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ORIGINAL ARTICLE

Antibody reactivity of conformational peptide mimics of a conserved H5N1 neutralization site in different fusion proteins

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Abstract Several peptide mimics of a conserved H5N1 avian influenza virus neutralization site recognized by 8H5 mAb have been reported previously. In this study, the secondary and possibly higher structural orders of the peptide mimics 122 and 125 were investigated and found to be closely related to the specific binding with 8H5 mAb. These two peptide mimics were fused to three different carrier proteins, and the antibody binding activities were recovered in 4 of the 11 fusion proteins. HEV structural protein p239 and HBc were more suitable than the outer membrane protein T47 of the *Treponema pallidum* particle for the recovery of reactivity. The increase in the copy number of peptide mimics was important for the recovery of antibody-binding activity and the interaction between peptide and carrier protein may affect the spatial structure of both the peptide and the carrier protein. These results are likely to be of relevance for conformational peptide mimics in diagnostic tests, vaccine and inhibitors.

Introduction

The development of phage peptide libraries, where random peptide sequences are displayed on the surface of a phage, provide researchers with a highly effective tool for selecting peptide ligands for antibodies against linear as well as discontinuous protein epitopes [23, 27]. These peptides have been successfully applied to or have potential application in several different areas, such as epitope mapping, diagnostic testing, vaccine development and inhibitors [2, 10, 24, 28]. When an antibody recognizes a conformational B cell epitope, the peptide mimic is presumed to structurally mimic the epitope at the peptide level [8, 25], bearing no sequence homology to the natural epitope. A current challenge is to maintain the correct conformational peptide mimics.

Peptide mimics can come in four forms: phagotope, synthesized peptide, synthesized peptide chemically coupled with a carrier protein or fusion protein containing a peptide. A phagotope is a phage containing a peptide, and this is selected from a phage peptide library [6, 15, 34, 35, 39]. A synthesized peptide is constructed artificially and may be based on the phagotope. When a peptide is chemically coupled to carrier protein, this can enhance its immunogenicity and decrease its susceptibility to rapid proteolytic degradation. These carrier proteins include keyhole limpet hemocyanin (KLH), biotin, tetanus toxoid (TT) and bovine serum albumin (BSA) [3, 9–11]. As a peptide can have multiple conformations [21], conjugation to a carrier protein has implications for the structural integrity of the peptide mimic. For example, the conjugation chemistry itself may alter the configuration of the peptide mimic, while the carrier protein may induce epitope suppression [14, 19].

To overcome these potential problems, peptide mimics can be fused with other proteins using genetic-engineering methods. This again enhances immunogenicity [29] and increases affinity [26]. This approach is commonly used in native-epitope-based vaccine research [1, 22, 36, 38]. Short peptides with important neutralizing epitopes for influenza virus, FMDV, or HCV have been displayed on different carrier proteins, and these keep their correct conformation.

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Previously, we reported on several peptide mimics of a conserved H5N1 avian influenza virus neutralization site recognized by 8H5 mAb [18]. It was inferred that 8H5 mAb recognizes discontinuous sites presented by secondary and possibly higher structural orders in spatially favorable positions for binding with the antibody. In the present study, two of the peptide mimics, 122 (ETQLTTAGLRLL) and 125 (DTPLTTAALRLV), are investigated for their reactivity with 8H5 mAb in 11 fusion proteins. The peptide mimics were fused to HEV structural protein p239 [16] and the outer membrane proteins T47 of the *Treponema pallidum* particle or inserted at positions 79 and 80 of the 1–149 fragment of the HBc protein [21].

Materials and methods

Materials and reagents

Eight murine monoclonal antibodies (MAb) were used: 8H5 and 2F2 were generated against the H5N1 influenza virus [4, 30]. MAb 13D8, 1E40, 8C11, 8H3, and 15B2 were generated against a recombinant structural protein of HEV as described by Zhang et al. [37]. 18H6 was generated against the HBc protein. 21C4 serum was collected from a patient. Peptide mimics 122 and 125 were synthesized at GL Biochem (Shanghai, China). Taq polymerase, restriction enzymes, and T4 DNA ligase were purchased from TaKaRa (Dalian, China). DNA purification kits were purchased from Tiangen (Beijing, China). Horseradish peroxide (HRP)-conjugated goat anti-mouse IgG was developed in our laboratory. Oligo primers were synthesized by Sangon (Shanghai, China).

Virus strains

The avian influenza virus strains Chicken/HongKong/YU22/2002(YU22), HongKong/213/2003(HK213), Rosybilled Pochard/HongKong/821/2002 (HK821), Chicken/Jiangxi/6151/2003(6151), Indonesia/2A/2004(2A), and Indonesia/5/2005(5) were kindly provided by State Key Laboratories of Emerging Infectious Diseases, Department of Microbiology, The University of Hong Kong.

Construction and expression of fusion proteins

Two peptide mimics were fused to the C-terminus of HEV structural protein p239, to the outer membrane T47 proteins of the *T. pallidum* particle, or inserted at positions 79 and 80 of the 1–149 fragment of the HBc protein. Eleven different constructs were made, as shown in Table 1. One or two copies of peptide mimics were fused with carrier proteins, and a flexible linker, GGGGS, was inserted into tandem peptides. Three fusion proteins with two copies of peptide mimics were fused to p239 and HBc [18]. The reverse sequences were also fused to the C-terminus of HEV structural protein p239.

Genes coding for the fusion proteins were cloned into the plasmid pTO-T7 [37] and resultant recombinant vectors were used to transform ER2566 competent cells. Fusion p239 proteins and T47 proteins were recovered in the pellet of the cell lysate, treated with 2% Triton X-100 at 37°C for 30 min and then dissolved in 8 M urea. The proteins were renatured by dialysis against phosphate-buffered saline, pH 7.45, at room temperature. The fusion HBc protein was recovered from the supernatant of the cell lysate. The fusion proteins were precipitated from a 33% ammonium sulfate solution and re-suspended by shaking in CB buffer containing 5% β-mercaptoethanol at 37°C for 30 min followed by dialysis in PB buffer (pH 7.4). The resulting purified fusion proteins were kept at 4°C until use.

Indirect ELISA test of fusion proteins and synthesized peptides

The purified fusion proteins were then made to a concentration of 10 μg/ml or synthesized peptides were made to a concentration of 50 μg/ml. These solutions were individually coated onto 96-well plates at 37°C for 2 h. The plates were washed and then blocked with ED buffer (2% gelatin, 5‰ casein, 1‰ ProClin 300, in PBS) at 37°C for 2 h, after which 100 μl of mouse monoclonal antibody (1.0 μg/ml) or 21C4 anti-T47 serum was added to each microtiter well and incubated at 37°C for 1 h.

In a dose-dependence experiment, twofold dilutions of 8H5 mAb starting from 40 μg/ml were tested in fusion-protein-coated microtiter plates. 100 μl of antibody solution was added to each microtiter well and incubated at 37°C for 1 h. The plates were then washed five times with PBST (0.05% Tween20 in PBS). Then, 100 μl GAM-HRP (1:20,000 dilution) or GAH-HRP (1:10,000 dilution) was added to each well and incubated at 37°C for a further 30 min. The plates were then washed again three times with PBST. Colorimetric development was done by adding 3,3′,5,5′-tetramethylbenzidine (TMB) Liquid Substrate System, and color intensity was measured in a microplate reader.

Blocking ELISAs

The spatial relationships of epitope recognition by each of the MAbs were investigated in a "blocking ELISA" experiment in which 5 ng/well p239 antigen was coated to the microplate and then the solid surface was incubated with saturating levels of unlabeled MAbs prior to the addition of HRP-conjugated MAbs. The HRP-conjugated

Table 1 Construction of fusion proteins

Fusion proteins	Construction form
p239-s122, p239-s125	p239 peptide
P239-r122,p239-r125	p239 r-peptide
p239-122	p239 peptide peptide
T47-122, T47-125	T47 peptide peptide
HBc-s122, HBc-s125	HBc peptide HBc
HBc-122, HBc-125	HBc peptide peptide HBc

The peptide mimics 122 (ETQLTTAGLRLL) and 125(DTPLTTAALRLV) were fused to the C-terminus of p239 and T47, or inserted at positions 79 and 80 of the HBc protein. One or two copies of peptide mimics were fused with carrier proteins, and a flexible linker, GGGGS, was inserted into tandem peptides. The reverse sequences were also fused to the C-terminus of p239

s single copy, *r* reverse sequence

mAbs were diluted in PBS containing 20% bovine sera to a concentration that resulted in a final OD value between 1.0 and 1.5 in an indirect ELISA. Samples of unlabeled MAbs (100 µl, 20 µg/ml in PBST) were added to a p239-coated microplate for 30 min at 37°C, and then removed by aspiration. Then, 100 µl of suitably diluted HRP-conjugated MAbs was added for a further 30 min at 37°C. The wells were washed, and color development was carried out as described above. The blocking rate was calculated as the percent decrease in the OD value of the blocked well compared to the unblocked well.

Competitive ELISA test

The 8H5 mAb, at a concentration of 10 µg/ml in PBS buffer (pH 8.0), was coated onto 96-well plates at 37°C for 2 h. The plates were washed once and then blocked with ED buffer. Then, the purified fusion protein (10 µg/ml) or synthesized peptide (50 µg/ml) and H5 avian influenza virus (4HA of HK213, HK812,YU22,6151,2A, or 5) were added to the microtiter wells and incubated at 37°C for 30 min [18]. HA is a "unit" of hemagglutination defined as the amount of virus needed to agglutinate an equal volume of a standardized red blood cell suspension. The highest dilution of virus that causes complete hemagglutination is considered the HA titration end point. The HA titer is the reciprocal of the dilution of virus in the last well with complete hemagglutination [31]. In a dose-dependent reactivity experiment, fusion protein or synthesized peptide with different concentrations was mixed with H5N1 virus Chicken/HongKong/YU22/2002 (4HA). The mixture was reacted with 8H5 coated on a microplate at 10 µg/ml. HBc protein, p239 protein, synthesized peptide 122 and PBS were used as negative controls. The plates were washed five times with PBST (0.05% Tween 20 in PBS). Then, 100 µl secondary antibody 2F2-HRP (2 µg/ml) against H5 avian influenza virus was added to each well and incubated at 37°C for 30 min with subsequent washing. Color development was done as described above.

Results

Binding of fusion proteins containing peptide mimics to 8H5 mAb

Two peptide mimics, 122 and 125, were fused as shown in Table 1 to the C-terminus of HEV structural protein p239 and the outer membrane protein T47 of the *T. pallidum* particle, or inserted at positions 79 and 80 of the 1–149 fragment of the HBc protein. This produced 11 fusion proteins (Fig. 1).

The 11 fusion proteins and synthesized peptides were tested by ELISA for their ability to bind with 8H5 mAb. Figure 2 shows that among the 5 fusion proteins with p239 as carrier protein, only p239-122 and p239-s125 were reactive with 8H5. Among the four fusion proteins with HBc as carrier protein, only HBc-122 and HBc-125 reacted with 8H5. Two fusion proteins with T47 as carrier protein showed no reactivity. Two peptide mimics fused to the C terminus of p239 in their reverse orientation and synthesized peptides did not bind to 8H5. Interestingly, p239-s122, HBc-s122 and HBc-s125, which contained one copy of the peptide mimics, also did not bind to 8H5, but p239-122, HBc-122 and HBc-125, which contained two copies of peptide mimics, showed 8H5 binding activity. The

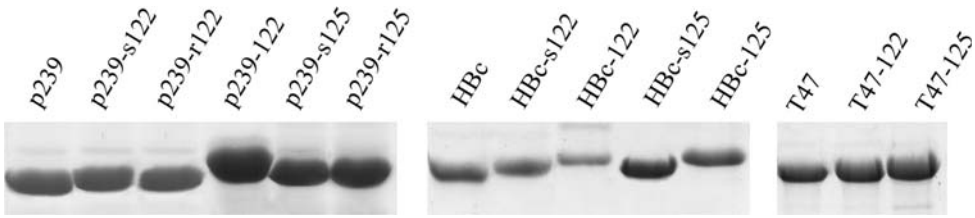

Fig. 1 Purified fusion proteins containing peptide mimics of conserved H5N1 avian influenza virus neutralization sites. Two peptide mimics were fused to p239, T47 or HBc protein. Eleven fusion proteins were expressed and purified as described in "Materials and methods" and subjected to SDS-PAGE in a 12% polyacrylamide gel

results in Fig. 3 show that the fusion proteins reacted with 8H5 mAb in a dose-dependent manner. p239-s125 and HBc-125 had similar reactivity with 8H5, but p239-122 had stronger binding activity than HBc-122.

Those results implied that the carrier protein, the copy number and orientation of peptide mimics may have a determinable influence on the peptide's activity.

Effects of fused peptide mimics on the conformation of p239 carrier protein

Five mAbs against a recombinant structural protein of HEV E2 were used to test the effects of fused peptide mimics on the conformation of p239. Previous studies have shown that 13D8, 8c11 and 8H3 recognize a conformational epitope located at aa 459–606 of HEV ORF2, and 15B2 recognizes a linear epitope located at aa 408–423 of HEV ORF2 [32]. p239-s122, p239-122, p239-s125 and p239 at a concentration of 10 μg/ml were coated onto an ELISA plate and incubated separately with the 5 mAbs coupled with HRP. Figure 4 shows that p239-122 and p239-s125 bind to all five of the mAbs with similar intensity as p239. Nevertheless, p239-s122 showed lower reactivity with 8C11 mAb, and much lower reactivity with 13D8 and 1E40.

The relationship between the five p239 antibodies was analyzed in a cross-blocking study. ELISA was carried out using microplates coated with p239 and pre-incubated with buffer or buffer containing mAb p239, 13D8, 1E40, 8C11, 8H3 or 15B2, which were then allowed to react with the indicated HRP-labeled antibodies as described in "Materials and methods". Binding of the labeled antibodies was measured in OD units. The results showed that 13D8, 1E40 and 8C11 blocked the binding of one another to a similar extent, raising the speculation that the epitopes recognized by them may be located in close proximity, while 8H3 and 15B2 recognized different sites on p239 (Table 2).

The interaction of peptide 122 with p239 carrier protein may have caused mutual spatial structural changes leading to the loss of its own activity and dramatic decreases of 13D8 and 1E40 binding to p239.

Competition of fusion proteins and H5N1 viruses for 8H5 mAb

The four fusion proteins that showed 8H5 mAb binding activity and two synthesized peptides were further analyzed for their capacity to compete with avian influenza viruses for binding to 8H5 mAb. The fusion proteins and synthesized peptides were mixed with different strains of H5N1 virus, and the mixture was added to microplates that had been coated with 8H5 mAb. The amount of virus bound was determined by ELISA using a secondary antibody, 2F2-HRP, against H5N1 HA. Compared to the results concurrently obtained with viruses alone, the presence of fusion proteins and peptides was found to reduce the amount of virus bound to 8H5-coated plates by 18–76%, whereas carrier proteins HBcAg and p239 and synthesized peptide 121 did not affect virus binding (Fig. 5).

The four fusion proteins and two synthesized peptides were further tested at different concentrations for their competition with H5N1 virus Chicken/HongKong/YU22/2002 (4HA) for 8H5 mAb binding. We found that the competition was dose-dependent, and p239-122 showed the strongest activity (Fig. 6). The concentration of p239-122 for 50% competition was 10 μg/ml, but that of synthesized peptide 122 was 160 μg/ml (Table 3). We also found that the molar concentrations of peptides in fusion proteins for 50% competition were much lower than those of the synthesized peptides. The molar concentration of peptide in p239-122 for 50% competition was 0.69 nmol/ml, which was 173.9-fold lower than that of the synthesized peptide 122. The molar concentration of peptide in p239-s125 for 50% competition was 1.65 nmol/ml, which was 18.8-fold lower than that of the synthesized peptide 125 (Table 3).

Discussion

We previously reported several peptide mimics of a conserved H5N1 avian influenza virus neutralization site recognized by 8H5 mAb [16]. In the present study,

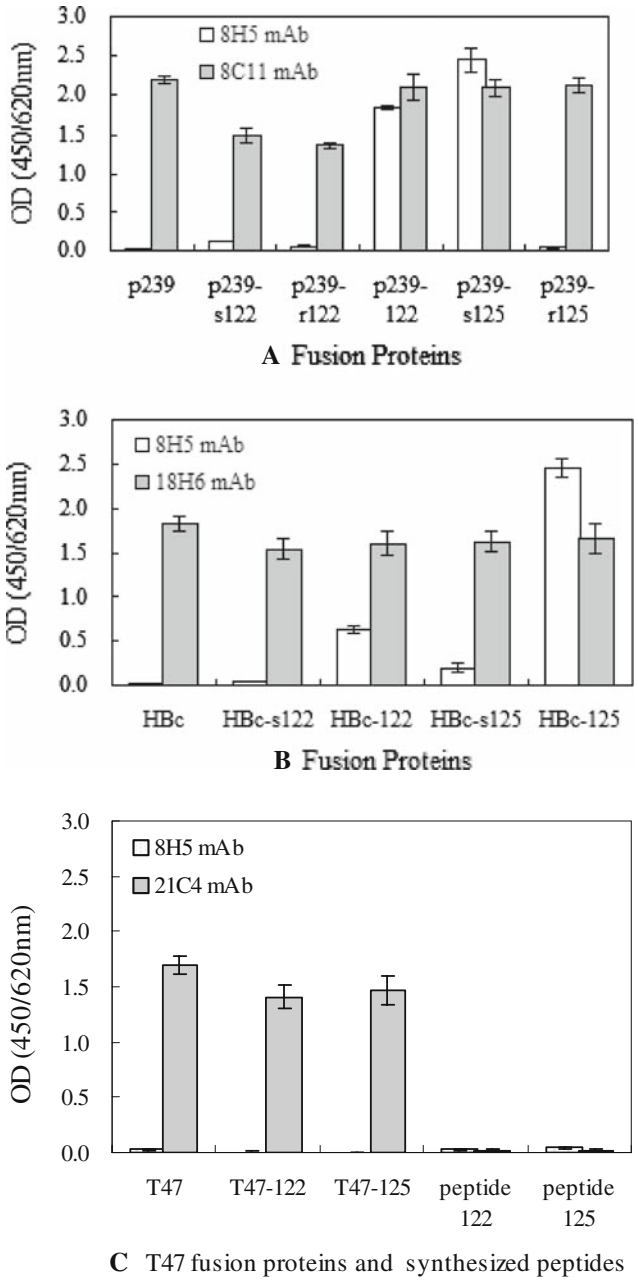

Fig. 2 Binding of 8H5 mAb to p239, HBc and T47 fusion proteins and synthesized peptides. Fusion proteins of p239 (**A**), HBc (**B**) and T47 carrying the different peptides and synthesized peptides (**C**) were tested by ELISA for binding with 8H5 mAb. Anti-p239 mAb 8C11, anti-HBc mAb 18H6 and anti-T47 serum 21C4 were used as controls. Tests were done in triplicate, and results are shown as mean absorbance ± SD

synthesized peptides of two peptide mimics, 122(ETQLTTAGLRLL) and 125(DTPLTTAALRLV), lost their reactivity with 8H5 mAb in ELISA, but could still compete with H5N1 viruses for binding to 8H5 mAb in a competitive ELISA assay. This suggests that their structures may be damaged when they are immobilized on a microtiter plate or nitrocellulose membrane, but not when in solution.

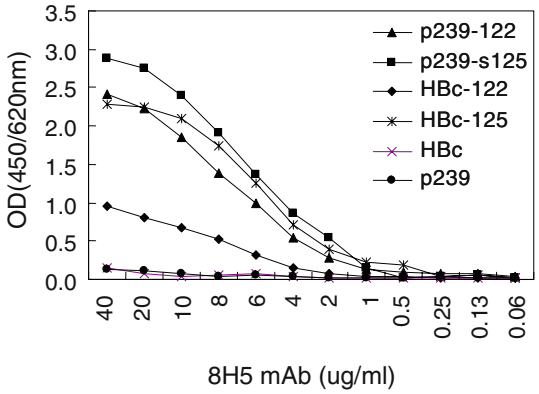

Fig. 3 Dose-dependent reactivity of fusion proteins with 8H5 mAb in ELISA test. Twofold dilutions of 8H5 mAb starting from 40 μg/ml were observed for reaction with fusion proteins coated on a microplate at 10 μg/ml

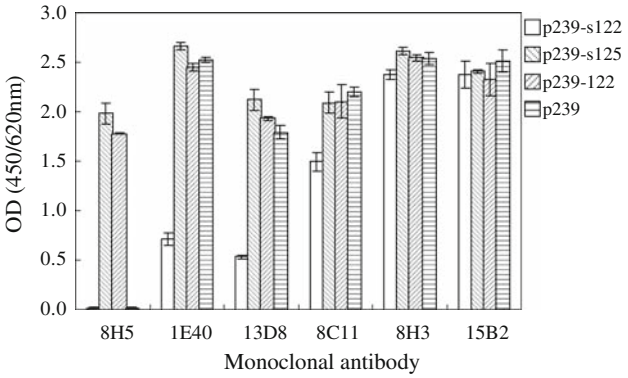

Fig. 4 Binding of p239 fusion proteins to five anti-p239 mAbs. Fusion proteins p239-s122, p239-122. p239-s125 were tested by ELISA for binding with five anti-p239 mAbs. p239 was used as a control. Tests were done in triplicate, and results are shown as mean absorbance ± SD

Table 2 Blocking results for 13D8, 1E40, 8C11, 8H3 and 15B2

mAbs	13D8 (%)	1E40 (%)	8C11 (%)	8H3 (%)	15B2 (%)
13D8	97.0	97.0	98.0	56.0	30.0
1E40	93.0	95.0	97.0	26.0	20.0
8C11	98.0	96.0	98.0	22.0	26.0
8H3	40.0	40.0	30.0	93.0	16.0
15B2	20.0	18.0	38.0	50.0	85.0

The spatial relationships of epitope recognition by each of the mAbs were investigated by "blocking ELISA" experiments. p239 antigen was coated to the microplate, after which the microtiter well was incubated with saturating levels of unlabeled mAbs prior to the addition of HRP-conjugated mAbs. The blockage rate was calculated as the percent decrease in the OD value of the blocked microtitre well compared with the the OD value of the unblocked microtiter well

These results also confirm that the secondary and possibly higher structural orders of the peptides were closely related to the specific binding of 8H5 mAb. These two peptide

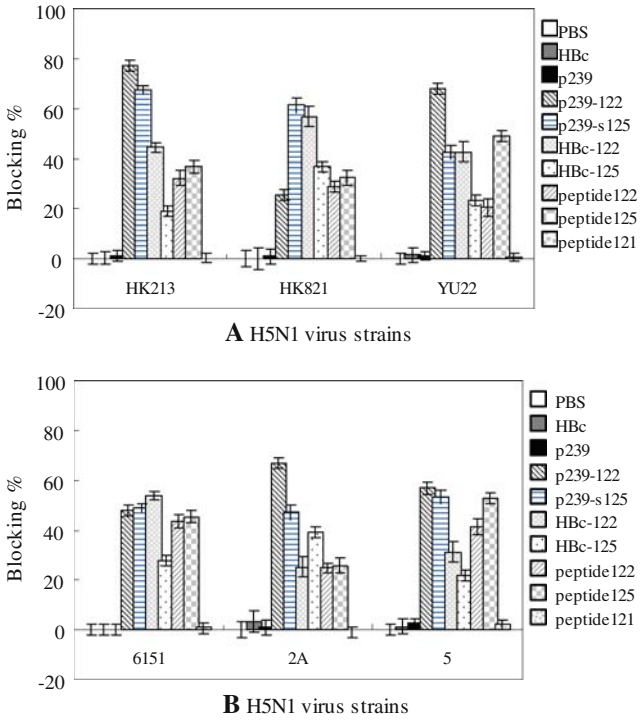

Fig. 5 Fusion proteins and synthesized peptides compete with H5N1 virus for 8H5 mAb binding. Fusion proteins carrying different peptides or the parental protein (10 μg/ml), or synthesized peptides (50 μg/ml) were mixed with H5N1 avian influenza virus (4HA of HK213, HK812, YU22, 6151, 2A, 5). The mixtures were added to microtiter plates that had been coated with 10 μg/ml of 8H5. The plates were incubated for 30 min and washed, and amount of virus bound to 8H5 was determined using another H5N1 monoclonal antibody, 2F2-HRP. Experiments were done in triplicate. Mean residual virus bound (R), where R = mean virus bound (absorbance) in the presence of fusion protein/mean virus bound in the absence of fusion protein. Mean % blocking of virus binding by fusion protein = 100 − R

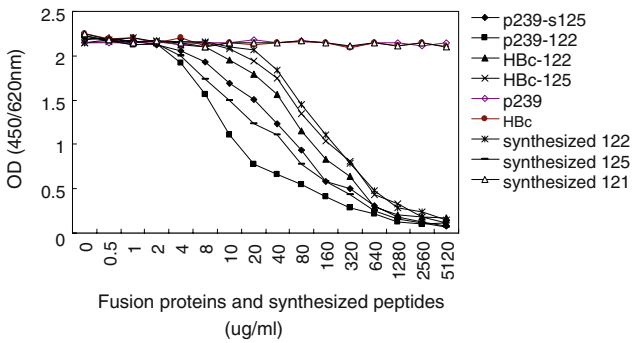

Fig. 6 Dose-dependent reactivity of fusion proteins and synthesized peptides competing with H5N1 virus for 8H5 mAb binding. Fusion protein or synthesized peptide at different concentrations was mixed with H5N1 virus Chicken/HongKong/YU22/2002 (4HA). The mixture then incubated with 8H5 coated onto microplate well (10 μg/ml). The amount of virus bound to 8H5 was determined using another H5N1 monoclonal antibody, 2F2-HRP

Table 3 Concentrations of peptide or fusion protein for 50% competition with H5N1 virus Chicken/HongKong/YU22/2002 for 8H5 mAb binding

Peptide or fusion protein	Concentration of peptide or fusion protein (μg/ml)	Molar concentration of peptide (nmol/ml)
Synthesized 122	160	120
p239-122	10	0.69
HBc-122	80	7.08
Synthesized 125	40	30
p239-s125	50	1.65
HBc-125	140	12.44

mimics are similar to other peptides whose structures exert a great effect on their functions. These peptides included antibacterial peptides [12, 13, 33], a beta-amyloid-plaque specific epitope [19], a peptide determinant of gp120 [14], and a peptide inhibitor [5, 20].

Peptide mimics 122 and 125 were fused to HEV structural protein p239 and the outer membrane protein T47 of the *T. pallidum* particle or inserted at positions 79 and 80 of the 1–149 fragment of HBcAg protein. These peptide mimics retained 8H5 binding activity when fused with p239 and HBc, but not when fused with T47. The two peptide mimics fused to the C terminus of p239 in the reverse orientation did not bind to 8H5. These results suggest that the carrier protein and orientation of peptide mimics influence their activity. Similar results have been reported by De Filette et al. [7], studying an M2e-based human influenza A vaccine, and Nuzzaci et al. [22], who used cucumber mosaic virus as a presentation system for a double hepatitis C virus-derived epitope. Significant changes in peptide activity may occur when the relative position or direction of peptides or their carrier protein is changed.

In the present study, when one or two copies of peptide mimics were fused to p239 and HBcAg, only 1 of 4 fusion proteins containing a single copy of peptide mimic p239-s125 was reactive with 8H5, while three of the five fusion proteins containing two copies of peptide mimics p239-122, HBc-122 and HBc-125 were reactive with 8H5. Thus, it can be tentatively concluded that the copy number of peptide mimics also influences their activity. Although repeats of B cell or T cell epitopes have often been used to increase the activity or immune response and were also used by Liu et al. [17] in M2e fused to glutathione-*S*-transferase and by Rotzschke et al. [26] in oligomerized T cell epitopes, there is no similar report on peptide mimics.

To further investigate the effects of fused peptides on carrier protein, five different MAb were used to react with p239 carrier protein and p239 fusion proteins. Previous studies have shown that five MAb were generated against recombinant structural protein E2 of HEV, and all of them

but 15B2 recognized a conformational epitope located at aa 459–606 of HEV ORF2 [33]. In the present study, we show that p239-122 and p239-s125 bind to all five of the mAbs with similar intensity as the p239 carrier protein. Nevertheless, p239-s122 showed lower reactivity with 8C11 mAb and much lower reactivity with 13D8 and 1E40. Peptide 122 in p239-s122 might interact with p239 carrier protein such that the spatial structures of peptide 122 and p239 are changed. Manea et al. [19] reported similar research on antigenic bioconjugates comprising a beta-amyloid-plaque specific epitope. They determined the antigenic properties of some constructs by ELISA using an anti-Abeta (1–17) monoclonal antibody and found that the epitope topology and the presence of a spacer moiety between the carrier and the epitope peptide influenced antibody binding of the Abeta (4–10) epitope.

In conclusion, secondary and possibly higher structural orders of the peptide mimics 122 and 125 were closely related to the specific binding with 8H5 mAb, and this was probably responsible for the antibody reactivity of the peptide mimics of the four fusion proteins. The carrier protein, orientation and copy number of peptide mimics are key factors for the peptide's reactivity in fusion proteins. These results should be useful for applying conformational peptide mimics in diagnostics, vaccines and inhibitors.

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