and 1% Triton X-100. Eluted proteins were precipitated with acetone and analyzed by SDS-PAGE gel and Western blot.

Production of hyperimmune antiserum in rabbits. Rabbits were immunized with 100 μg of different eluted recombinant H5N1 hemmaglutinin (HA0; HA1/HA2) mixed with Freund's complete adjuvant by intradermal injection followed by subcutaneous booster injection 3 weeks later with the same dose in Freund's incomplete adjuvant. Rabbits were bled 2 weeks later. The serum was collected and stored at –20°C until use.

Immunoperoxidase Monolayer Assay (IPMA). *Sf9* cells were seeded into 12-well cell culture plates (10^6 cells/ml) and infected with the recombinant baculoviruses at the multiplicity of infection of 1. After incubating for 48 hours, the medium was removed and the cells

Table 2. Description of different antibodies against group 2 hemagglutinins used in this study.

Group 2. Hemagglutinins				
	Strain	Immunogen		Antibody description
Human	H3N2	HA	Purified human-cell derived recombinant H3N2 A/Brisbane/10/2007 HA. Clone ID MM03	Mouse Monoclonal Antibody Sino Biological Inc. cat. no 11056-MM03
Avian/Human	H7N9	HA	Purified recombinant Influenza A H7N9/A/Shanghai/1/2013 HA 1–524 aa	Rabbit Polyclonal Antibody Sino Biological Inc. cat. no 40104-RP02
Avian/Human	H7N9	HA	Purified recombinant Influenza A H7N9 /A/Shanghai/1/2013 HA 1–524 aa	Mouse Monoclonal Antibody Sino Biological Inc. cat. no 11082-MM04
Avian	H7Nx	H7Nx avian influenza	Whole virus- H7/Poland	Chicken Polyclonal Antibody National Veterinary Research Institute PIWet
Avian	H4N6	HA	Purified recombinant Influenza A H4N6/A/mallard/Ohio/657/2002 HA 1–524 aa	Rabbit Polyclonal Antibody Sino Biological Inc. Cat.no 11714-RP01
Human	FI6	HA	H1N1 v	Highly specific humanised synthetic antibodies based on neutralizing antibody selected from plasma cells that bind to group 1 and group 2 Influenza A HAs

were fixed with 70% methanol. Cells were then incubated with primary antibodies (Tables 1–4) in a binding buffer (PBS – 0.05% Tween 80, 5% FBS) for 1 h, at RT. Cells were then washed three times with PBS — 0.05% Tween 80, horseradish peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, USA) was added at a dilution 1:1000 and plates were incubated for 1 h, at RT. After a final wash, NovaRED Peroxidase Substrate Kit (Vector) was used in order to detect positive red reaction products. The color reaction was allowed to develop for 5–15 minutes and the plates were then washed with H_2O and examined under a light microscope.

Western blot analysis. Proteins were separated by 10% SDS-PAGE followed by samples transfer to PVDF membranes. After protein electrotransfer in a Tris-glycine buffer at 25V overnight, membranes were blocked with 3% non-fat milk at RT for 1h and incubated with different antibodies against HA proteins (Tables 1–4) at RT for 1 h. Subsequently, alkaline phosphatase-conjugated secondary antibody (1:1000, Santa Cruz Biotechnology, USA) was added and it was incubated for 1h at RT. After three washes with TBS, a substrate solution containing NBT and BCIP was added. After developing the bands, the membrane was washed with water.

Flow cytometry. *Sf9* cells were seeded into 6-well cell culture plates (1.5×10^6 cells/ml) and infected with the recombinant baculoviruses at the multiplicity of infection of 1. Presence of full and stalk recombinant HA proteins at the cell surface was determined by indirect immunofluorescence as follows. Cells (5×10^5 cells/sample) were collected into 100 µl of a PBA buffer (PBS + 1% BSA + 0.02% sodium azide) and placed separately into the wells of 96-well polystyrene V-bottom microplate (Greiner Bio-One, Germany). Next, the cells were centrifuged for 3 min at 1300 rpm.

Cells were resuspended in the primary antibody solution in PBA (Tables 3–4) and stained for 1 hour on ice. After two rounds of washing with PBA by 3 min centrifugation at 1300 rpm, 4°C, discarding the antibody solution and resuspending the cell pellet in the PBA washing buffer, cells were incubated with goat anti-mouse IgG-PE (1:1000, Becton-Dickinson, USA) for 45 min on ice. After double washing with PBA, cells were resuspended in 120 µl of PBA and transferred to 0.65 ml tubes. Cells were analyzed using the FACS Calibur flow cytometer (Becton-Dickinson, USA) and the CellQuest software (Becton-Dickinson, USA).

Antibodies recognizing various hemagglutinins. Different monospecific rabbit polyclonal antibodies (anti HA0 H5N1, anti HA1/HA2 H5N1) were obtained in our laboratory (unpublished data). Chicken polyclonal antibodies (anti H5Nx, H1N1, H7Nx) were kindly provided by National Veterinary Research Institute (PIWet). The rest of the used antibodies were purchased from Santa Cruz Biotechnology Inc., Sino Biological Inc. and Veterinary Laboratories Agency (VLA). The details are presented in Tables 1 and 2.

RESULTS

Expression of the hemagglutinin recombinant protein genes in Sf9 insect cells

Our aim was to investigate whether the approach based on presentation to the host immune system of a stalk region of hemagglutinin of the human influenza viruses (H1 and H3) described by Steel and coworkers, 2010 can be applied also to the HPAI strains. For this purpose we planned to characterize the conserved, truncated form of hemagglutinin (HA stalk) from the Polish H5N1 strain. To compare the results obtained for truncated form of HA we decided to express the full length HA gene as well. Recombinant viruses bearing the full length and stalk form of hemagglutinin were used to infect insect cells under optimized conditions. A major polypeptide band of full-length HA0 with a molecular mass of 70 kDa was identified in cell lysates by SDS-PAGE followed by either Coomassie brilliant blue staining or Western blot analysis using monoclonal antibodies anti-HA H5N1 (1:1000, Sino Biological Inc.) (Fig. 2).

All the HPAI hemagglutinins possess multiple arginine residues at the site of cleavage and are cut intracellulary by ubiquitously occurring proteases (Steinhauer, 1999; Gamblin & Skehel, 2010). Apart from recombinant HA0 we could also observe HA1 with molecular mass of 53 kDa and HA2 subunits of 30 kDa which were the re-

Table 3. Reactivity of H5N1 HA stalk recombinant protein with antibodies against group 1 hemagglutinins.

Group 1. Hemagglutinins			IPMA				Western Blotting				FACS			
			H5N1 antigen				H5N1 antigen				H5N1 antigen			
		ANTIBODY	Bac HA stalk	Bac HA full	Bac WT	Mock (*Sf9*)	Bac HA stalk	Bac HA full	Bac WT	Mock (*Sf9*)	Bac HA stalk	Bac HA full	Bac WT	Mock (*Sf9*)
H1N1	anty HA	Mouse MAb anty HA H1N1 Sino Biological Inc. 1:1000	+++	–	–	NT	+++	NT	–	–	++	–	–	–
H1N1	anty HA	Mouse MAb anty HA H1N1 Santa Cruz Biotech. 1:1000	+++	–	–	NT	+++	–	–	–	+++	+	–	–
H1N1	anty HA	Monospecific Rabbit PAb 1:500	+++	+	–	NT	+++	NT	–	–	NT	NT	NT	NT
H1N1	anty H1N1 avian influenza	Chicken PAb National Veterinary Research Institute PIWet 1:500	NT	NT	NT	NT	++	++	–	–	NT	NT	NT	NT
H5N1	anty HA0	Monospecific Rabbit PAb 1:1000	+++	+++	+	NT	+++	+++	+	–	NT	NT	NT	NT
H5N1	anty HA1	Monospecific Rabbit PAb 1:1000	–	+++	–	NT					NT	NT	NT	NT
H5N1	anty HA1/ HA2	Monospecific Rabbit PAb 1:500	+++	+++	+	NT	+++	+++	+	NT	NT	NT	NT	NT
H5N1	anty H5N1 — reference	Chicken PAb VLA 1:1000	+++	+++	–	NT	+++	+++	–	NT	NT	NT	NT	NT
H5N1	anty HA	Mouse MAb anty HA H5N1 #11048–MM01 Sino Biological Inc. 1:1000	+	–	–	–	+++	–	–	–	–	+	–	–
H5N1	anty HA	Mouse MAb anty HA H5N1 #11048–MM06 Sino Biological Inc. 1:1000	+++	–	–	–	NT	+++	–	–	NT	NT	NT	NT
H5N1	anty HA	Mouse MAb anty HA H5N1 #11048–MM10 Sino Biological Inc. 1:1000	–	–	–	–	NT	–	–	–	NT	NT	NT	NT
H5N2	anty H5N2 — reference	Chicken PAb VLA # RAA7003 1:500	+++	+++	–	NT	++	+++	–	–	NT	NT	NT	NT
H5Nx	anty H5Nx	Chicken PAb National Veterinary Research Institute PIWet 1:500	+	+	–	NT	+++	++	–	–	NT	NT	NT	NT

NT, not tested; –, negative reaction; +++, the strongest positive reaction; +, the weakest positive reaction

sult of a proteolytic cleavage by insect cells protease — furin (Fig. 2b). The recombinant HA stalk protein was also detected using anti-HA0 H5N1 rabbit polyclonal serum (1:800, obtained in our laboratory). We detected HA stalk protein mainly in the membranes which is similar to cell localization of full length HA (Fig. 3). In western blot analysis under reducing conditions we observed three major bands of different forms of recombinant HA stalk protein. The observed three forms of expressed recombinant protein most likely represent: HA stalk composed of N- and C-terminal parts of HA1 subunit plus the whole HA2 subunit with molecular mass of 45 kDa, single HA2 subunit (37 kDa) and N- and C-terminal parts of HA1 subunit linked by four glycines (27 kDa).

We wanted to investigate whether absence of the globular head domain would disrupt transport through the Golgi complex to the cell surface. We performed

Table 4. Reactivity of H5N1 HA stalk recombinant protein with antibodies against group 2 hemagglutinins.

Group 2. Hemagglutinins			IPMA				Western Blotting				FACS			
			H5N1 antigen				H5N1 antigen				H5N1 antigen			
		Antibody	Bac HA stalk	Bac HA full	Bac WT	Mock (*Sf9*)	Bac HA stalk	Bac HA full	Bac WT	Mock (*Sf9*)	Bac HA stalk	Bac HA full	Bac WT	Mock (Sf9)
H3N2	anty HA	Mouse MAb Sino Biological Inc. # 11056–MM03 1:1000	–	–	–	–	NT	NT	NT	NT	+	–	–	–
H7N9	anty HA	Rabbit PAb Sino Biological Inc. #40104–RP02 1:1000	–	–	–	–	NT	NT	NT	NT	NT	NT	NT	NT
H7N9	anty HA	Mouse MAb Sino Biological Inc. #11082–MM04 1:1000	–	–	–	–	NT	NT	NT	NT	++	–	–	–
H7NX	anty H7Nx avian influenza	Chicken PAb National Veterinary Research Institute PIWet 1:500	++	+	–	NT	+	–	–	–	NT	NT	NT	NT
H4N6	anty HA	Rabbit PAb Sino Biological Inc. #11714–RP01 1:1000	++	–	–	–	NT	NT	NT	NT	NT	NT	NT	NT

NT, not tested; –, negative reaction; +++, the strongest positive reaction; +, the weakest positive reaction

fluorescence-activated cell sorter analysis of infected insect cells with recombinant baculoviruses (Bac HA, Bac HA stalk) following surface staining with HA specific antibodies (Table 3). We could detect the homotrimeric form of HA stalk protein (130 kDa) in the membrane fraction (Fig. 4a) and on the surface of the infected cells (Fig. 4c), which indicates that disulfide bond formation between Cys 52 and 277 is probably conserved among all avian and human influenza viruses.

Production of hyperimmune antiserum in rabbits

Analyses of the human antibody repertoire after infection with pandemic H1N1 strain, revealed presence of

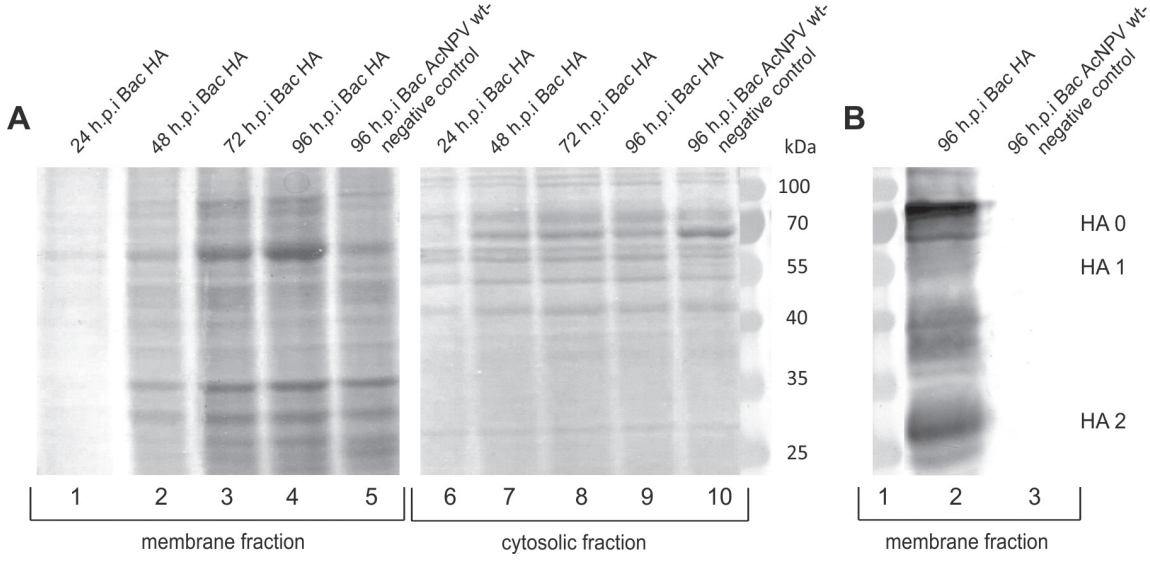

Figure 2. Analysis of the time course of protein production of Influenza Virus H5N1 full length hemagglutinin in insect cells .
Cell membrane fraction and cytoplasm were examined by SDS-PAGE under reducing conditions (0.5×10^6 cells/well). The fractions were collected in 24, 48, 72 or 96 hours post infection. (**A**) 10% SDS-PAGE gel stained with Coomassie blue-R 250. Lane 1, 2, 3, 4, 5 insoluble fraction; Lane 6, 7, 8, 9, 10, soluble fraction. (**B**) Western blot analysis of rHA H5N1 using monoclonal antibodies anti-HA H5N1 (1:1000 Sino Biological Inc.) HA0 — 70 kDa; HA1 — 53 kDa; HA2 — 30 kDa; Lane 1 Protein Ladder — PageRuler Prestained Protein Ladder (Fermentas); Lane 2 insoluble fraction; Lane 3 insoluble fraction of negative control.

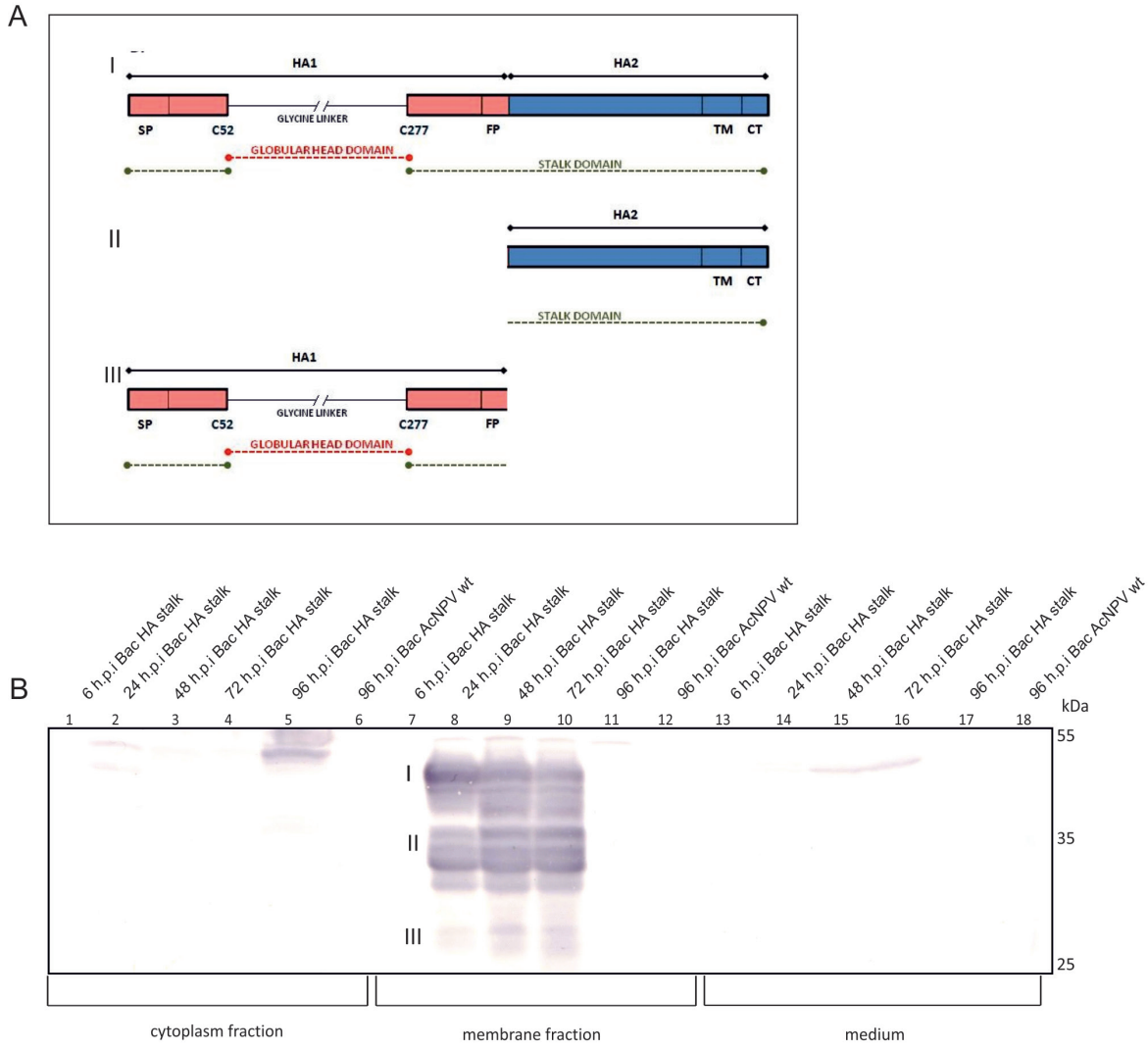

Figure 3. Analysis of the production time course of Influenza Virus H5N1 hemagglutinin stalk domain in insect cells.
Cell membrane fraction, cytoplasm and medium were examined by SDS-PAGE under reducing conditions (0.5×10^6 cells/lane). Fractions were collected in 6, 24, 48, 72 or 96 hours post infection. (**A**) Schematic description of three different forms of HA stalk protein. (**B**) Western Blot analysis of the hemagglutinin stalk domain expressed in Sf9 cells was performed. Anti-HA0 H5N1 rabbit polyclonal serum (1:800 dilution; anti-HA0 H5N1) was used to detect the recombinant protein — HA stalk.

broadly neutralizing antibodies against influenza hemagglutinin.

With the purpose of investigation whether the vaccination with full length recombinant hemagglutinin from H5N1 strain elicits similar antibodies recognizing the conserved epitopes localized in the HA stalk domain we decided to examine sera that were obtained earlier (unpublished data).

Rabbits were immunized with recombinant full-length HA obtained in different forms: uncleaved HA0 protein and two subunits HA1/HA2 together. The sera obtained had high titers of antibodies against full length HA when tested in Western blotting, comparable to the ones commercially available. (Fig. 5). As shown in Fig. 3, the anti-HA0 H5N1 serum reacted with linear epitopes localized in the conserved HA stalk region (HA2 subunit). Positive results were also obtained for conformational antigen – HA stalk in IPMA test (Fig. 6).

As many scientist described the cross reactivity of antibodies raised against a pandemic recombinant hemagglutinin from H1N1 strain with different HAs, here we report for the first time the reactivity of antibodies to HA0 H5N1 with conserved region of HA2 subunit (H5N1 HA stalk). We suppose that specific structure of pandemic HA H1N1 triggers the production of antibodies recognizing conserved epitopes but in this study we present that the HA H5N1 also stimulates production of antibodies that recognized universal epitopes (Table 3).

Reactivity of HA stalk with different antibodies

Expression of the recombinant H5N1 HA stalk protein was confirmed by SDS-PAGE and Western blot as described above. Recombinant protein was characterized by its reactivity with various antibodies in the IPMA test, confirmed by Western blotting and FACS (Tables 1–4). Exposure of the HA stalk domain from the avian influenza virus H5N1 strain resulted in higher reactivity with different antibodies directed against various hemagglutinins.

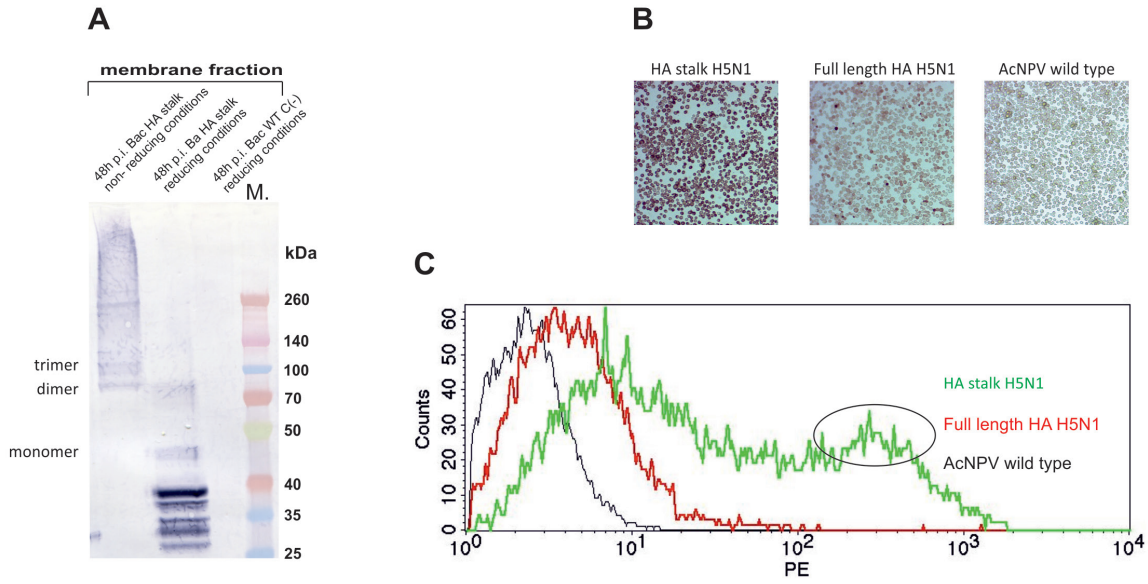

Figure 4. Example of analysis of HA stalk H5N1 recombinant protein reactivity with different antibodies (anti-H1N1 MAbs) in Western blotting (A), IPMA (B) and FACS (C).
(**A**) Western blotting analysis of cytosolic and membrane fractions containing HA stalk recombinant protein with H1N1 MAbs. Sf9 cells were infected with recombinant baculovirus HA stalk with m.o.i. = 1 and collected after 48 h post infection. Cells were lysed and soluble and insoluble fractions were separated under reducing and non-reducing conditions. Western blotting was performed in order to examine the reactivity with H1N1 antibodies (1:1000 Sino Biological Inc.). (**B**) Cells were seeded in a 12-well culture plate and infected with recombinant baculovirus HA stalk with m.o.i. = 1. After 48h IPMA test was performed using anti-H1N1 MAbs (1:1000 Sino Biological Inc.). (**C**) Cells were seeded in a 6-well culture plate and infected with recombinant baculovirus HA stalk with m.o.i. = 1. After 48h cell surface expression of HA stalk recombinant proteins was determined using anti-H1N1 MAbs (1:1000 Sino Biological Inc.) by indirect immunofluorescence and analyzed using the FACS Calibur flow cytometer.

We observed that HA stalk recombinant protein reacted with polyclonal antibodies raised against influenza virus type A strain H5N1, against HA2 subunit of H5N1, against H5N1 reference strain, H5N2 VLA and H5Nx chicken polyclonal antibody.

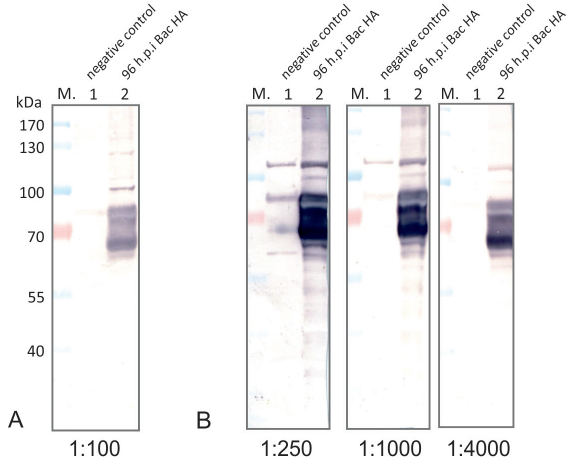

Figure 5. Western blot analysis of rHA H5N1 using polyclonal serum obtained after immunization of a rabbit with purified recombinant HA0 protein.
A rabbit was immunized with recombinant HA0 protein purified from PVDF membrane. First immunization was with 100 µg of protein and the second after 3 weeks with 100 µg of protein. (**A**) reference anti-H5N1 from VLA, (**B**) anti- rHA- H5N1; Western Blot analysis of the reactivity of obtained polyclonal serum with different recombinant antigens. Lane M — Protein Ladder, lane 1 — negative control — WT AcNPV, lane 2 — recombinant full length HA (70 kDa), membrane fraction after 96 h.p.i.

HA stalk was able to react with rabbit polyclonal as well as mouse monoclonal antibodies against H1N1 pandemic strain. In full length hemagglutinin, HA2 subunit is thought to be masked by the membrane-distant portion of HA1 subunit, the globular head domain. Thus, epitopes localized in the stalk domain were not recognized by anti H1N1 mAbs.

H5N1 HA stalk protein also reacted with antibodies against HAs from phylogenetic group 2 (Tables 2 and 3) which implies that the antigen described in this study contains the conserved, probably universal epitopes.

Reactivity of HA stalk with universal human neutralizing antibody FI6

FI6 antibody recognizes all subtypes of the influenza A virus hemagglutinin. As the FI6 antibody recognized only conformational epitopes we decided to investigate its reactivity with HA stalk antigen. In the IPMA test we examined whether recombinant H5N1 HA stalk protein reacts with FI6 human neutralizing antibody. FI6 specifically recognized HA stalk protein (Fig. 6).

DISCUSSION

Influenza virus causes 250 000–500 000 deaths worldwide annually. Potential global pandemic could kill millions (Kang *et al.,* 2012). Current seasonal influenza vaccines are designed to provide strain-specific protection against two circulating subtypes of influenza A virus (H1N1 and H3N2) and one influenza B virus (Doyle *et al.,* 2013). The rapid spreading of the 2009 pandemic H1N1 influenza virus was a signal that universal influenza vaccines are necessary, so that they would broadly protect against many mutated strains (Lu *et al.*, 2014).

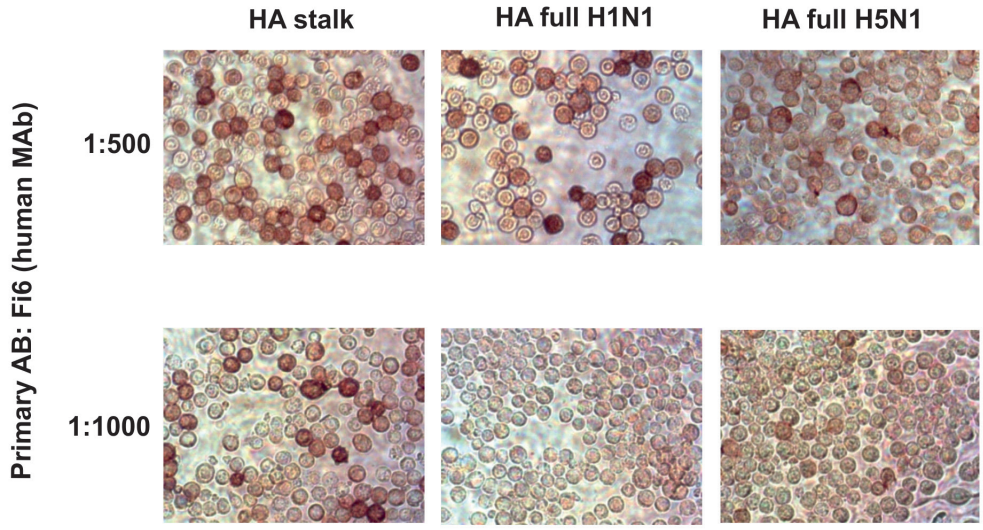

Figure 6. Analysis of immunoreactivity of H5N1 HA stalk recombinant protein with conformational, universal human FI6 MAb. Cells were seeded in a 12-well culture plate and infected with recombinant baculovirus HA stalk with m.o.i. = 1. After 48h IPMA test was performed using anti-FI6 MAbs dilutions 1:500,1:1000.

Current efforts for development of a universal influenza virus vaccine are mostly based on two strategies: the application of new approaches for delivery of existing antigens constructs (nanoplatforms) and the design of novel immunogens. Because of their great potential of cross-reactivity, HA stalk domain, the M2 protein and neuraminidase were identified as the universal antigenic targets (Pica & Palese, 2013).

Recent studies describe the expression and characterization of HA stalk domain from human influenza viruses produced in mammalian cells (Steel *et al*., 2010; Krammer *et al*., 2012a). Based on current knowledge we decided to express the recombinant hemagglutinin stalk domain gene from H5N1 influenza A virus in the baculovirus system with the aim to determine its potential as a universal antigen for vaccine design. To produce homotrimeric "headless" HA in insect cells, we retained Cys 52 and Cys 277 (H3 numbering) (Steel *et al.,* 2010).

Cleavage site of the headless HA of HPAI strain was recognized by insect cells proteases. We observed three major bands most likely representing three forms of the expressed recombinant protein: HA stalk composed of N- and C-terminal parts of HA1 subunit plus all of HA2 subunit, single HA2 subunit and N- and C-terminal parts of HA1 subunit linked by four glycines.

The universal HA stalk antibodies and "headless" HA protein from H1N1 strain obtained in mammalian cells are currently being studied. (Steel *et al*., 2010; Krammer *et al*., 2012a). Therefore, we planned to investigate whether the H5N1 "headless" construct produced in insect cells would also be recognized by different antibodies. We assumed that hemagglutinin of the avian viruses would probably contain conserved epitopes which were previously described for hemagglutinins of human Influenza viruses (Krammer *et al*., 2012b). Our results show that recombinant H5N1 HA stalk domain cross-reacts with various antibodies raised against group 1 and group 2 HAs, which confirms this hypothesis.

Previously, it has been shown that infection in humans with the pandemic swine influenza virus induces antibodies with specificity to the stalk domain of the viral hemagglutinin (Krammer *et al*., 2012a). In accordance with this result we observe the strongest immunoreactivity of pandemic H1N1 antibodies with the HA stalk domain, which suggests that HA from H1N1 has a distinct structure that results in intensive production of antibodies that recognize conserved epitopes within HA stalk domain. We also planned to test whether immunization of rabbits with full length H5N1 hemagglutinin would elicit the anti-stalk antibodies. The results achieved demonstrate that obtained polyclonal antibodies reacts with recombinant hemagglutinin stalk domain protein, confirming that a pool of antibodies against HA stalk domain is raised after immunization with H5N1 influenza.

Our results showing that HA H5N1 vaccination elicits cross-reactive stem-directed antibodies were in accordance with those of Whittle JR and co-workers (Whittle *et al.,* 2014).

In the last few years many research groups were involved in the identification and characterization of the universal antibodies that recognize different HAs (Corti *et al*., 2011). The HA stalk domain is responsible for fusion of viral and host membranes so that the virus can enter human cells. The FI6 antibody makes extensive contacts with conserved epitopes of the stalk, preventing viral entry (Lu *et al.,* 2014). We were curious whether the lack of globular head may have an impact on the efficacy of FI6 binding. In this report we show that exposure of HA stalk domain results in elevated immunoreactivity with FI6 as compared to full length HA (H5N1 as well as H1N1) (Fig. 6). Therefore, we suggest that in the absence of immunodominant globular head domain, the vaccination with HA stalk may elicit larger pool of antibodies against universal epitopes localized in the HA2 subunit.

Summary, there is an urgent need for formulation of universal vaccine against influenza virus. Many scientists investigate which antigen has the ability to give a broad rage protection. Our results confirm the presence of universal epitopes within the HA2 subunit in the stalk domain. It leads us to conclusion that with properly engineered HA stalk antigen we may obtain a potentially universal antigen.

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