Design and Structure of an Engineered Disulfide-Stabilized Influenza Virus Hemagglutinin Trimer

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We engineered a disulfide-stabilized influenza virus hemagglutinin (HA) trimer, termed HA₃-SS, by introducing cysteine residues into the HA stem to covalently bridge the three protomers. HA₃-SS has increased thermostability compared to wild-type HA, and binding of head- and stem-targeted antibodies (Abs) is preserved; only minor structural changes are found in the vicinity of the additional disulfide. This platform has been applied to H1 and H3 HAs and provides prospects for design of intact, stabilized influenza virus HA immunogens.

Influenza viruses cause significant and unpredictable human disease on an annual basis. To combat seasonal infections, vaccination remains the best countermeasure. However, due to continual antigenic drift of circulating viruses, the vaccine formulations require nearly annual updates to match the circulating strains that are predicted to infect humans that year. The vaccines are composed of a combination (tri- or tetravalent) of different subtypes and types of the influenza virus hemagglutinin (HA) surface glycoprotein, which is the primary target of the adaptive immune response. Recent discoveries of broadly neutralizing antibodies (bnAbs) against the HA have advanced knowledge in the field and have provided renewed optimism for discovery of a universal influenza vaccine (reviewed in references 1 and 2).

The HA is a type I fusion glycoprotein and is the major surface glycoprotein on influenza viruses (3). It is synthesized as a single polypeptide precursor protein (HA0), and three copies assemble into a noncovalent trimer. Host proteases cleave HA0 to generate the mature prefusion HA (HA1 or HA2), which is sensitive to low pH and hence metastable. The globular HA “head” is composed of HA1 residues and contains the receptor binding sites, whereas the helical HA “stem” that houses the fusion machinery is made up of HA2 and some HA1 residues. The HA contains six intraprotomer disulfide bonds, which include four HA1-HA1, one HA2-HA2, and one HA1-HA2 linkages (Fig. 1A).

The HA from the 2009 H1N1 pandemic strain has a propensity to dissociate into monomers (4–6), and this instability has been linked with subpar immune response in vaccines (7). As such, creating a more stable, trimeric HA immunogen may enhance elicitation of a protective antibody response. This notion has been demonstrated for the respiratory syncytial virus (RSV) viral glycoprotein, where a combination of cavity-filling mutations and an introduced disulfide stabilized its prefusion antigenic structure (8). In addition, human immunodeficiency virus type 1 (HIV-1) Env glycoprotein prefusion trimers have been successfully engineered, through addition of a disulfide between gp120 and gp41, and properly display neutralizing epitopes, thereby giving promise as vaccine candidates (9). Disulfides have also been incorporated into the measles F glycoprotein and inhibit its fusion activity (10). Dissociation of the influenza virus HA protomers has also been remedied by introducing disulfides on the HA head (6). Here, we report an HA that was stabilized by introducing a novel disulfide into the HA stem to link neighboring protomers while preserving its antigenic structure.

Two cysteine residues were incorporated in the HA stem at HA1 residue 30 and HA2 residue 47 (H3 numbering) in the H1N1 A/Puerto Rico/8/1934 (PR8/H1) and H3N2 A/Hong Kong/1/1968 (HK68/H3) strains, which we term HA₃-SS (Fig. 1A). These residues are in close proximity between neighboring HA protomers and are located in a γ -turn of HA1 and in the A-helix of HA2; the C β atoms are ~ 4.4 Å apart (PDB code 4FNK [11]), which is stereochemically suitable for disulfide formation (12). The mature wild-type (wt) and HA₃-SS HAs were produced in insect cells, as previously described (13), and the two HAs have similar expression profiles and elute at identical elution volumes by gel filtration. No oxidizing agents were added at any point during purification. Under reducing conditions, wt HA and HA₃-SS dissociate into their HA1 and HA2 subunits by SDS-PAGE (Fig. 1B). However, under nonreducing conditions, the HA₃-SS runs at a higher molecular weight corresponding to three times that of wt HA, suggesting that it is completely converted to a disulfide-linked species (Fig. 1B). These results show that the introduced cysteine residues spontaneously form disulfide bridges between the HA protomers of the trimer.

To confirm the location of the disulfide bond between the HA protomers, the crystal structure of the HK68/H3 HA₃-SS was determined at 3.0-Å resolution (see Table S1 in supplemental material). The asymmetric unit of the crystal contains three HA copies that form a biological trimer (Fig. 2A). The crystal structure reveals that the incorporated cysteine residues indeed link the HA

Received 25 March 2015 Accepted 25 April 2015

Accepted manuscript posted online 29 April 2015

Citation Lee PS, Zhu X, Yu W, Wilson IA. 2015. Design and structure of an engineered disulfide-stabilized influenza virus hemagglutinin trimer. *J Virol* 89:7417–7420. doi:10.1128/JVI.00808-15.

Editor: R. W. Doms

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This is The Scripps Research Institute manuscript number 29057.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/JVI.00808-15>.

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doi:10.1128/JVI.00808-15

FIG 1 Design and SDS-PAGE analysis of the engineered HA₃-SS. (A) Schematic of the engineered HA₃-SS. Connecting black lines under the HA1 and HA2 boxes indicate the six native intraprotomer disulfide bonds. The thick lines above the boxes indicate the incorporated cysteines at positions HA1 30 and HA2 47. SH, cysteine. (B) SDS-PAGE analysis of the mature PR8/H1 and HK68/H3 wild-type HA and trimerized HA₃-SS under reducing and non-reducing conditions. Each lane contains 5 μg of each HA or HA₃-SS. βME, 2-mercaptoethanol.

protomers as designed (Fig. 2B). The overall structure of the HA₃-SS is very similar to the wt prefusion HA structure (PDB code 4FNK; HA trimer Cα root mean square deviation [RMSD] of 1.0 Å). However, some minor local structural deviations occur at the top of the HA2 A-helix and B loop (HA2 residues 50 to 58); HA₃-SS loses one turn at the carboxyl end of the A-helix (Fig. 2C). Although no structurally characterized antibodies are known to directly interact with these residues on the HA stem, we assessed if the antigenic structure of the HA₃-SS is perturbed for the PR8/H1 and HK68/H3 strains as well as for the H1N1 A/California/04/2009 strain by measuring antibody affinities to the HAs by Bio-Layer interferometry (Table 1). The binding affinities of conformation-interferometric HA head- or stem-targeted bnAbs (14–18) to wt HA and HA₃-SS are equivalent and, thus, show that the antigenic structure and broadly neutralizing epitopes are maintained.

To investigate if the engineered disulfides provided additional

FIG 2 Crystal structure of the HK68/H3 HA₃-SS. (A) Overview of the HA₃-SS structure. One of three engineered disulfides in the HA stem is circled in dashed lines and is shown as spheres. Each HA protomer is colored red, blue, or green. (B) Top view of the HA₃-SS, with the disulfides displayed as spheres. (C) Zoomed-in view of a disulfide bridge that connects HA protomers. The wt HA is overlaid and colored gray and shows some minor structural deviations at the top of the A-helix connecting to the B loop.

stability to HA₃-SS, the melting temperatures (T_m) of these constructs were measured by differential scanning calorimetry. The introduction of the disulfides to PR8/H1 HA increased the T_m from 61.2°C to 63.5°C (Fig. 3A). More significantly, the HK68/H3 HA₃-SS increased the thermal transition from 61.5°C to 72.8°C, a difference of 11.3°C (Fig. 3B). These data demonstrate that the disulfide-stabilized HA₃-SS is more thermostable than wt HA, particularly for the H3 subtype. The native H1 and H3 HA structures have differences in the positioning of the head group and fusion peptides, which have been suggested to correlate with differences in the stability of the HAs (19, 20). Thus, the disulfide of the HA₃-SS may be more structurally favorable for H3 than for H1 HAs.

The pH stability of the HA₃-SS HAs was also assessed, using protease susceptibility assays (13), to test if the design prevents the large rearrangements triggered at low pH which transform the metastable prefusion conformation to a postfusion structure. Al-

TABLE 1 Comparison of antibody binding affinities of wild-type and disulfide-stabilized HAs^a

Fab fragment	Epitope	K_d (nM)					
		PR8/H1 wt HA	PR8/H1 HA ₃ -SS	Cali04/H1 wt HA	Cali04/H1 HA ₃ -SS	HK68/H3 wt HA	HK68/H3 HA ₃ -SS
5J8	Head	—	—	10 ^b	9	—	—
CR6261	Stem	0.6	0.3	0.6	0.8	—	—
CR9114	Stem	0.2	0.1	0.4	0.2	33	40
F045-092	Head	—	—	—	—	31	27
CR8043	Stem	—	—	—	—	2.5	1.2

^a K_d , dissociation constant; —, no detectable binding.

^b The Cali04 HA contains an engineered, HA trimer-stabilizing HA2 Glu47Gly mutation (14).

FIG 3 Differential scanning calorimetry (DSC) studies of the wt and HA₃-SS HAs. The melting profiles show that HA₃-SS has higher thermostability than wt HA for the PR8/H1 (A) and HK68/H3 (B) strains. The raw data are in black, and the fitted curves from which the T_m values were calculated using a non-two-state model are in red. T_{onset} indicates the temperature at which the peak can first be differentiated from baseline, and the $T_{1/2}$ value is the width at half of the peak height. Cp, heat capacity.

though the fusion transition for HK68/H3 HA₃-SS is stabilized by 0.2 pH units, the disulfides have no such pH-stabilizing effect for PR8/H1 HA₃-SS as it is still susceptible to protease degradation after exposure to low pH (data not shown). In contrast, engineered disulfides that bridge HA head protomers have been shown to inhibit membrane fusion activity (21). Thus, as the experiment detects susceptibility to proteases at low pH, it appears that the introduced disulfide does not protect the HA against trypsin at acidic pH. However, these proteolysis results do not directly demonstrate whether HA₃-SS retains fusion competency. Since the disulfide bond is formed within the A-helix, which is dramatically rearranged between the pre- and postfusion forms (22), it is unlikely that the HA₃-SS can fully rearrange and extend to form the native postfusion six-helix bundle. Membrane fusion assays using HA-transfected cells (23) or fluorescently labeled viruses (24) would better assess the fusion competency of the HA₃-SS.

Recently, many groups have been creating “headless” HA constructs to focus bnAb elicitation against the highly conserved stem (25–30). However, since the stem domain can spontaneously adopt the postfusion conformation in the absence of the HA head (31), removal of the head component may impact the integrity of

some of these immunogens. Incorporation of the disulfide bridges described in this study, as well as potential space-filling mutations, may help stabilize the native, trimeric, prefusion HA stem conformation. Thus, this protein-engineering design can be utilized as a platform for the stabilization and trimerization of HA immunogens.

Protein structure accession numbers. The atomic coordinates and structure factors of the HK68/H3 HA₃-SS have been deposited in the Protein Data Bank (PDB) under accession code [4ZCJ](#).

ACKNOWLEDGMENTS

We thank Henry Tien and Dagart Allison of the Robotics Core at the JCSG for automated crystal screening, the staff of the Advanced Photon Source (APS) GM/CA CAT beamline 23ID-D for support, and Marc Elsliger and Robyn Stanfield for computational and crystallographic support.

The work was funded by NIH R56 AI099275 (to I.A.W.) and T32AI007244 (to P.S.L.). The GM/CA CAT has been funded in whole or in part with federal funds from the National Cancer Institute (Y1-CO-1020) and the National Institute of General Medical Sciences (Y1-GM-1104). Use of the Advanced Photon Source was supported by the U.S.

Department of Energy, Basic Energy Sciences, Office of Science, under contract no. DE-AC02-06CH11357.

The content of this publication is solely our responsibility and does not necessarily represent the official views of NIGMS or the NIH.

We declare that we have no competing financial interests.

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