Structural Definition of Duck Major Histocompatibility Complex Class I Molecules That Might Explain Efficient Cytotoxic T Lymphocyte Immunity to Influenza A Virus

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ABSTRACT
A single dominantly expressed allele of major histocompatibility complex I (MHC I) may be responsible for the duck's high tolerance to highly pathogenic influenza A virus (HP-IAV) compared to the chicken's lower tolerance. In this study, the crystal structures of duck MHC I (Anpl-UAA*01) and duck β2-microglobulin (β2m) with two peptides from the H5N1 strains were determined. Two remarkable features were found to distinguish the Anpl-UAA*01 complex from other known MHC I structures. A disulfide bond formed by Cys95 and Cys112 and connecting the β5 and β6 sheets at the bottom of peptide binding groove (PBG) in Anpl-UAA*01 complex, which can enhance IAV peptide binding, was identified. Moreover, the interface area between duck MHC I and β2m was found to be larger than in other species. In addition, the two IAV peptides that display distinctive conformations in the PBG, B, and F pockets act as the primary anchor sites. Thirty-one IAV peptides were used to verify the peptide binding motif of Anpl-UAA*01, and the results confirmed that the peptide binding motif is similar to that of HLA-A*0201. Based on this motif, approximately 600 peptides from the IAV strains were partially verified as the candidate epitope peptides for Anpl-UAA*01, which is a far greater number than those for chicken BF2*2101 and BF2*0401 molecules. Extensive IAV peptide binding should allow for ducks with this Anpl-UAA*01 haplotype to resist IAV infection.

IMPORTANCE
Ducks are natural reservoirs of influenza A virus (IAV) and are more resistant to the IAV than chickens. Both ducks and chickens express only one dominant MHC I locus providing resistance to the virus. To investigate how MHC I provides IAV resistance, crystal structures of the dominantly expressed duck MHC class I (pAnpl-UAA*01) with two IAV peptides were determined. A disulfide bond was identified in the peptide binding groove that can facilitate Anpl-UAA*01 binding to IAV peptides. Anpl-UAA*01 has a much wider recognition spectrum of IAV epitope peptides than do chickens. The IAV peptides bound by Anpl-UAA*01 display distinctive conformations that can help induce an extensive cytotoxic T lymphocyte (CTL) response. In addition, the interface area between the duck MHC I and β2m is larger than in other species. These results indicate that HP-IAV resistance in ducks is due to extensive CTL responses induced by MHC I.

KEYWORDS
crystal structure, duck, MHC class I, influenza A virus, disulfide bond

Influenza A virus (IAV) poses a large threat for both animal and human health and is a growing problem (1–4). Ducks play a pivotal role in its epidemiology because they are natural reservoirs of IAV (5). Although all subtypes of IAV are perpetuated in ducks...
they typically do not show serious signs of the disease, even that caused by highly pathogenic IAVs (HP-IAVs), such as the H5N1 strain, which is lethal to chickens (8, 9). Since 2002, many Asian lineage H5N1 HP-IAVs have been shown to produce similar symptoms and mortality, although clinical outcomes are also affected by the age and species of the ducks and the strains of IAVs (10, 11). Moreover, ducks have more active and robust cellular immune responses than chickens (12). These findings suggest that at least some species of duck show efficient immune responses against IAV infection.

Studies have indicated that major histocompatibility complex class I (MHC I) plays a role in the anti-IAV response (13–15). MHC I can present viral epitope peptides to specific T cell receptors (TCRs), resulting in the proliferation of cytotoxic T lymphocytes (CTLs) and eventual clearance of the virus from the host (16–18). CTL responses mediated by MHC I play a significant role in primary IAV infection and provide cross-protection against different IAV strains in chickens, mice, and humans (19–23). Suppressive subtractive hybridization libraries have been constructed to enrich differentially expressed genes from the lungs of ducks infected with IAVs of high or low pathogenicity compared to mock-infected controls (13). These data showed that the MHC I gene was upregulated in ducks under both conditions, especially in the highly pathogenic group, in which the MHC I gene was increased by more than 1,000-fold (13).

Another study showed that there was an increase in MHC I gene expression in two different duck species after injection with a commercial inactivated vaccine (24). These results suggest that duck MHC I may play a role similar to that of the human HLA against infection.

MHC I molecules are encoded by polymorphic alleles from several loci. These loci make up the peptide binding groove (PBG) and contain epitope peptides against...

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**TABLE 1 Data collection and refinement statistics for pAnpl-UAA*01–MVM9/RL9**

| Parameter                              | Value for:       | pAnpl-UAA*01–MVM9 | pAnpl-UAA*01–RL9 |
|----------------------------------------|------------------|-------------------|-----------------|
| Data collection                        |                  |                   |                 |
| Space group                            | P1211            | P1211             |                 |
| Cell dimensions                        |                  |                   |                 |
| \( a, b, c \) (Å)                      | 46.27, 64.39, 77.92 | 46.49, 63.54, 79.95 |                 |
| \( \alpha, \beta, \gamma \) (°)       | 90.00, 105.74, 90.00 | 90.00, 106.10, 90.00 |                 |
| Resolution (Å)                         | 50.00–1.80 (1.86–1.80) | 50.00–2.05 (2.09–2.05) |                 |
| No. of reflections                     |                  | 112,298           | 180,543         |
| Total                                  |                  | 41,752            | 27,433          |
| \( R_{	ext{sym}} \) or \( R_{	ext{merge}} \) b | 0.063 (0.542) | 0.092 (0.568) |                 |
| \( I/|I| \)                            | 20.759 (2.667) | 24.100 (3.691) |                 |
| Completeness (%)                       | 96.2 (98.2) | 99.8 (99.9) |                 |
| Redundancy (f)                         | 4.5 (4.7) | 4.1 (4.1) |                 |
| Refinement                             |                  |                   |                 |
| Resolution (Å)                         | 50.00–1.71 | 50.00–2.06        |                 |
| No. of reflections                     | 40612            | 26122             |                 |
| \( R_{	ext{work}}/R_{	ext{free}} \) c | 18.27/21.06 | 20.80/25.33 |                 |
| Root mean square deviation             |                  |                   |                 |
| Bond length (Å)                        | 0.019            | 0.006             |                 |
| Bond angle (°)                         | 1.931            | 1.057             |                 |
| Avg B factor                           | 32.794           | 33.678            |                 |
| Ramachandran plot quality (%)          |                  |                   |                 |
| Most favored region                    | 98.33            | 98.92             |                 |
| Allowed region                         | 1.67             | 1.08              |                 |
| Disallowed                             | 0.00             | 0.00              |                 |

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aValues in parentheses are for the highest-resolution shell.
b\( R_{	ext{merge}} = \sum_{i=1}^{n} |I_i(hkl) − \langle I_i(hkl)⟩| / \sum_{i=1}^{n} \langle I_i(hkl)⟩\) where \( I_i(hkl) \) is the observed intensity and \( \langle I_i(hkl) \rangle \) is the average intensity from multiple measurements.
c\( R = \sum_{i=1}^{m} |F_{\text{calc}}(i) - k|F_{\text{obs}}(i)| / \sum_{i=1}^{m} |F_{\text{obs}}(i)| \), where \( R_{\text{free}} \) is calculated for a randomly chosen 5% of reflections and \( R_{\text{work}} \) is calculated for the remaining 95% of reflections used for structure refinement.
multiple pathogens. In humans and mice, there are three functional MHC I loci that provide polymorphic alleles for peptides (25, 26); however, many birds predominantly express only one MHC class I gene, such as in chickens and ducks (27). Chickens express only one dominant MHC I locus, referred to as BF2, which is adjacent to the TAP gene (28). Although there are five different loci in the duck MHC I genome region (named the UAA to UEA loci), only UAA, which lies adjacent to the polymorphic TAP2 gene, is predominantly expressed for the CTL immune response (29, 30). To date, more than 400 structures of the peptide-MHC I-β2-microglobulin β2m) protein complexes (here called pMHC I) of different species have been determined, and most of them are from human and mouse. Structural studies revealed that the epitope peptides are fixed in the PBG of the MHC I heavy chain by six pockets (A to F) (31). Some pockets, typically the B and F pockets, play a critical role to bind the peptides and determine the peptide binding motif of a certain MHC I molecule. In recent years, pMHC I structures of other species have been solved, while in avian species, only chicken MHC I (BF2) was resolved (32–34). These structures have greatly facilitated the identification of MHC I-restricted CTL epitopes.

Several studies have indicated that “minimal MHC” is related to resistance and the susceptibility of chickens to viruses, such as the Rous sarcoma virus and Marek’s disease.

FIG 1 Structural overview and additional disulfide bond of the pAnpl-UAA*01 complex. (A and B) The overall structures of pAnpl-UAA*01–RLI9 (A) and pAnpl-UAA*01–MVM9 (B). pAnpl-UAA*01 chains are shown with distinct colors: for pAnpl-UAA*01–RLI9, H in red and L in olive, and for pAnpl-UAA*01–MVM9, H in marine and L in green). Peptides are shown as spheres using the same colors with their light chains. (C to E) The additional disulfide bond of pAnpl-UAA*01 compared with chicken and human pMHC I complexes. Disulfide bonds are marked with spheres. The additional disulfide bond formed by Cys95-Cys112 of pAnpl-UAA*01 is labeled in a box and amplified to show its position and electronic density map. Both chicken pBF2*2101 (green, Protein Data Bank code 3BEV) and human pHLA-A*0201 (lemon, Protein Data Bank code 3PWN) have just three disulfide bonds distributed in the α2 and α3 domains and β2m chains.
virus (MDV) (32, 35, 36). Chickens expressing BF2*0401 (B4 haplotype) are susceptible to MDV, and chickens expressing BF2*2101 (B21 haplotype) are resistant to MDV. Crystal studies of BF2 structures have illustrated its resistance or susceptibility to MDV (32). The MHC I complex presents viral peptides to CTLs, depending on the six (A to F) pockets in its peptide binding groove (PBG). The MHC I polymorphisms determine the distinct three-dimensional (3D) structure of the MHC I PBG, and each classical MHC I molecule has a specific peptide binding motif. Crystal studies of B21 have shown that it can remodel its pockets to accommodate different peptides based on its wide binding groove and interplay of two charged residues (Arg⁹ and Asp⁺²⁴); B21 could bind multiple peptides from MDV and activate extensive T cell repertoires to clear MDV (32). In contrast, B4 has a narrow peptide binding groove and strong polar pockets that restrict its peptide binding motif and the presented MDV peptides (33). Therefore, the CTL response to MDV induced by B4 is not strong enough to prevent infection and suggests that resistance is controlled by the type of MHC I and its 3D structure.

MHC I could also play a role in the susceptibility of viruses. For IAV, the higher resistances of some species of duck indicate that their MHC I alleles would present more IAV peptide epitopes than those of chickens (11, 14). Our previous data, as well as a recently published study, confirmed the presence of two or more cysteines, which have never been found in humans or other species, in the PBGs of many duck MHC I alleles (30, 37). To date, little is known about the 3D structure and peptide presentation of virus (MDV) (32, 35, 36). Chickens expressing BF2*0401 (B4 haplotype) are susceptible to MDV, and chickens expressing BF2*2101 (B21 haplotype) are resistant to MDV. Crystal studies of BF2 structures have illustrated its resistance or susceptibility to MDV (32). The MHC I complex presents viral peptides to CTLs, depending on the six (A to F) pockets in its peptide binding groove (PBG). The MHC I polymorphisms determine the distinct three-dimensional (3D) structure of the MHC I PBG, and each classical MHC I molecule has a specific peptide binding motif. Crystal studies of B21 have shown that it can remodel its pockets to accommodate different peptides based on its wide binding groove and interplay of two charged residues (Arg⁹ and Asp⁺²⁴); B21 could bind multiple peptides from MDV and activate extensive T cell repertoires to clear MDV (32). In contrast, B4 has a narrow peptide binding groove and strong polar pockets that restrict its peptide binding motif and the presented MDV peptides (33). Therefore, the CTL response to MDV induced by B4 is not strong enough to prevent infection and suggests that resistance is controlled by the type of MHC I and its 3D structure.

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duck MHC I. In this study, we determined two crystal structures of the dominantly expressed duck MHC I \((\text{Anpl}}-\text{UAA}^*01)\) molecules with two IAV peptides. The crystal structures showed that \(\text{Anpl}}-\text{UAA}^*01\) exhibits characteristics consistent with the duck MHC I-peptide-H\(_9252\) complex (pMHC I) architecture; however, we also identified a disulfide bond in its PBG. Unlike the chicken MHC I, only B and F pockets of \(\text{Anpl}}-\text{UAA}^*01\) are the primary anchor sites for peptide binding. The two pockets, whose compositions are similar to those of the B and F pockets of HLA-A2, could accommodate diverse residues and make \(\text{Anpl}}-\text{UAA}^*01\) an extensive peptide binding motif. By screening the entire sequences of IAV strains, approximately 600 nonapeptides fit the identified peptide binding motif of \(\text{Anpl}}-\text{UAA}^*01\). Our study demonstrates that \(\text{Anpl}}-\text{UAA}^*01\) could present more IAV epitope peptides, suggesting that ducks with this MHC I haplotype may be protected from infection.

**RESULTS**

The \(\text{Anpl}}-\text{UAA}^*01\) structure shows an unexposed disulfide bond in the PBG and a large interface between heavy and light (H and L) chains. Two trimer complexes formed by \(\text{Anpl}}-\text{UAA}^*01\), duck \(\beta\text{2m} (\text{Anpl}}-\beta\text{2m})\), and the MVM9 and RLI9 peptides were crystallized in the P12\(_1\)1 space group at resolutions of 1.71 Å and 2.06 Å, respectively (Table 1). Both of the complexes displayed a canonical pMHC I structure, in which the peptide was located in the platform formed by the \(\alpha1\) and \(\alpha2\) domains of \(\text{Anpl}}-\text{UAA}^*01\) above the \(\alpha3\) domain and light-chain \(\text{Anpl}}-\beta\text{2m} \) (Fig. 1A and B). The most remarkable features of the p\(\text{Anpl}}-\text{UAA}^*01\) complexes are the two disulfide bonds formed by 4 cysteines in the PBG (Fig. 1C). In other known pMHC I structures, there is only one
A disulfide bond formed by a pair of cysteines in their PBGs, such as in chicken pBF2*2101 (32) and human pHLA-A*0201 (38) (Fig. 1D and E). These two cysteines have been found only in ducks (Fig. 2). This novel disulfide formed by Cys95 and Cys112 of pAnpl-UAA*01 complexes connects the /H92525 and /H92526 sheets at the bottom of the PBG.

The no-peptide refolding product (only pAnpl-UAA*01 and pAnpl-/H92522m) was used as a negative control to judge the refolding efficiencies of different peptides; however, a peak (approximately 300 milli-absorbance units [mAU]) eluted at the position representing the pAnpl-UAA*01. The gel filtration and SDS-PAGE results showed that the peak represented the complex of pAnpl-UAA*01 and pAnpl-/H92522m (Fig. 3), which is different from results of studies on cattle, pig, chicken, and horse pMHC I refolding (33, 39–41). This result could be due to the Cys95-Cys112 disulfide bond of pAnpl-UAA*01. In order to clarify the impact of this disulfide bond, Cys95 and Cys112 were mutated to alanine (A), alone and together. However, the results for refolding of the mutated pAnpl-UAA*01s were similar to those for the wild type (Fig. 3), indicating that the additional disulfide bond is not directly related to the stability of pAnpl-UAA*01 without peptide binding.

The formation of a complex without the peptide may be due to the strong binding affinity between H and L chains. There are a total of 18 hydrogen bonds and salt bridges between pAnpl-UAA*01 and pAnpl-/beta2m (Fig. 4A), which is greater than the numbers found in the pMHC I structures of chickens (13) (pBF2*0401, PDB code 4E0R) and pigs (16) (pSLA-1*0401, PDB code 3QQ3). In addition, the size of the interface between pAnpl-UAA*01 and pAnpl-/beta2m is 1,554.2 Å², which is the largest among the known pMHC I structures of different species (Fig. 4B), including human (PDB code 3QWN), monkey (PDB code 1ZVS), mouse (PDB code 3TID), rat (PDB code 1ED3), swine (PDB code 3QQ3), cattle (PDB code 3PWU), horse (PDB code 4ZUV), and chicken (PDB code 3BEV) (33, 38–44). The interactions and interface area between pAnpl-UAA*01 and pAnpl-/beta2m likely contribute to the stability of the complex without binding peptides.

The distinct peptide conformations presented by the Anpl-UAA*01 molecule. The conformations of IAV-MVM9 and IAV-RLI9 presented by Anpl-UAA*01 are distinct,
especially in the middle region from the residue at position 3 (P3) to that at P6 (Fig. 5).

The IAV-RLI9 peptide is clear on the electronic density map and adopts an "M" overall conformation, which is common to other pMHC I structures. The B factors of the IAV-RLI9 middle part are relatively higher than at the N and C termini, indicating that P4 to P6 are more flexible than other residues (Fig. 5A). In IAV-MVM9, the electronic density map is missing at P3 and P4, which means that these two residues are quite flexible (Fig. 5B). The side chain orientations of P5 are obviously different between the IAV-MVM9 and IAV-RLI9 peptides. In IAV-MVM9, the side chain of P5 stretches to the α1 helix but in RLI9, its side chain stretches upward and most parts are exposed from the PBG (Fig. 5C).

The conformations of the MVM9 and RLI9 peptides were significantly distinct when the two pAnpl-UAA*01 structures were superimposed (Fig. 6A and B); their deviation is focused mainly at the center, where the distance between the Ca atoms of MVM9 and RLI9 P5 residues can reach 4.4 Å. The N and C termini of the two peptides matched well. Structural analysis showed that the N termini of the MVM9 and RLI9 peptides (P1 and P2 residues) form the same hydrogen bonds with the PBG (Fig. 6C). From the P3 to P7 residues, there are no hydrogen bonds between RLI9 and the PBG. Although the P3 and P4 residues are missing in MVM9, three hydrogen bonds were found between P5, P7, and the PBG (Fig. 6D). These distinct peptide conformations of this part should be related to the differences in hydrogen bonds found here. In the C termini, the hydrogen bonds between the main chain of peptides and the PBG are the same; the only

![FIG 5 Divergent presentations of RLI9/MVM9 peptides presented by Anpl-UAA*01. (A and B) Electron densities and overall conformations of the structurally defined peptides RLI9 and MVM9. Simulated annealing omit maps (CNS) calculated for the two peptides are shown in blue at a contour of 1.5, and the coloration of the two peptides is according to the isotropic B factors. (C) General side chain orientations and the different interfacing areas of RLI9/MVM9 presented by Anpl-UAA*01 PBG, as viewed in profile from the peptide N terminus toward the C terminus. Black arrows indicate the directions in which the residues point: up is toward the T cell receptor, down is toward the floor of the peptide binding groove, left is toward the α1 helix domain, and right is toward the α2 helix domain. Pockets accommodating each residue are listed under the corresponding anchors within the peptide binding groove. ASA, accessible surface area of each residue; BSA, buried surface areas of the residues.](http://jvi.asm.org/Downloaded from http://jvi.asm.org/)

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difference is that the Lys\textsuperscript{142} of pAnpl-UAA*01 forms 3 hydrogen bonds with the side chains of the P8 and P9 residues in the RLI9 peptide but does not form a bond with the MVM9 peptide (Fig. 6E).

These data suggest that the conformation of the middle section of the peptide presented by Anpl-UAA*01 is flexible and is determined by the peptide’s specific amino acid sequence.

**Anpl-UAA*01 selects peptides by relying on the B and F pockets.** The compositions of the six pockets of Anpl-UAA*01 are shown in Fig. 7, and the interactions between peptides and these pockets are listed in Table 2. The RLI9 peptide was selected to illustrate the interactions with the six pockets in Fig. 6 because of its completeness.

The A pocket of Anpl-UAA*01, composed of Leu\textsuperscript{5}, Tyr\textsuperscript{7}, Tyr\textsuperscript{58}, Glu\textsuperscript{62}, Tyr\textsuperscript{156}, Thr\textsuperscript{160}, Cys\textsuperscript{161}, Trp\textsuperscript{164}, and Tyr\textsuperscript{168}, fixes P1-Arg by hydrogen bonds and strong van der Waals forces (VDWs) (Fig. 7A; Table 2). As in the known pMHC I structures, P1-Arg forms the hydrogen bonds with the A pocket by its main-chain atoms, and its side chain stretches upward out of the A pocket (45, 46). Therefore, although the binding between the A pocket and P1 residue is strong, the A pocket does not play a restrictive role in the PBG of Anpl-UAA*01.

The B pocket is a primary anchor site and plays a restrictive role in peptide binding. The B pocket of Anpl-UAA*01 accommodates P2-Leu (Fig. 7B) The charged Glu\textsuperscript{62}, which is on the top of the B pocket, can form a hydrogen bond with the main chain of P2-Leu.
The side chain of the P2 residue inserts into the B pocket and is fixed by the VDWs provided by the surrounding residues (Table 2).

The C, D, and E pockets usually connect with residues in the middle part of the binding peptides. The amino acid compositions of these three pockets of Anpl-UAA\(^*\)01 are shown in Fig. 7C to E. The C and D pockets can interact with the side chains of the P3 and P6 residues, respectively, but the E pocket can only interact with the main chain of the P7 residue, as the orientation of the P7 side chain faces upward (Table 2).

The additional disulfide bond formed by Cys\(^95\) and Cys\(^{112}\) is in the C pocket of Anpl-UAA\(^*\)01. The impact of this disulfide bond on peptide binding was checked by the refolding of MVM9 and RLI9 peptides with the C95A, C112A, and C95A-C112A double mutant heavy chains. We found that mutant H chains can still form a complex with peptides, but the refolding is worse than that of the wild-type H chain, especially for the C112A mutant H chain (Fig. 8A and B). The reduced refolding efficiencies of the mutants indicated that the disulfide bond in the C pocket helps with the peptide binding of Anpl-UAA\(^*\)01; however, the distances between the peptide residues and disulfide bond are over 5.0 Å, which means that their direct interactions are negligible (Fig. 6C). Previous studies have suggested that Cys\(^{95}\) and Cys\(^{112}\) do not meet the preferred geometry to form the disulfide bond (30), and our Anpl-UAA\(^*\)01 structures show that the distance between Cys\(^{95}\) and Cys\(^{112}\) is less than that for the corresponding positions in HLA-A2 and B21 (Fig. 8C). To some extent, the additional disulfide bond of Anpl-UAA\(^*\)01 alters the bottom of the C pocket and strengthens the stability. Improved peptide binding efficiency may strengthen the stability of the C pocket with the additional disulfide bound.

The F pocket is the most important anchor site at the C terminus of PBG. The F pocket of Anpl-UAA\(^*\)01 is composed of Asn\(^{76}\), Thr\(^{77}\), Ala\(^{80}\), Arg\(^{83}\), Tyr\(^{84}\), Trp\(^{93}\), Phe\(^{119}\), and Ala\(^{135}\), and it has a strong binding affinity with P9-I (Fig. 7F). The main chain of P9-I can form 2 salt bridges with Arg\(^{83}\) and Lys\(^{142}\) and 2 hydrogen bonds with Asn\(^{76}\) and
Thr\textsuperscript{139} in the F pocket. The side chain of P9-I inserts into the F pocket and is fixed by the strong hydrophobic forces (Table 2).

In order to determine the primary anchor residues of Anpl-UAA\textsuperscript{*}01-presenting peptides and the vital restriction pockets for peptide binding, the RLI9 peptide was mutated by alanine scanning and circular dichroism (CD) spectral analysis was used to test the stabilities of Anpl-UAA\textsuperscript{*}01 complexes with these mutant peptides (Fig. 9). The wild-type RLI9 peptide was used as a control, and its midpoint transition temperature (T_m) value was 43.3°C. Among all of the alanine mutant peptides, only the T_m values of the P2-Ala and P9-Ala mutant peptides were significantly lower than that of the wild-type RLI9 peptide, indicating that the side chains of P2 and P9 play key roles in RLI9 peptide binding and that these two residues are the primary anchor residues. The pockets (B and F) at the two termini of Anpl-UAA\textsuperscript{*}01 PBG anchor the peptides and determine the peptide binding motif of Anpl-UAA\textsuperscript{*}01.

Table 2 Interactions between the RLI9/MVM9 peptide and Anpl-UAA\textsuperscript{*}01

| Complex              | Peptide Residue | Heavy chain Residue | Hydrogen bonds and salt bridges | van der Waals contact residues* |
|----------------------|-----------------|---------------------|--------------------------------|--------------------------------|
| pAnpl-UAA\textsuperscript{*}01–RLI9 | P1-Arg N | Tyr168 OH | Leu5, Tyr7, Tyr58, Glu62, Tyr156, Thr160, Trp164, Thr168 (59) |
|                      | N               | Tyr7 OH |  |
|                      | O               | Tyr156 OH | |
|                      | P2-Leu N        | Glu62 OE1 | Tyr7, Tyr9, Thr24, Met43, Glu62, Thr65, Ser66, Tyr156 (46) |
|                      | P3-Ile          | Thr65, His97, Trp153, Tyr156 (42) |
|                      | P4-Ile          | Arg61, Thr65 (18) |
|                      | P5-Asn          | (0) |
|                      | P6-Ser          | Asn69, Ile72 (5) |
|                      | P7-Ile          | Trp143, Lys142, Glu146, Val149, Ile72 (21) |
|                      | P8-Thr OG1      | Lys142 NZ | Lys142, Trp143, Asn76, Thr139, Phe119, Thr79, Ala80, Arg83, Trp93 (69) |
|                      | O               | Lys142 NZ |  |
|                      | O               | Trp143 NE1 |  |
|                      | P9-Ile N        | Asn76 OD1 | Lys142, Trp143, Asn76, Thr139, Phe119, Thr79, Ala80, Arg83, Trp93 (69) |
|                      | O               | Lys142 NZ |  |
|                      | O               | Trp143 NE1 |  |
|                      | OXT             | Arg83 NH2 (S) |  |
|                      | OXT             | Thr139 OG1 |  |
|                      | O               | Lys142 NZ (S) |  |
| Total                | 11              |                           | 291 |

| pAnpl-UAA\textsuperscript{*}01–MVM9 | P1-Met N | Tyr7 OH | Leu5, Tyr7, Tyr58, Arg61, Glu62, Tyr156, Trp164, Thr168 (56) |
|                                    | N       | Tyr156 OH | |
|                                    | N       | Tyr156 OH | |
|                                    | P2-Val N | Glu62 OE1 | Tyr7, Tyr9, Thr62, Thr65, Asn69, His97, Tyr156 (37) |
|                                    | P5-Leu O | Trp153 NE1 | Gln152, Trp153, Tyr156 (33) |
|                                    | P6-Ile O | Asn76 OD1 | Tyr9, Asn69, Ile72, Phe73, Trp153 (26) |
|                                    | P7-Arg O | Glu146 NE2 | Ile72, Asn76, Trp143, Glu146, Val149, Gln152 (31) |
|                                    | P8-Met O | Trp143 NE1 | Ile72, Val75, Asn76, Lys142, Trp143 (22) |
|                                    | P9-Ile N | Asn76 OD1 | Asn76, Thr79, Ala80, Arg83, Trp93, Phe119, Thr139, Lys142, Trp143 (70) |
|                                    | OXT     | Arg83 NH2 (S) |  |
|                                    | OXT     | Thr139 OG1 |  |
| Total                | 11              |                           | 275 |

*Numbers in parentheses are the amounts of van der Waals force.
P9-I are almost the same (Fig. 10B). The peptide presentations and pocket compositions make the peptide conformations of pAnpl-UAA*01 and pHLA-A2 almost identical (Fig. 10C and D).

Previous studies have shown that the B pocket of HLA-A2 prefers hydrophobic anchor residues, such as I, L, V, A, and F, and can also accommodate the same polar residues, such as T. The most frequently occurring anchor residues in the F pocket of HLA-A2 are I, V, and L. The two peptides presented by pAnpl-UAA*01, MVM9 and RLI9, perfectly fit the peptide binding motif of HLA-A2. Eighteen additional peptides from IAV and 11 mutant peptides were used to verify the peptide binding motif of pAnpl-UAA*01 (Table 3). The results confirmed that the peptide binding motifs of these two MHC I molecules from different species are mostly overlapping (Fig. 10E and F). In brief, the nonapeptide binding motif of pAnpl-UAA*01 is x-(A, V, I, L, M, T, S, or F)-x-x-x-x-x-x-(A, V, I, L, M, or F).

Many IAV peptides match the motif of pAnpl-UAA*01. Using the motif of Anpl-UAA*01 that we identified, the proteomes of several IAV strains were screened to identify the epitope peptides that could be presented by pAnpl-UAA*01, including the H1N1, H3N2, H3N8, H4N6, H5N1, H7N9, and H9N2 subtypes (47–53). There were

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FIG 8 Mutants of Anpl-UAA*01 influence the in vitro refolding efficiency with L chain and RLI9/MVM9 peptides. The refolding products were analyzed by chromatography on a Superdex 200 10/300 GL column (GE Healthcare). Curves in different colors indicate different H chains. (A) Refolding results for the wild-type Anpl-UAA*01 and three mutants with the MVM9 peptide. (B) Refolding results with the RLI9 peptide. (C) The bottom β sheet of Anpl-UAA*01 is more compact than those in B21 and HLA-A2. All the structures are in ribbon models. The distances between C95 and C112 in Anpl-UAA*01 and their corresponding positions in B21 and HLA-A2 are labeled by dashed lines.
approximately 600 candidate nonapeptides from different IAV strains matching the peptide binding motif of Anpl-UAA*01 (Fig. 11). Most selected peptides also fit the motif of HLA-A2, and 11 nonapeptides were proven to activate HLA-A2-restricted CTL responses efficiently according to IEDB data (http://www.iedb.org) (54). The in vitro refolding results for 31 peptides confirmed that the predicted IAV epitopes could be bound by Anpl-UAA*01 (Table 3).

Based on the motifs of chicken B4 and B21, the longer peptides from IAV strains were also screened. There are fewer than 20 octapeptides for B4 and 24 to 27 decapeptides for B21 (Fig. 11), which are much less than the numbers of peptides found in Anpl-UAA*01.

**DISCUSSION**

The most distinguishable feature of Anpl-UAA*01 was the additional disulfide bond in the PBG. The mutant experiments showed that this disulfide bond located in the C pocket can increase the efficiency of peptide binding to pAnpl-UAA*01. Although all of the mutants could still form complexes with the RL9/MVM9 peptide and β2m, their refolding efficiencies were significantly lower than that of the wild type (Fig. 8A and B). Without the peptide, this disulfide bond did not increase the refolding production of
the complex of Anpl-UAA*01 and β2m (Fig. 3). These data indicate that this additional disulfide bond can improve peptide binding to Anpl-UAA*01 and lead to the increased formation of a trimer complex; however, no significant contacts between the presented peptide and disulfide bond were identified. We also discovered that the additional disulfide bond makes the bottom β sheet of the C pocket of Anpl-UAA*01 more compact than in other MHC I molecules. The refolding effect of the C112A mutant indicated that the free side chain of Cys95 could disturb peptide binding in the C pocket region (Fig. 8A and B). Therefore, we hypothesize that this additional disulfide bond provides a more stable C pocket, which helps with peptide presentation of Anpl-UAA*01.

Another characteristic of the Anpl-UAA*01 structure is the intensive interaction

FIG 10 Alignments of B pocket, F pocket, and binding peptides indicate similar peptide binding motifs of Anpl-UAA*01 with HLA-A*0201 (PDB code 3PWN). Residues making up these pockets and binding peptides are as follows: for pAnpl-UAA*01, C, red; N, blue; O, red; and for pHLA-A*0201, C, lemon; N, blue; O, red). (A and B) B and F pocket alignments of Anpl-UAA*01 with HLA-A*0201. The same compositions of P2-Leu and P9-Ile in both RLI9/HuDG2L peptides are shown using the same color as their H chains. Hydrogen bonds formed by P2/P9 and their H chains are represented as dashed lines with each corresponding color. (C and D) Side and top views of the RLI9 peptide alignment with HuDG2L peptide presented by HLA-A*02. Both of the peptides’ main chains are shown as bold sticks. Shared P2-Leu and P9-Ile in RLI9/HuDG2L peptides are colored black in their sequences in the upper left corner. (E and F) Residues fitting the B and F pockets of Anpl-UAA*01 and HLA-A*0201 can mostly overlap. Residues within the solid red circle are motifs for Anpl-UAA*01, and those within the circle are motifs for HLA-A*0201.
between its H and L chains. In comparison with other known pMHC I structures, pAnpl-UA^A*01 has the greatest interface area and many interchain bonds (Fig. 5). The interface area in ducks and chickens is significantly larger than that in mammals. Compared with BF2^2101 (32), pAnpl-UA^A*01 had a 100-Å²-larger interface area and 4 more hydrogen bonds; this relatively stronger interaction could lead to the formation of complexes containing only Anpl-UA^A*01 and β2m through in vitro refolding, which has never been identified in pMHC I structures of other species (33, 39–41).

Anpl-UA^A*01 is similar to human HLA in peptide presentation (but not to chicken BF2), although the amino acid identities among Anpl-UA^A*01 and BF2 molecules are much higher than those between Anpl-UA^A*01 and HLA (>60% versus <50%, respectively). There are only two primary anchor pockets in pAnpl-UA^A*01, the B and F pockets, which is common in HLA molecules (Fig. 10A and B). Using structural analysis and mutant peptide refolding tests, we found that the peptide binding motif of pAnpl-UA^A*01 overlaps with HLA-A^*0201 to a large degree. The B pocket of Anpl-UA^A*01 can accommodate extensive uncharged residues, and the F pocket can accommodate multiple hydrophobic residues (Fig. 10E). However, BF2 exhibits peptide-presenting strategies that differ from those of HLA. B4 has a narrow and highly charged PBG, which limits the binding peptides that must fit its B, C, and F pockets together (33). B21 has a large central cavity and flexible Arg9, which makes its binding motif promiscuous with three anchor residues (32). The moderate limits of the peptide binding motif suggest that ducks can accommodate more peptides from pathogens than chickens.

The role of MHC I mediating the CTL response in clearance of IAV has been confirmed in human and mouse. Although substantial data on anti-IAV CTL responses in duck and chicken are scarce, some evidence indicates that duck MHC genes respond
Peptide predictions from different influenza virus subtypes according to the distinct binding motifs of Anpl-UAA*01, BF2*0401, and BF2*2101. Genome-wide scanning results of peptides matching the motifs of Anpl-UAA*01 (A), BF2*0401 (B), and BF2*2101 (C) are as follows: Anpl-UAA*01, x-(A, V, I, L,

(Continued on next page)
to infection with IAV (13, 55). The studies showed that duck MHC locus genes (MHC I/II and TAP) are overexpressed after infection with IAV, especially the duck MHC I gene, and under the condition of HP-IAV infection its expression could be increased by about 1,000 times in the lung (13). Unlike mammals, both ducks and chickens have a “minimal MHC” region with a limited set of genes and express only one dominant MHC I allele. The “minimal MHC” in chickens is critical to defense against a particular pathogen because it is completely dependent on whether or not it can load peptides from that pathogen. The best illustrated example is that chickens of the BF2*2101 genotype could defend against MDV, while chickens of the BF2*0401 genotype could not (32, 33, 56). The difference in duck versus chicken responses to IAV might also relate to the different peptide loading abilities of duck and chicken MHC I. Approximately 600 nonapeptides from different IAV strains matching the motif of pAnpl-UAA*01 were identified in this study, which is much higher than the numbers of BF2*2101 and BF2*0401 from chicken (Fig. 11; see Data Set S1 in the supplemental material). Therefore, the duck Anpl-UAA*01 should exhibit stronger resistance to IAV strains than chicken BF2*2101 and BF2*0401 (57, 58). In addition, the Anpl-UAA*01 structure showed that it can present IAV peptides in distinct conformations, which is believed to be useful for activating the T cell repertoire and inducing stronger CTL responses. Our study illustrates the structural basis of duck MHC I and provides a novel approach for explaining why ducks are more resistant to HP-IAV.

MATERIALS AND METHODS

Prediction and synthesis of IAV-derived peptides. All nonamer peptides that potentially bound to Anpl-UAA*01 were predicted using the NetMHCpan 3.0 server (http://www.cbs.dtu.dk/services/NetMHCpan/) (59, 60). The 31 peptides used in this study (Table 3) were synthesized and purified to 90% by reverse-phase high-performance liquid chromatography (HPLC) and mass spectrometry (SciLight Biotechnology). These peptides were stored in lyophilized aliquots at ~80°C after synthesis and dissolved in dimethyl sulfoxide (DMSO) before use.

Protein preparation. DNA fragments encoding extracellular domains of Anpl-UAA*01 (GenBank accession no. AB115245, residues 1 to 270 of the mature protein with EcoRI and HindIII restriction sites) (37) and Anpl-b2-microglobulin (Anpl-b2m) (GenBank accession no. AB2346408, residues 1 to 101 of the mature protein) were synthesized by Shanghai Invitrogen Life Technologies and then cloned into pET21a (+) vectors (Novagen) and expressed in Escherichia coli BL21 (DE3). The recombinant Anpl-UAA*01 and b2m were expressed in inclusion bodies and purified as previously described (61, 62). Finally, the Anpl-UAA*01 heavy chain and Anpl-b2m bodies were separately dissolved in 6 M guanidinium chloride buffer to a protein concentration of 30 mg/ml.

Assembly of the pAnpl-UAA*01 complex. To assemble the pAnpl-UAA*01 complex, the peptide, Anpl-UAA*01, and Anpl-b2m inclusion bodies were refolded (in a 1:1:1 molar ratio) according to the gradual dilution method that we described previously (39, 63). After a 24-h refolding step at 277 K, the remaining soluble portion of the complex was concentrated and purified using a Superdex 200 16/60 column (GE Healthcare), followed by Resource Q anion-exchange chromatography (GE Healthcare). Purified proteins were buffer exchanged with 10 mM Tris-HCl and 50 mM NaCl at a pH of 8.0.

Crystallization and data collection. The purified pAnpl-UAA*01 complex was ultimately concentrated to 10 mg/ml. After mixing with reservoir buffer at a 1:1 ratio, the purified protein was crystallized using the sitting-drop vapor diffusion technique at 277 K. Index, Crystal Screen I/II, and Crystal Screen Cryo I/II kits (Hampton Research, Riverside, CA) were used to screen for optimal crystal growth conditions. After several days, crystals (pAnpl-UAA*01–MVM9 and pAnpl-UAA*01–RLI9) were observed with solutions NO.7 from the Crystal Screen Cryo II kit (8% polyethylene glycol 1000, 8% [wt/vol] polyethylene glycol 8000, and 20% [vol/vol] glycerol) and NO.43 from the Crystal Screen Cryo I kit (24% [wt/vol] polyethylene glycol 1500 and 20% [vol/vol] glycerol), respectively. Diffraction data for pAnpl-UAA*01 crystals were collected to resolutions of 1.71 Å (pAnpl-UAA*01–MVM9) and 2.06 Å (pAnpl-UAA*01–RLI9) at the Shanghai Synchrotron Radiation Facility (SSRF) using beamline BL17U at a wavelength of 1.5418 Å (Shanghai, China) (64). The crystals were first soaked in reservoir solution containing 25% glycerol as a cryoprotectant and were then flash-cooled in a stream of gaseous nitrogen at 100 K (65). The collected
nant mutants were expressed as inclusion bodies and further purified as described above. The mutant purified by gel filtration and anion-exchange chromatography as described above (39,63).

In addition, all of the mutant pAnpl-UAAs were solved by molecular replacement with the chicken BF2 (PDB code 4E0R) as the search model using the program CONTACT and were defined as residues containing an atom within 3.3 Å of the target partner (69). Structural illustrations and electron density-related figures were generated using the PyMOL molecular graphics system (http://www.pymol.org/). Solvent-accessible surface areas and the B factor were calculated with CCP4.

Preparation of Cys-to-Ala mutants of pAnpl MHC I. To investigate the function of the additional disulfide bond in Anpl-UAAs, Cys95 and Cys112 were mutated to Ala by overlap PCR (the primers used for Cys95-to-Ala mutation were 5’-GGCAGGGATGCTGGCGTG-3’ and 5’-GCCATGCTGCGCTGCCATG-3’ and those for Cys112-to-Ala mutation were 5’-CAACAGGGGCTATGAGGG-3’ and 5’-CCCCATCGTAGCGGTTGCT-3’; where the underlined sequences mutated the codon encoding Ala). These mutants were inserted into the pET21a vector and expressed in BL21(DE3) cells. The mutants were termed Anpl-UAAs-C95A, Anpl-UAAs-C112A, and Anpl-UAAs-C95A-C112A, respectively. Recombinant mutants were expressed as inclusion bodies and further purified as described above. The mutant inclusion bodies were refolded with Anpl-β2m using the in vitro gradual dilution method as described above (61, 62). In addition, all of the mutant pAnpl-UAAs were formed by refolding were further purified by gel filtration and anion-exchange chromatography as described above (39, 63).

CD spectra and thermal unfolding. Circular dichroism (CD) experiments for the pAnpl-UAAs with parental RL19 or mutant peptides were performed on a Jasco J-810 spectropolarimeter equipped with a water-circulating cell holder. The CD spectra were collected at a protein concentration of 8 mM in pH 8.0 Tris buffer (20 mM Tris and 50 mM NaCl), using a 1-mm-optical-path-length cuvette with ellipticity at 218 nm. Thermal denaturation curves were determined as the temperature was raised from 25 to 80°C at a linear rate of 1°C/min. The temperature of the sample solution was directly measured with a thermistor. The fraction of unfolded protein was calculated from the mean residue ellipticity (θ) using the standard method. The unfolded fraction (%) was expressed as (θ - θN)/θH - θN), where θN and θH are the mean residue ellipticity values in the fully folded and fully unfolded states, respectively. The midpoint transition temperature (Tm) was determined by fitting data to the denaturation curves using the Origin 9.1 program (OriginLab) (73).

Accession number(s). The coordinates and structure factors for pAnpl-UAAs-RL19 and pAnpl-UAAs-MVM9 have been deposited in the Protein Data Bank (http://www.rcsb.org/pdb/home/home.do) under accession numbers 5GJX and 5GJY, respectively.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JVI.02511-16.

SUPPLEMENTAL FILE 1, XLSX file, 0.1 MB.

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We declare no conflict of interest.

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