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Short communication

A novel linear epitope crossing Group 1 and Group 2 influenza A viruses located in the helix A of HA2 derived from H7N9

Zhanping Li[a,b,c,d,1], Zhimin Wan[a,b,c,1], Tuofan Li[a,b,c], Quan Xie[a,b,c], Haiwei Sun[e], Hongjun Chen[e], Guangchen Liang[a,b,c], Hongxia Shao[a,b,c,d], Aijian Qin[a,b,c,d,*], Jianqiang Ye[a,b,c,d,*]

[a] Key Laboratory of Jiangsu Preventive Veterinary Medicine, Key Laboratory for Avian Preventive Medicine, Ministry of Education, College of Veterinary Medicine, Yangzhou University, Yangzhou, Jiangsu, 225009, China
[b] Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou, Jiangsu, 225009, China
[c] Joint International Research Laboratory of Agriculture and Agri-Product Safety, The Ministry of Education of China, Yangzhou University, Yangzhou, Jiangsu, 225009, China
[d] Institutes of Agricultural Science and Technology Development, Yangzhou University, Yangzhou, Jiangsu, 225009, China
[e] Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, 518 Ziyue Road, Shanghai 200241, China

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ABSTRACT

In this research, four monoclonal antibodies (mAbs) were first generated as an immunogen by using the GST fusion protein that carries the fusion peptide and helix A derived from H7N9 influenza A virus (IAV). These mAbs could react with HA of H7N9, H3N2, and H9N2 with neutralizing activity. A novel linear epitope recognized by these mAbs was identified by peptide-based ELISA, and this epitope was located in TAADYKSTQSAIDQITGKLN at the C terminus of the helix A of H7N9. 3 A11, which is one of the four mAbs, could efficiently recognize the corresponding epitopes derived from H9, H7, H5, H3, and H1. Analysis of sera against the corresponding epitope from different HAs revealed that the C terminus of helix A in H9, H7, and H3 possessed dominant B cell epitopes that cross both Group 1 and Group 2 IAV, whereas the C terminus of helix A in H5 possessed only dominant B cell epitopes that cross subtypes in Group 1 virus. All these results demonstrated that the linear epitope identified in the helix A of H7N9 could be a novel target for developing broad-spectrum influenza diagnostics or vaccine candidates.

1. Introduction, methods, and results

Although vaccination and antiviral drugs significantly reduce the burden of influenza disease, seasonal human influenza epidemics, avian influenza endemics, and occasional human influenza pandemics continue to be a threat for both human health and the sustainable development of animal husbandry because of the antigenic variation and genome reassortment of influenza A virus (IAV) (Noda, 2012). On the basis of the two major surface antigenic proteins HA and NA, IAV can be currently subtyped into 18 HA and 11 NA serotypes, respectively (Tong et al., 2012, 2013). All these 18 HAs could be further clustered into two structurally and antigenically different groups (Group 1 and Group 2) (Russell et al., 2004). Notably, current commercial vaccines of IAV are strain specific and show only limited protective efficacy against the emerging strains with antigenic drift or shift (Russell et al., 2008; Osterholm et al., 2012). In addition, the frequent emergence of drug-resistant IAV narrows the therapeutic windows for the current anti-influenza drugs (Moscona, 2005; Dharan et al., 2009; Baz et al., 2009). Therefore, a cross-protective or universal vaccine against all 18 HAs or both Group 1 and Group 2 IAV is highly required for protection against the epidemics or pandemics of influenza viruses in both human and animals. To identify the cross-protective epitopes among these multiple subtypes of IAV is critical for the development of efficient broad-spectrum vaccines against IAV. For this goal, several neutralizing monoclonal antibodies (mAbs) cross-reacting with Group 1 or Group 2 IAV or with both Groups have been generated (Corti et al., 2010; Sui et al., 2009; Throsby et al., 2016; Wrammert et al., 2011; Dunand et al., 2015; Ekiert et al., 2011; Friesen et al., 2014; Tan et al., 2014; Corti and

* Corresponding authors at: Ministry of Education Key Lab for Avian Preventive Medicine, Yangzhou University, No.12 East Wenhui Road, Yangzhou, Jiangsu, 225009, China.
E-mail addresses: 462407484@qq.com (Z. Li), 723694341@qq.com (Z. Wan), 2030579924@qq.com (T. Li), 809322407@qq.com (Q. Xie), 845861718@qq.com (H. Sun), vetchj@shvri.ac.cn (H. Chen), 1561181511@qq.com (G. Liang), 987137092@qq.com (H. Shao), aijian@yzu.edu.cn (A. Qin), jqye@yzu.edu.cn (J. Ye).
[1] Equal contribution.

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Lanzavecchia, 2011; Dreyfus et al., 2012; Nakamura et al., 2013; Wu et al., 2015; Kallewaard et al., 2016; Joyce et al., 2016). Studies from these broadly neutralizing antibodies (bnAbs) have revealed that the cross-protective epitopes were mainly located in the fusion peptide and helix A region from the stem of HA. However, these exact epitopes need to be further refined, and little is known whether these epitopes are linear or have conformation.

To identify the potential linear cross-protective epitopes in the fusion peptide and helix A, in this study, a gene fragment for the coding region of the fusion peptide and helix A derived from H7N9 was first amplified using the forward primer 5′GTTCCAGGGGCCCATGGGA AGCggcctatttggtgctat3′ and the reverse primer 5′GTTTTCACCGTCA TTAgtttaattttcctgt3′. In this PCR analysis, the plasmid pDP2002-H7 containing the HA gene from A/Shanghai/1/2013 (H7N9) was used as the template. The PCR product was then cloned into the pGEX-6P-1 vector for analyzing GST fusion expression using a ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China) as previously described (Shao et al., 2015). The positive recombinant confirmed by sequencing was designated as GST-PEP-HEX. The expression of GST-PEP-HEX was then induced by 1 mM IPTG in the transformed BL21 cells incubated for 4 h at 37℃, and protein expression was analyzed by SDS-PAGE. As described in Fig. 1A, a specific band with 33 kD molecular weight corresponding to the size of the GST-PEP-HEX fusion protein could be mainly found in the pellet from the lysates of BL21 cells transformed with GST-PEP-HEX in comparison with the lysates of BL21 cells transformed with the GST vector pGEX-6-P-1. To evaluate the antigenicity of GST-PEP-HEX and generate specific mAbs against the fusion peptide and helix A, purified GST-PEP-HEX was immunized into 6 week old mice at an interval of 10 days for three times. Seven days after the third immunization, the mouse sera were collected for detecting a specific antibody against the fusion peptide and helix A by immunofluorescence assay (IFA). In IFA, DF1 cells transfected with pDP2002-H7 were used as the antigen, and the sera (1:1000 dilution) from mouse immunized with the purified GST-PEP-HEX were used as the primary antibody. As shown in Fig. 1B, the bright and specific fluorescence signals could be found in DF1 cells transfected with pDP2002-H7 but not in the control DF1 cells. This indicated that the fusion peptide and helix A in the GST-PEP-HEX protein generated in this study showed efficient antigenicity.

To generate mAbs for determining B cell epitopes in the fusion peptide and helix A, the spleen cells from the mouse immunized with the purified GST-PEP-HEX protein were fused with sp2/0 cells by a method as previously described (Wang et al., 2018). After selection in the hypoxanthine-aminopterin-thymidine (HAT) medium, the hybridoma cells that secrete antibody specific to the fusion peptide or helix A were screened by IFA. The positive hybridoma cells were then subcloned. In IFA, the 293 T cells transfected with pDP2002-H7 were used as the antigen, and supernatant from the hybridoma cells was used as the primary antibody. Finally, four mAbs, designated as 1 A9, 1H8, 3 A11, and 4D11, showed a strong positive reaction with the 293 T cells transfected with pDP2002-H7 as described in Fig. 2A. The subclass of these four mAbs was IgG2b, which was identified using a mouse mAb isotyping kit (Thermo, Massachusetts, USA) according to the manufacturer's protocol. The mAbs in ascites were generated by a method as previously described and purified using protein G columns (GE Healthcare Life Sciences, Uppsala, Sweden) (Wang et al., 2018). To further confirm the specificity of these mAbs, western blot assay was performed. In the western blot assay, the denatured lysates of 293 T cells transfected with pDP2002-H7 were used as the antigen. The blots in the nitrocellulose (NC) membrane were developed using a fully automatic chemiluminescence image analysis system (Tanon 5200). As shown in Fig. 2B, all these four mAbs could efficiently recognize the HA protein expressed in 293 T cells transfected with pDP2002-H7 but not in the control 293 T cells. Therefore, IFA and western blot assay clearly demonstrated that the four mAbs generated in this study could efficiently react with the linear HA derived from the A/Shanghai/1/2013(H7N9) isolate.

To evaluate the potential cross-strain and cross-group reaction, two IAVs, one from Group 2 (A/Duck/Jiangsu/YZ/2016(H3N2)) and another from Group 1 (A/Chicken/Jiangsu/WS1/2012(H9N2)), were tested by the western blot and viral neutralization assays. In the western blot assay, the lysates of the MDCK cells infected with H3N2 and H9N2 were used as the antigen, and the ascites of the four mAbs (1:1000) were used as the primary antibody. Interestingly, all the four mAbs could efficiently recognize the HA protein in the MDCK cells infected with H3N2 or H9N2 with a different pattern but not in the control MDCK cells as described in Fig. 2C, thus indicating their potential broadly reactive patterns for both Group 1 and Group 2 IAVs. The different reactive pattern obtained in the western blot also indicated that the four mAbs might react with different epitopes or show different affinity to the HA of H3N2 and H9N2.

To test the neutralization assay, 100$TCID_{50}$ of H3N2 or H9N2 viruses were mixed with the serial diluted mAb and the mixture was incubated at 37℃for 1 h. Then, the mixture was inoculated into MDCK cells in 96-well plates. After 2 h post inoculation, the infected MDCK cells were washed with PBS twice and then cultured in opti-MEM with 1ug/ml TPCK-treated trypsin at 37℃for four days. And then the neutralizing titers were measured by hemagglutination assay (HA)

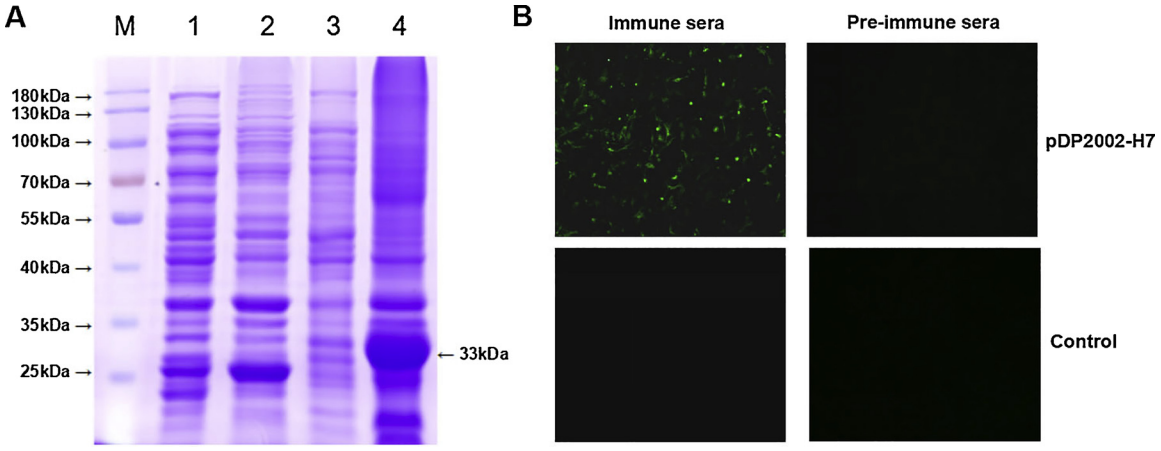

Fig. 1. Expression of the GST-PEP-HEX fusion protein and its antigenicity. (A) SDS-PAGE analysis of the expression of the GST-PEP-HEX fusion protein. Lane M: protein marker; Lanes 1 and 2: the supernatant and pellet of the lysates of BL21 cells transformed with the vector pGEX-6P-1, respectively; Lanes 3 and 4: the supernatant and pellet of the lysates of BL21 cells transformed with recombinant GST-PEP-HEX, respectively; (B) Immunofluorescence assay for polyclonal antibodies from mouse immunized with purified GST-PEP-HEX. a, DF1 cells transfected with pDP2002-H7; b, control DF1 cells.

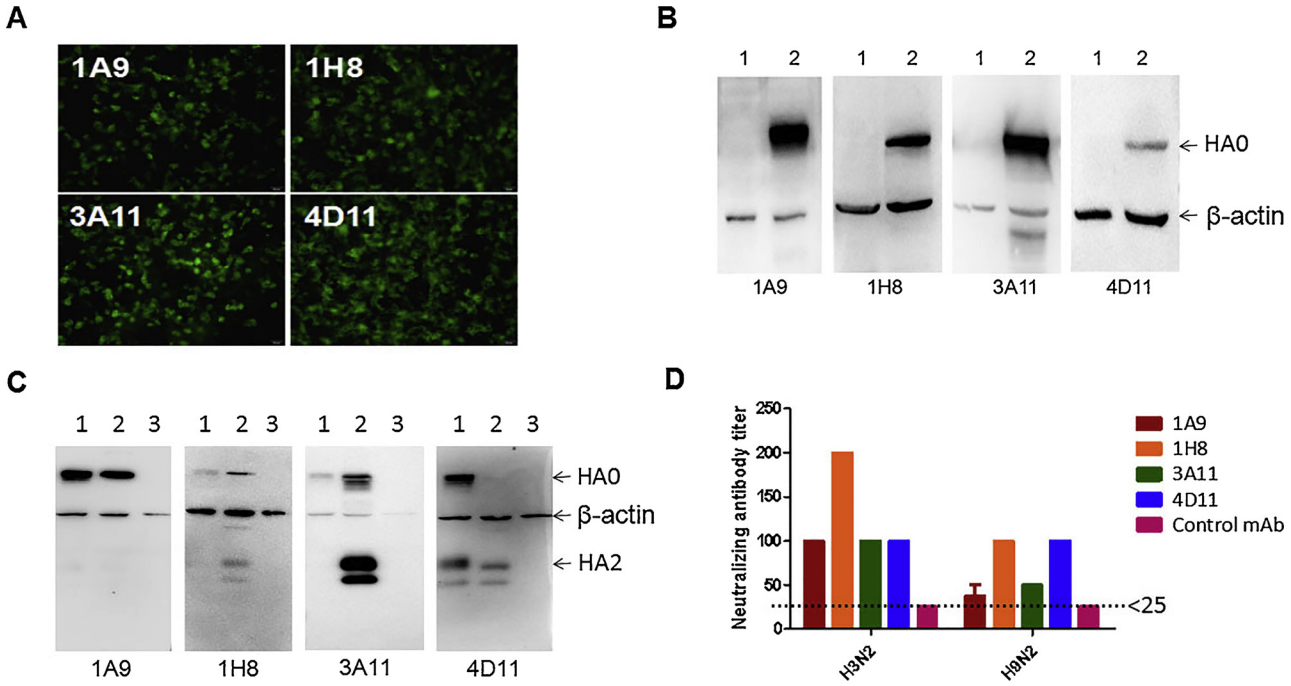

Fig. 2. Characteristic of the four mAbs. (A and B) Immunofluorescence assay and western blotting were performed for the four mAbs using 293 T cells transfected with pDP2002-H7. Lanes 1 and 2: the lysates of control 293 T cells and 293 T cells transfected with pDP2002-H7, respectively; (C) Western blot assay for the four mAbs using MDCK cells infected with H3N2 and H9N2. Lanes 1 and 2: The lysates of MDCK cells infected with H3N2 and H9N2, respectively; Lane 3: The lysates of the control MDCK cells. (D) Neutralizing activity test for the four mAbs.

according to previous report. As described in Fig. 2D, the titer of the neutralizing antibody of mAb 1 A9, 1H8, 3 A11 and 4D11 was 1:100, 1:200, 1:100 and 1:100 for H3N2, and 1:37.5, 1:100, 1:50 and 1:100 for H9N2 respectively whereas that of the control mAb was < 1:25 for both H3N2 and H9N2.

To map the epitopes recognized by the four mAbs, five peptides (designated as SH-PEP1, SH-PEP2, SH-PEP3, SH-PEP4, and SH-PEP5) derived from the amino acids of the fusion peptide and the helix A of HA of H7N9 were synthesized, and the detailed sequences of the five peptides are shown in Fig. 3A. The epitopes of mAbs were determined by ELISA. In ELISA, the five synthesized peptides were coated onto the plate. After blocking with 5% skimmed milk in PBST at 37 ℃ for 60 min, mAbs, with a dilution of 1:1000, were added into the plate, and the plate was incubated at 37 ℃ for 60 min, followed by peroxidase-conjugated goat-anti-mouse IgG at 37 ℃ for 30 min. Finally, 100 μl/well of freshly prepared substrate solution TMB was added for color development; the plate was incubated for 10 min, and then, the reaction was stopped by the addition of 50 μl of 2 M sulfuric acid. The absorbance value at 450 nm (OD450) was measured using an ELISA reader (TECAN Infinite M200 Pro). Surprisingly, all these four mAbs could react with the SH-PEP5 with a high OD450 value but not with the other four peptides tested as shown in Fig. 3A. Interestingly, the polyclonal mouse sera (positive sera) against the GST-PEP-HEX protein showed a high OD450 value with SH-PEP5 and low OD450 values with SH-PEP4, SH-PEP3, and SH-PEP2, but it did not show reaction with SH-PEP1, thus indicating that the fusion peptide does not carry a strong dominant B cell epitope, whereas the helix A of HA2 has such epitope. The location of the sequence of SH-PEP5 (TAADYKSTQSAIDQITGKLN) in the C terminus of the helix A highlights that the C terminus of helix A in the HA of H7N9 possesses dominant, linear, cross-group neutralizing epitopes.

To further test the reaction profiles for these mAbs, four corresponding epitopes SH9-PEP, SH5-PEP, SH3-PEP, and SH1-PEP derived, respectively, from H9, H5, H3, and H1 IAVs were synthesized, and the detailed sequences of the four peptides are shown in Fig. 3C. At the same time, five KLH-conjugated peptides, namely, SH-PEP5-KLH, SH9-PEP-KLH, SH5-PEP-KLH, SH3-PEP-KLH, and SH1-PEP-KLH, were also synthesized. Mice sera against SH-PEP5, SH9-PEP, SH5-PEP, SH3-PEP, and SH1-PEP were generated by immunizing the mice with the corresponding KLH = conjugated peptides. The four mAbs and sera from these mice were tested by peptide-based ELISA using the peptides SH-PEP5, SH9-PEP, SH5-PEP, SH3-PEP, and SH1-PEP. As shown in Fig. 3C, the mAb 3 A11 showed efficient reaction with all these five peptides derived from different HAs with a high OD450 value. The mAbs 1 A9, 1H8, and 4D11 showed efficient reaction with the peptides SH-PEP5 and SH3-PEP. Different from mAb 4D11, mAb 1 A9 and 1H8 also showed reaction with the peptide SH9-PEP. These results further highlighted that the epitopes recognized by these mAbs were variant or their binding affinity was different.

Notably, in comparison with other KLH-conjugated peptides, SH1-PEP-KLH could not induce efficient antibody against either SH1-PEP or other peptides tested, namely, SH-PEP5, SH9-PEP, SH5-PEP, and SH3-PEP, as shown in Fig. 3C, thus indicating low antigenicity of the C terminus of helix A in H1 IAVs. Mice sera against SH-PEP5-KLH, SH9-PEP-KLH, and SH3-PEP-KLH showed efficient reaction with the peptides SH-PEP5, SH9-PEP, SH3-PEP, and SH1-PEP but not with the peptide SH5-PEP, whereas sera against SH5-PEP-KLH showed efficient reaction with the peptides SH9-PEP, SH5-PEP, and SH1-PEP (derived from Group 1) but not with the peptides SH-PEP5 and SH3-PEP (derived from Group 2).

To evaluate the variation in the corresponding sequence of SH-PEP5 in Group 1 and Group 2 IAVs, 18 HAs representing 18 subtypes of IAVs were analyzed using WebLogo and Mega 6 (Crooks et al., 2004; Tamura et al., 2013). The related sequence of SH-PEP5 (TAADYKSTQSAIDQITGKLN) in Group 2 was less variable than that in Group 1 as shown in Fig. 4. The identity of SH-PEP5 in Group 2 was approximately 80–100%, whereas that in Group 1 was 45–95%. Notably, the epitope of the subtypes H1N1, H8N4, H9N2, and H12N5 from Group 1 was clustered together compared with the epitope of those from Group

2. Discussion

The current influenza vaccines mainly elicit strain-specific

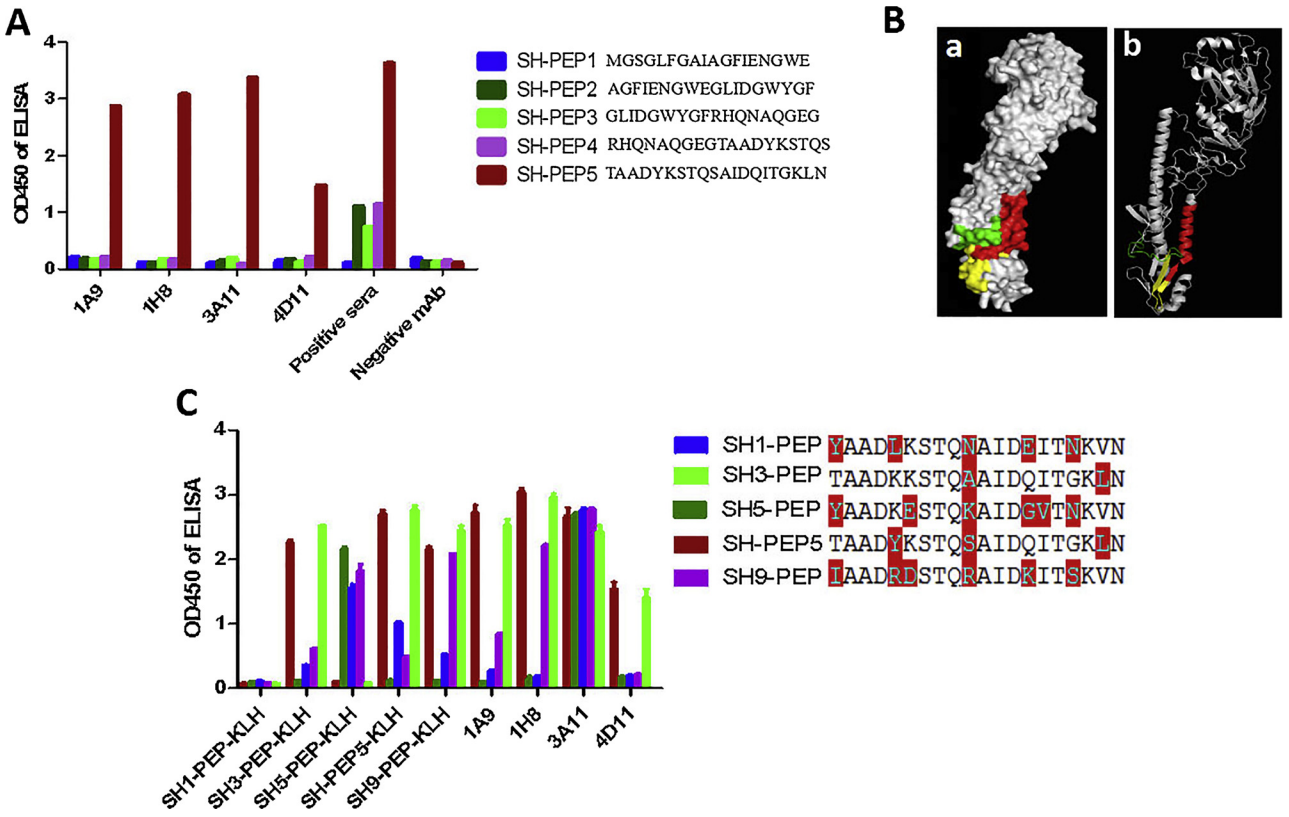

Fig. 3. Epitope mapping and reaction pattern for the four mAbs. (A) The epitopes recognized by the four mAbs were identified by peptide-based ELISA; (B) The location of the epitope recognized by the four mAbs in the HA molecule was determined by PyMOL using the PDB 1TI8 (H7). The green color indicates the sequence of the fusion peptide; the red color indicates the peptide SH-PEP5 in the helix A; the yellow color indicates the N-terminal sequence of the helix A; (C) Reaction patterns of the four mAbs were analyzed using different peptides derived from different HAs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

neutralizing antibodies against the two surface proteins HA and NA and generally show inefficient protection against strains with antigenic drift or shift (Russell et al., 2008; Osterholm et al., 2012). Such influenza vaccines need to be updated annually because of the altered antigenicity of the epidemic influenza viruses. Notably, the updated process for the generation of antigenic-matched influenza vaccine is time consuming and laborious, and hence, it needs international collaboration from the global influenza research centers (Osterholm et al., 2012). To overcome the limitation of the current influenza vaccines, the development of broad-spectrum vaccines against multiple or all subtypes of IAVs is an ideal strategy. Different from the variant and antigenic dominant head part of HA, which is the major target for current vaccines, several regions in the stem of HA are conserved across HA subtypes or two HA groups. The fusion peptide in the HA stem plays a crucial role during viral entry into cells, and antibodies targeting this region could provide broad protection against HA subtypes (Corti et al., 2010; Sui et al., 2009; Throsby et al., 2016; Wrammert et al., 2011; Dunand et al., 2015; Ekiert et al., 2011; Friesen et al., 2014; Tan et al.,

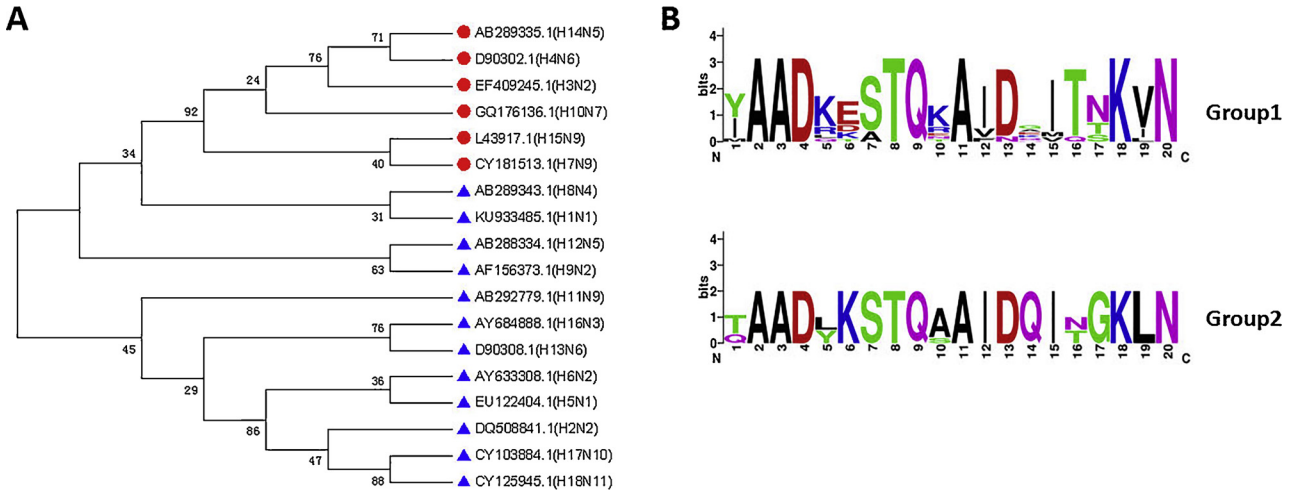

Fig. 4. Variation in epitope in Group 1 and Group 2 influenza viruses. (A) Variation in epitope in Group 1 and Group 2 influenza A viruses was analyzed using WebLogo; (B) Phylogenic tree for the epitope in Group and Group 2 influenza A viruses was constructed using Mega 6.

2014; Corti and Lanzavecchia, 2011; Dreyfus et al., 2012; Nakamura et al., 2013; Wu et al., 2015; Kallewaard et al., 2016; Joyce et al., 2016). Thus, influenza universal vaccine strategies based on the HA stem have been extensively studied (Yewdell, 2013; Margine et al., 2013; Sutton et al., 2017). Although such vaccine candidates could induce cross-protection against heterogenic strains, the neutralizing titers elicited were significantly lower than that induced by the head part of HA due to the weak antigenicity of the stem region of HA. Therefore, identification of such B cell epitopes in the stem region and enhancement of their antigenicity are critical for generating efficient broad-spectrum vaccines against influenza. Recently, the generation of several broadly neutralizing mAbs across HA subtypes has uncovered epitopes that are located in the fusion peptide and helix A region from the HA stem (Corti et al., 2010; Sui et al., 2009; Throsby et al., 2016; Wrammert et al., 2011; Dunand et al., 2015; Ekiert et al., 2011; Friesen et al., 2014; Tan et al., 2014; Corti and Lanzavecchia, 2011; Dreyfus et al., 2012; Nakamura et al., 2013; Wu et al., 2015; Kallewaard et al., 2016; Joyce et al., 2016; Quiñones-Parra et al., 2014). Epitope mapping using these bnAbs reveals that such broadly neutralizing epitopes have mainly conformation dependence.

In this study, a linear neutralizing epitope was found in the helix A region derived from H7N9. This linear epitope could be efficiently recognized by four mAbs (1 A9, 1H8, 3 A11, and 4D11) generated in this study. Peptide-based ELISA revealed that the epitope was located in the C terminus of helix A (TAADYKSTQSAIDQITGKLN, peptide SH-PEP5). To our knowledge, this is the first demonstration of the linear cross-reactive epitope TAADYKSTQSAIDQITGKLN, which is located in the C terminus of the helix A of HA2. Although the epitopes recognized by other bnAbs such as CR6261, CR9114 and MED18852 were also mainly located in the helix A of HA2, previous studies did not point out the exact amino acids in the epitope of the helix A for these mAbs and did not clarify whether the epitope was linear or had conformation dependence (Dreyfus et al., 2012; Kallewaard et al., 2016; Quiñones-Parra et al., 2014). Our study clearly demonstrated that the linear C terminus of helix A with 20 amino acids, represented as TAADYKSTQSAIDQITGKLN, was identified as a novel linear cross-reactive epitope that could be easily engineered into universal vaccine candidates.

Although we did not test all the 18 HA subtypes using these four mAbs, the reaction pattern of these mAbs against different peptides derived from H9, H7, H5, H3, and H1 demonstrated that the mAb 3 A11 could react with all these five peptides derived from H9, H7, H5, H3, and H1, whereas the other three mAbs mainly reacted with peptides derived from H7 and H3 but not with peptides from H1 and H5, thus highlighting the broad reaction pattern of the mAb 3 A11 against both Group 1 and Group 2 HA. It also should be noted that the sera from the mice immunized with the KLH-conjugated peptide SH1-PEP-KLH derived from H1 did not show efficient reaction with the peptides SH-PEP5, SH9-PEP, SH5-PEP, SH3-PEP, and SH1-PEP. In comparison, mice sera against SH-PEP5-KLH, SH9-PEP-KLH, and SH3-PEP-KLH cross-reacted with the peptides SH-PEP5, SH9-PEP, SH3-PEP, and SH1-PEP, whereas sera against SH5-PEP-KLH only reacted with the peptides SH9-PEP, SH5-PEP, and SH1-PEP. All these data highlighted that the C terminus of helix A in H9, H7, and H3 possessed dominant B cell epitopes that cross Group 1 and Group 2, whereas that in H5 only possessed dominant B cell epitopes that cross subtypes in Group 1. The low antigenic activity of the C terminus of helix A in H1 IAV might have contributed to the low cross-reaction with different H1 seasonal influenza vaccines. Therefore, refining and engineering the epitope in TAADYKSTQSAIDQITGKLN identified in H7 in this study might provide novel linear targets for generating peptide-based broad-spectrum influenza vaccine candidates.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

ZL and JY conceived and designed the experiments. ZL, ZW, QX, GL, and HC performed the experiments. ZL, ZW, AQ, HS, and JY analyzed the data. ZL, QX, HC, and AQ contributed reagents/materials/analysis tools. TL and JY contributed to the writing of the manuscript. ZW and JY prepared the figures. All authors read and approved the final manuscript.

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