·基础研究·

以 HA2 为基础的流感病毒候选疫苗同时与 H5N1、H1N1 和 H3N2 亚型毒株产生交叉反应

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摘 要：【目的】探讨以流感病毒(IAV)血凝素蛋白茎部(HA2)保守表位为免疫原来诱导针对不同血清型流感病毒的广谱体液免疫应答。【方法】我们构建了一个重组的 IAV 疫苗 IR30-Fd，由 A/Viet Nam/1203/2004(H5N1)病毒株 HA2 的 30 个保守的氨基酸残基(IR30)和一个连在碳端的三聚体基序(foldon，Fd)组成。原核表达该蛋白，圆二色谱和 Western Blot 验证 IR-30-Fd 的室间结构之后，分别免疫 BALB/c 小鼠和新西兰白兔，并同时免疫 IR30 多肽为对照，制备血清抗体。然后应用 ELISA 和 Western Blot 方法检测抗体的滴度，应用细胞 ELISA 和 Western Blot 方法检测抗体与不同的 IAV 菌株 HA2 蛋白的交叉反应性。【结果】IR30-Fd 能够形成具有 α 螺旋的三聚体空间结构。用 IR30-Fd 和 IR30 免疫 BALB/c 小鼠和新西兰白兔后获得了高效价的抗 IR30 的特异性抗体。鼠抗 IR30-Fd 抗体能与 H3N2 和 H1N1 的 HA 蛋白反应而鼠抗 IR30 抗体不能。鼠抗 IR30-Fd 抗体稀释 6 400 倍、兔抗 IR30-Fd 抗体稀释 8.2×10^5 倍均可以与 H5N1 的 HA 蛋白反应。细胞 ELISA 表明兔抗 IR30-Fd 抗体与天然状态下的 H5N1 和 H3N2 的 HA 蛋白的结合亲和力高于兔抗 IR30 抗体。【结论】本研究结果表明 IR30-Fd 蛋白能够模拟 H5N1 HA2 蛋白保守序列的天然三聚体结构，并且能够诱导产生针对 H5N1 H1N1 和 H3N2 亚型 HA 蛋白的广谱交叉反应抗体，为研制通用的流感病毒疫苗提供了结构基础。

关键词：甲型流感病毒；通用疫苗；HA2；三聚体
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An Influenza A Virus Vaccine Candidate Containing the Conserved Sequence in HA2 of H5N1 Induces Broadly Cross-reactive Antibody Responses to HA of H5N1, H1N1, and H3N2 Subtypes

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Abstract:【Objective】This study aimed to find epitopes in HA2 that can induce broadly cross-reactive antibody responses against divergent influenza A viruses (IAVs).【Methods】We constructed a recombinant IAV vaccine containing a 30 amino acid conserved sequence (IR30) from HA2 of the IAV strain A/Viet Nam/1203/2004(H5N1) plus a trimeric motif (foldon), designated as IR30-Fd, which was expressed in Escherichia coli (E.coli) using prokaryotic fusion protein expression system. The secondary structures of IR30-Fd and IR30 were analyzed by circular dichroism spectroscopy and Western blot. We immunized mice and rabbits with IR30-Fd protein or IR30 peptide (as a control), and detected antibody titers of the sera using ELISA and Western Blot. We then employed cell ELISA and Western blot to determine the cross-reactivity of antibodies against divergent IAV strains.【Results】IR30-Fd can form an

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α-helical trimer.We found that both anti-IR30-Fd and anti-IR30 antibodies were highly effective in binding to IR30. However, mouse anti-IR30-Fd antibodies could interact with HA of H3N2 and H1N1, while anti-IR30 antibodies could not. Mouse anti-IR30-Fd antiserum at the dilution as high as 1:6,400 could react with HA of H5N1. Rabbit anti-IR30-Fd antiserum at the dilution as high as 1:8.2x105 could react with HA of H5N1. Furthermore, rabbit anti-IR30-Fd antibodies showed significantly higher affinity than anti-IR30 antibodies to the native conformation of HA of H5N1and H3N2 expressed on Hela cells.【Conclusion】These results suggest that IR30-Fd, which could mimic the native trimeric form of the conserved sequence in HA2 region of H5N1, was able to induce broadly cross-reactive antibody against HA of H5N1, H1N1, and H3N2 subtypes, thus serving as a basic structure for developing universal influenza vaccines.

Key words: Influenza A virus; universal vaccine; HA2; trimeric

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As the influenza A virus (IAV) has strong ability to evade the immune system by recombination and mutation, the flu vaccines specific for new IAV strains have to be developed before every flu outbreak season, causing a great burden to governments and research scientists worldwide. Even so, nobody can guarantee that the pre-developed vaccines will be effective against the emerging viral strains predominating in the coming flu outbreak season[1]. Nevertheless, several different subtype viral strains often circulate simultaneously in an area at a given time point. Thus, development of a broadly cross-reactive vaccine against different serum subtypes of IAV is urgently needed [2]. It has been reported that the conserved IAV proteins nucleocapsid protein (NP) and matrix protein 2 (M2) can be used for designing broadly protective flu vaccine[3-6]. However, recent evidence suggests that without the combinatorial vaccination of hemagglutinin (HA), neither NP nor M2 alone is insufficient to induce broadly protective immunity, suggesting that HA may serve as the most important target for developing broadly protective flu vaccines[7]. HA, which can be divided into two major phylogenetic groups and further divided into 16 subtypes[8], is initially synthesized as a precursor, HA0, in a trimeric form in the endoplasmic reticulum and transferred through the Golgi apparatus to the cell surface[9]. HA0 is then proteolytically cleaved into the functional subunits, HA1 and HA2, which remain linked by a disulfide bond and associated with each other to constitute the mature HA spike on the viral surface. HA1 as a globular head is responsible for receptor-binding, and HA2 as a stalk structure mediates membrane fusion between the viral envelope and the endosomal membrane of the host cell[10]. The antibodies targeting HA2 stalk can prevent the membrane fusion of IAV with the host cell[11-13] The HA subtypes are classified into group 1 and group 2 based on their antigenicity and their major structural features[14-16]. As the group 1 and group 2 IAVs have similar stalk structure, the stalk has been selected to be a target for designing broadly protective vaccine against IAV infection. Previous studies have shown that the relatively conserved stalk structure of HA can protect mice from IAVs and the antiserum of stalk showed binding affinity to different subtypes of IAVs, indicating the possibility of developing a broadly protective IAV vaccine [17-18]. Recently, Palese and colleagues reported that an HA stalk peptide-based vaccine could induce protection against distinct subtypes of IAVs[18]. This group also identified a broadly-neutralizing mAb against H3 influenza viruses, 12D1, which bound to a continuous region (residue 76-106) of HA2[11]. In this study, we designed a recombinant IAV vaccine, designated IR30-Fd, which consists of a 30-mer conserved sequence (IR30) in the HA2 of H5N1, which corresponds to the continuous epitope of 12D1 in the H3 HA[11], and a trimeric motif, foldon (Fd)[19]. After immunizing mice and rabbit with IR30-Fd or IR30 peptide (as a control), we found that the antibodies against IR30-Fd and IR30 could react with the conserved sequence in HA2 in ELISA and with H5N1 HA and HA2 in Western blot. But unlike IR30, anti-IR30-Fd antibodies could also bind to the HA of H3N2 and H1N1. These findings suggest that

IR30-Fd, which is different from the linear peptide IR30, can form a helical trimer, mimicking the native conformation of HA and induce broadly humoral immune responses against homologous (H5N1) and heterologous (H1N1 and H3N2) subtypes of IAV, providing a structure base for developing broadly protective IAV vaccines.

1 Materials and Methods

1.1 Reagents

Peptide IR30 (residues 77-106 IENLNKKMED GFLDVWTYNAELLVLMENER), which is derived from the HA2 region of A/Viet Nam/1203/2004 (H5N1), was synthesized by GL Biochem (Shanghai, China) using a standard solid-phase fluorenylmethyloxycarbonyl (FMOC) method. The peptide was acetylated at the N termini and amidated at the C termini and was >95% pure by high-performance liquid chromatography (HPLC) and identified by laser desorption mass spectrometry. Eight recombinant HA proteins from different subtypes of IAVs, including A/Anhui/1/2005(H5N1), A/Hong Kong/483/97(H5N1), A/Indonesia/5/2005(H5N1), A/Viet Nam/1194/2004(H5N1), A/Xingjiang/1/2006 (H5N1), A/Brevig Mission/1/1918 (H1N1), A/California/04/2009(H1N1), and A/Aichi/2/1968/(H3N2), and three anti-HA polyclonal antibodies (against influenza viruses A/Anhui/1/2005(H5N1), A/California/04/2009 (H1N1), and A/Brisbane/10/2007/ (H3N2) were purchased from Sino Biological Inc. (Beijing, China).

1.2 Expression and purification of IR30-Fd

The amplified gene encoding IR30-Fd was inserted into the vector pGEX-6P-1 by *Bam*H1 and *Eco*R1, respectively, to generate the expression vector, pIR30-Fd, which was then transfected into the *E. coli* strain BL21(DE3)pLysS. After induction with IPTG overnight, the bacteria was frozen at −20 ℃ and lysed with PBS plus 1% triton X-100 and sonication, followed by centrifugation. The expressed IR30-Fd in supernatants was purified with Glutathione-Sepharose 4B affinity column (Novagen, USA). After incubation with PreScission™ Protease (GE Healthcare, USA) at 4 ℃ overnight, the released IR30-Fd was further purified by fast protein liquid chromatography (FPLC) and precipitation with 70% saturated ammonium sulfate.

1.3 Circular dichroism (CD) spectroscopic analysis

The secondary structure of IR30-Fd and IR30 was analyzed by CD spectroscopy as previously described[20-21]. In brief, IR30-Fd and IR30 were diluted in phosphate buffer solution (PBS, pH 7.2). The CD spectrum of each sample at 10 μmol/L was obtained on a Jascospectropolarimeter (Model J-816, Jasco Inc., Japan) at 20°C with a 5 nm bandwidth, 0.5 nm resolution, 0.1 cm path length, and an average time of 5.0 s. Correcting the spectra was applied by subtraction of a blank corresponding to the solvent composition of each sample.

1.4 Immunization of mice and rabbits with IR30-Fd and IR30

BALB/c mice (6–8 weeks old, $n = 6$) were immunized with 30 μg of IR30-Fd or IR30 mixed with Freund's complete adjuvant (CFA), followed by 3 boosts with the same antigen plus Freund's incomplete adjuvant (IFA) at 14 and 28 d, and without adjuvant at 42 d, respectively. New Zealand White rabbits (2 kg of body mass, $n = 2$) were immunized with 500 μg of antigen mixed with CFA, followed by 3 boosts with the same antigen plus IFA at 14 and 30 d, and without adjuvant at 50 d. Both mouse and rabbit sera were collected one week after the last boost and incubated at 56 ℃ for 30 min before using. The animal study was approved by the Ethic Committee of the Zhongshan School of Medicine, Sun Yat-Sen University.

1.5 ELISA

IR30-Fd or IR30 was coated in wells of a 96-well plate. After blocking with 5% fat free milk, a serial diluted mouse or rabbit antisera were added, followed by addition of HRP-conjugated goat-anti-mouse or rabbit IgG, correspondingly, and substrate TMB, sequentially. The optical density was measured at a wavelength of 450 nm.

1.6 Western blots

HA proteins or purified antigens were boiled for 5 min at 100°C in loading buffer containing SDS and DTT. After centrifuge at 3 000 r/min for 3 min, supernatants were run in 15% SDS-PAGE gel. The proteins were then transferred onto PVDF membranes. The blots were blocked by 5% fat free milk and 0.1% Tween-20 diluted in Tris-buffered saline. The HA proteins were detected by addition of mouse antiserum, anti-mouse IgG-alkaline phosphatase (Sigma, USA), and BCIP/NBT (Sigma, USA), sequentially.

1.7 Cell ELISA

A cell ELISA was performed as previously described[22] to determine the binding activity of antibodies to the native HA expressed on the transfected cells. Briefly, Hela cells were planted in 96-well plate and transfected with the plasmids expressing HA protein of A/Hong Kong/483/97 (H5N1), A/Guangdong/1070/2009 (H1N1), and A/Swine/Guangdong/z5/2003 (H3N2), respectively. After incubation for 48 h, the cells were treated with TPCK-trypsin for 1 h, and cultured under pH 5.0 for 30 min. The cells were then fixed by polyformald-ehyde for 20 min at room temperature, followed by addition of serially diluted rabbit antisera, HRP conjugated goat-anti-rabbit IgG, and TMB, sequent-ially.

2 Results

2.1 Structural characterization of IR30-Fd and IR30

IR30 is a synthetic peptide corresponding to the conserved sequence (residues 77-106) in HA2 of H5N1 (Fig. 1A), while IR30-Fd is a recombinant protein consisting of IR30 and a trimeric motif foldon (Fd) (Fig. 1B and 1C). We added Fd at the C-terminus of IR30, in a hope to enhance the trimeric α-helical conformation[19], allowing IR30-Fd to mimic the native trimeric conformation of HA2.

In CD spectroscopic analysis, IR30 showed a random coiled secondary structure, suggesting that the free peptide IR30 cannot maintain its native α-helical conformation in HA2. However, IR30-Fd exhibited an α-helical coiled coil conformation, confirming that addition of Fd can indeed enhance the trimeric α-helical conformation of IR30 (Fig. 1D and 1E).

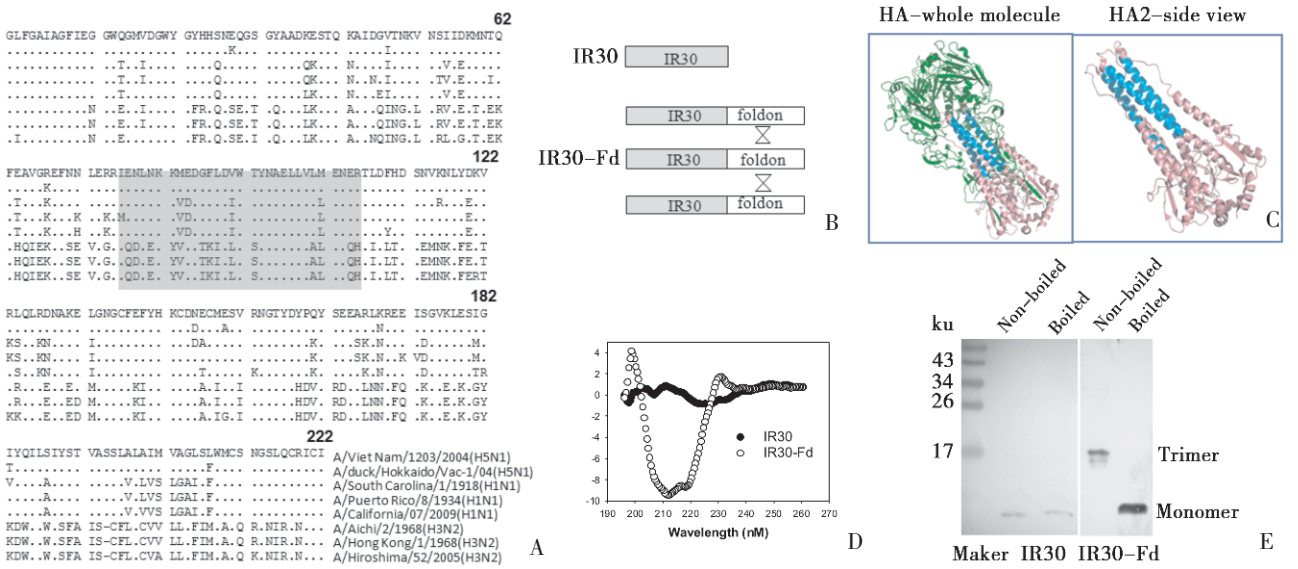

Fig.1 Structure of the two antigens

A: Alignment of the HA2 sequence of IAVs of different serum types. Sequences of antigens are marked in gray box; B: Schematic representation of the two antigens; C: The 3D scheme of the structure of HA1 (green) and HA2 (pink), the antigen IR30 in HA2 was colored in cyan; D: CD spectrographic analysis of secondary structures of the antigens; E: Western blot detects the boiled or non-boiled antigens, the anti-IR30 from mice was used as the first antibody. Homology-based structural models are from Protein Data Bank, PDB ID: 2FK0.

In Western blot, we generally dissociated an α-helical trimer formed by foldon, which cannot be de polymerized by SDS and reduced reagent by heating at 100 ℃ for 5 min. As shown in Figure1E, IR30 was in a monomeric form with and without heating, suggesting that IR30 peptide was unable to form a trimer. IR30-Fd displayed a trimeric conformation without heating and a monomeric conformation after heating at 100 ℃ for 5 min, confirming that with the assistance of Fd as a trimeric motif, IR30-Fd can form an α-helical trimer.

2.2 Binding of mouse and rabbit anti-IR30-Fd antibodies to the conserved sequence IR30 in HA2 of H5N1 virus

We immunized mice and rabbits with IR30-Fd and IR30 in the presence of adjuvant and then tested the binding activity of their antisera against peptide IR30 using ELISA. As shown in Figure 2A and 2B, the antibodies in both mice and rabbits immunized with IR30-Fd or IR30 could bind to the conserved HA2 sequence, IR30. Anti-IR30-Fd antibodies exhibited higher binding activity than anti-IR30 antibodies, suggesting that adding foldon to the IR30 sequence enhances its immunogenicity against the conserved sequence.

2.3 Recognition of anti-IR30-Fd antibodies to HA of IAVs with different serum subtypes

The binding activities of the anti-IR30-Fd and anti-IR30 antibodies to HA, HA1, and HA2 from different subtypes of IAVs were compared using Western blot assay. As shown in Figure 3A, both anti-IR30-Fd and anti-IR30 antibodies could react with HA and HA2 of H5N1 strains, including A/Anhui/1/2005(H5N1), A/Indonesia/5/2005(H5N1) and A/Viet Nam/1194/2004(H5N1)(the above HA preparations contained also partially cleaved HA, i.e., HA1 and HA2), and HA of other H5N1 strains, including A/Hong Kong/483/97 (H5N1)and A/Xingjiang/1/2006 (H5N1) (their HA preparations contained uncleaved HA only). Interestingly, anti-IR30-Fd antibodies could also bind to the HA of different heterologous subtypes, i.e. H3N2 and H1N1 strains (their HA preparations contained only uncleaved HA), while anti-IR30 antibodies could not. These results suggest that unlike the linear peptide IR30, HR30-Fd, which maintains helical trimeric form, could induce broadly cross-reactive antibodies that were specific for HA of both homologous and heterologous subtypes of IAVs (Fig. 3A).

Further study showed that the mouse anti-IR30-Fd antiserum at the dilution as high as 1:6 400 could react with both HA and HA2 of H5N1, and HA of H3N2 and at 1:3 200 with HA of H1N1, while the rabbit anti-IR30-Fd antiserum at the dilution as high as 1:8.2×10^5 could react with H5N1 and H1N1, and

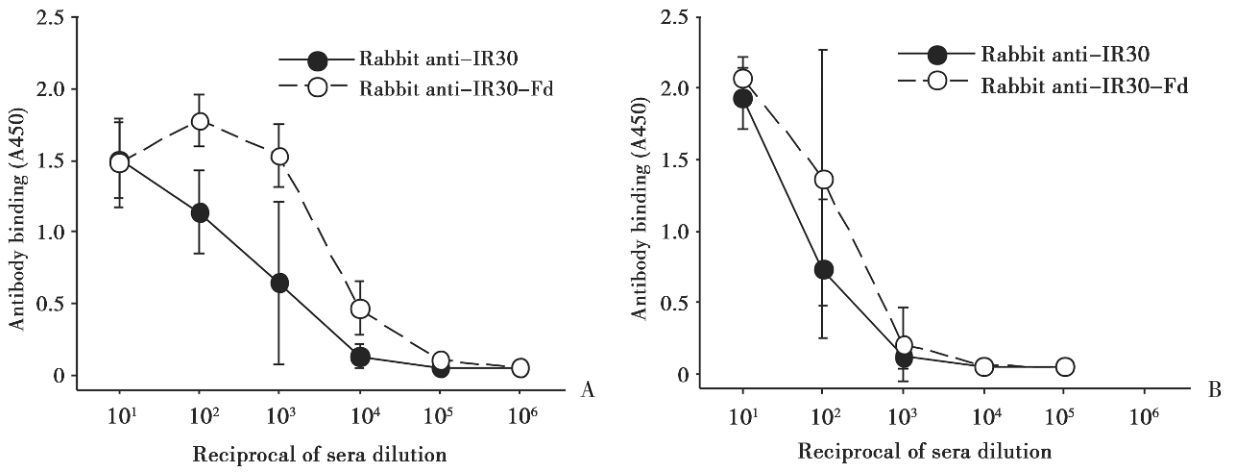

Fig.2　The titers of anti-IR30 and anti-IR30-Fd antisera against the conserved sequence in HA2 of H5N1

Binding activities of antibodies in mouse antisera (A) and rabbit antisera (B), respectively, were measured by ELISA. The experiment was repeated twice and similar results were obtained. The data were presented in mean titer ± SD of a representative experiment.

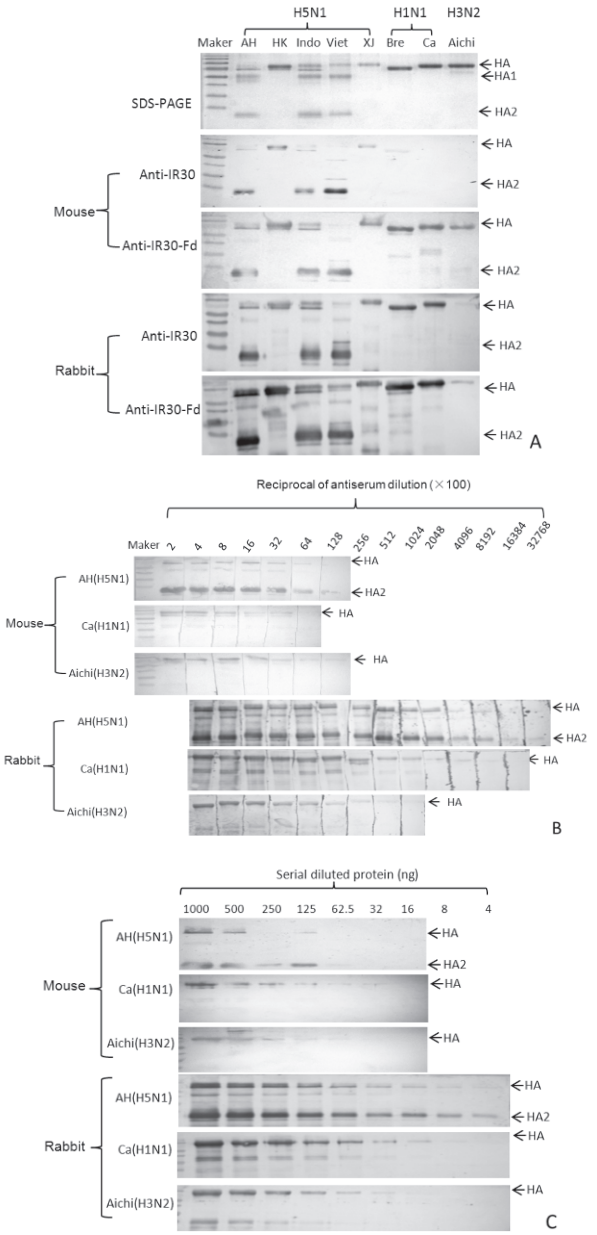

Fig.3 Binding of anti-IR30-Fd antibodies with HAs as detected by Western blot

A: The recognition of antibodies in the antisera to the recombinant HAs (at 500 ng respectively) of different strains of H5N1, H1N1 and H3N2 IAVs, including A/Anhui/1/2005(H5N1)(AH), A/Indonesia/ 5/2005(H5N1)(Indo), A/Viet Nam/1194/2004 (H5N1)(Viet), A/Hong Kong/483/97 (H5N1)(HK), A/Xingjiang/1/2006 (H5N1)(XJ), A/Brevig Mission/1/1918(H1N1)(Bre), A/California/04/2009(H1N1)(Ca), and A/Aichi/2/1968/(H3N2)(Aichi). The HA in the AH, Indo, and Viet preparations was partially cleaved, thus containing HA, HA1, and HA2, while other preparations contain only uncleaved HA; B: The binding ability of serially diluted anti-IR30-Fd antisera with HAs at 500 ng. The initial mouse and rabbit serum dilutions were 1:200 and 1:400, respectively, and were then 2-fold serially diluted; C: The binding ability of anti-IR30-Fd atdilution of 1:800 with serially diluted HAs.

at $1:5.1\times10^4$ with H3N2 (Fig.3B). Further, anti IR30-Fd antiserum at 1:800 dilution could react with HA and HA2 of H5N1 (125 ng for testing mouse antisera; 8 ng for testing rabbit antisera), HA of H1N1 (125 ng for testing mouse antisera; 16 ng for testing rabbit antisera) and HA of H3N2 (125 ng, mice; 32 ng for testing rabbit mouse antisera) (Fig. 3C). These results indicated that the IR30-Fd antibodies showed a strong binding affinity for IAV HA of different serum subtypes of IAVs with high efficiency.

2.4 Binding of rabbit anti-IR30-Fd and anti-IR30 antibodies to the native conformation of HA expressed on cell surface

We further tested the binding ability of rabbit anti-IR30-Fd and anti-IR30 antibodies with the native conformation of HA expressed on Hela cells by cell ELISA. The results showed that both anti-IR30-Fd and anti-IR30 antibodies in the rabbit antisera could effectively bind to HA of three distinct subtype of IAVs on the Hela cells (Fig.4), while they only had a background binding to the Hela cells expressing no HA. Interestingly, the relative binding affinity between anti-IR30-Fd antibodies and H3N2 HA was much higher than that between anti-IR30 antibodies and H3N2 HA. The relative binding affinity between anti-IR30-Fd antibodies and H5N1 HA was also higher than that between anti-IR30 antibodies and H5N1 HA, while the difference between anti-IR30-Fd and anti-IR30 antibodies for their binding to H1N1 HA was not significant. These results suggested that although both anti-IR30-Fd and anti-IR30 antibodies could recognize the native HAs expressed on cells, anti-IR30-Fd antibodies were more efficient than anti-IR30 antibodies to bind H5N1 and H3N2 HAs.

3 Discussion

In addition to the seasonal IAVs, the newly emerged IAVs, such as H1N1 and H7N9, have posed great threats to the public health. Therefore, development of an effective vaccine with broad cross-

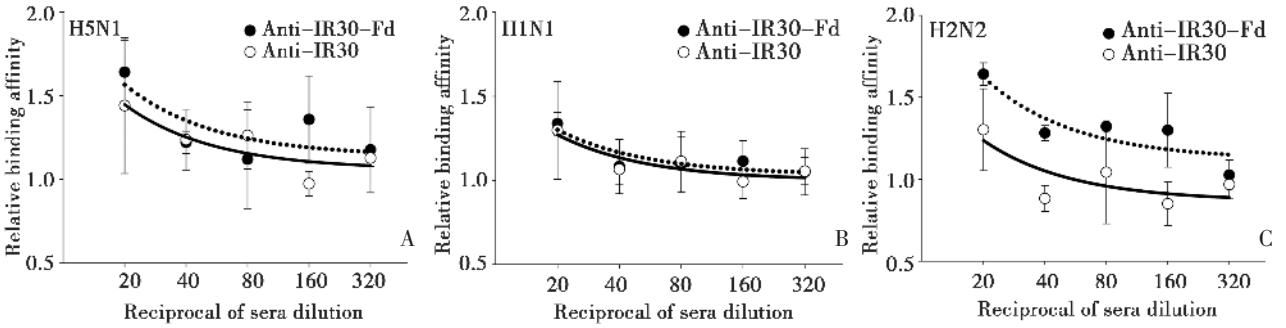

Fig.4　Binding of the rabbit anti-IR30-Fd antibodies to the native conformation of HAs

The relative binding affinity (the optical density at A450 ofHela cells expressing HA/the optical density at A450 of Hela cells expressing no HA) of anti-IR30-Fd antibodies(anti-IR30 antibodies as control) in rabbit antisera with the HAs of H5N1 (A), H1N1 (B), and H3N2 (C) expressed on the Hela cells (Hela cells expressing no HA as control) was determined by cell ELISA as described in Materials and Methods. The experiment was repeated once and similar results were obtained. The data were presented in mean relative binding affinity ± SD of a representative experiment

reactivity against divergent IAVs is urgently needed [2].

Since the epitope of 12D1 retains an α-helical conformation in the trimeric HA2 domains, we thus conjugated thetrimerization motif, foldon, to the C-terminus of 12D1's epitope, IR30, in a hope to keep the epitope in the vaccine in its native conformation. Our previous studies have shown that conjugation of foldon, to the C-terminus of N46 peptide resulted in significant increase of gp41 N46-trimer's α-helicity and induction of increased titer of neutralizing HIV-1 antibodies [19]. As expected, addition of Fd could significantly enhance the trimeric α-helical conformation of IR30.

It has been reported that the region of aa 38-175 is more immunogenic than other part of HA2, but is still a weak natural immunogen. In the present study, we found that anti-IR30-Fd antibodies exhibited significantly higher binding activity to IR30 than anti-IR30 antibodies,indicating that conjugation of foldon to the conserved epitope of 12D1 could enhances its immunogenicity. Further characterization of these two antibodies revealed that unlike anti-IR30 antibodies that only reacted with the HA of homologous subtype, anti-IR30-Fd antibodies could bind to the HA of both homologous subtype (H5N1) and heterologous subtypes (H3N2 and H1N1) of IAVs. Both mouse and rabbit anti-IR30-Fd antisera could strongly react with HAs of different subtypes of IAVs, with the antibody titer in the order of anti-H5N1 > anti-H1N1 > anti-H3N1. Furthermore, the rabbit anti-IR30-Fd antibodies could also bind to the native conformation of HAs of H5N1, H3N2, and H1N1 expressed on the cell surface. These results suggested that IR30-Fd, which maintained helical trimeric conformation, could induce broadly cross-reactive antibodies that were specific for HA of both homologous and heterologous subtypes of IAV.

Moreover, we will look for an experienced collaborator with appropriate facility, methods and animal models to test IR30-Fd for its potential cross-protection against divergent IAV strains of different subtypes.

Collectively, the results obtained from this study suggest that the antigens containing the conserved neutralizing epitope in HA2 domain with trimeric α-helices mimicking the natural structure of HA- stalk has great potential to be developed as an effective broadly protective IAV vaccine, providing a new strategy in developing universal influenza vaccines.

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