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Molecular Mechanisms of Influenza Inhibition by Surfactant Protein D Revealed by Large-scale Molecular Dynamics Simulation†

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Abstract

Surfactant protein D (SP-D), a mammalian C-type lectin, is the primary innate inhibitor of influenza A virus (IAV) in the lung. SP-D interactions with highly branched viral N-linked glycans on hemagglutinin (HA), an abundant IAV envelope protein and critical virulence factor, promote viral aggregation and neutralization through as yet unknown molecular mechanisms. Two truncated human SP-D forms, wild-type (WT) and double mutant D325A+R343V, representing neck and carbohydrate recognition domains are compared in this study. Whereas both WT and D325A+R343V bind to isolated glycosylated HA, WT does not inhibit IAV in neutralization assays; in contrast, D325A+R343V neutralization compares well with full-length native SP-D. To elucidate the mechanism for these biochemical observations, we have solved crystal structures of D325A+R343V in the presence and absence of a viral nonamannoside (Man9). Based on the D325A+R343V/Man9 structure and other crystallographic data, models of complexes between HA and WT or D325A+R343V were produced and subjected to molecular dynamics. Simulations reveal that whereas WT and D325A+R343V both block the sialic acid receptor site of HA, the D325A+R343V complex is more stable, with stronger binding due to additional hydrogen bonds and hydrophobic interactions with HA residues. Furthermore, the blocking mechanism of HA

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Supporting Information Available

This information addresses the treatment of calcium ion coordination in the lectin site for molecular dynamics simulations and more details on simulation DMNC. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Atomic coordinates have been deposited with the Protein Data Bank with code numbers 4M17 and 4M18.

differs for WT and D325A+R343V due to alternate glycan binding modes. The combined results suggest a mechanism through which the mode of SP-D/HA interaction could significantly influence viral aggregation and neutralization. These studies provide the first atomic-level molecular view of an innate host defense lectin inhibiting its viral glycoprotein target.

Influenza A virus (IAV) poses a major global biothreat to humans and animals. Newly emerging viral strains and viruses from animal reservoirs are responsible for IAV outbreaks that cause widespread illness before vaccines can be developed. Initial host defense is provided by the innate immune system, which can neutralize novel viral strains without prior recognition. One of the host's innate strategies against influenza relies on *collagenous lectins* (collectins), which are present in respiratory lining fluids where they participate in front-line defense against pathogens. Pulmonary collectins, including surfactant protein D (SP-D), are calcium-dependent (C-type) mammalian lectins that are involved in a wide range of immune functions¹⁻⁴. SP-D, a pattern recognition receptor present in mucosal secretions, targets glycoproteins on viruses, bacteria, fungi, yeast and allergens^{5, 6}. SP-D mediates a wide range of anti-influenza activities⁷⁻¹², including inhibition of hemagglutination, viral aggregation and neutralization. The major IAV target for SP-D is the viral glycoprotein hemagglutinin (HA), a major virulence factor which packs densely on the viral surface. HA plays a central role in influenza infection. It is responsible, through its sialic acid receptor site, for attaching IAV to sialylated glycoproteins on host cells and facilitating entry of the viral genome. HA on its own surface expresses high-mannose glycans, particularly a highly-branched nonamannose (Man9), which are recognized by SP-D through lectin activity. There is accumulating evidence that increased virulence of HA strains is correlated with evasion of SP-D innate surveillance through loss of HA glycosylation^{8, 9, 13, 14}.

Collectins are secreted multi-domain C-type (calcium-dependent) lectins with a short N-terminal domain, a collagen-like domain, an alpha-helical neck domain, and a carbohydrate recognition domain (CRD), which contains the lectin site. Recombinant production of the neck and carbohydrate recognition domain (NCRD) yields a trimeric unit with lectin activity, capable of binding saccharides and pathogens. Interestingly, whereas wild-type human SP-D NCRD (WT) binds saccharides and HA but exhibits poor antiviral activity¹⁵⁻¹⁷, gain-of-function NCRD mutants can be engineered to perform such activities at levels approaching or exceeding those of native collectins¹⁶. These mutants are able to aggregate IAV particles, which is surprising given that they lack the N-terminal and collagen domains of SP-D which are associated with oligomeric assembly, increased avidity, and the neutralizing and aggregating activity of native SP-D¹⁶. This attribute of these mutants was unexpected and up to now unexplained. The mutants have provided a valuable probe into correlations between HA interactions and antiviral activity *in vitro* and *in vivo*. For example, we have reported that unlike WT, D325A+R343V inhibits seasonal and poorly glycosylated pandemic strains of IAV and protects against influenza infection when administered intra-tracheally to mice¹⁶.

Molecular recognition between viral glycoproteins and lectins involved in the innate immune response is poorly understood; little is known beyond the basic knowledge that carbohydrate targets are involved. Although dozens of crystal structures of HA and of several complexes with antibody Fab fragments have been reported¹⁸⁻²¹, no protein-HA complexes with innate host defense proteins have been studied at atomic level detail. Previous computational studies of lectins with anti-influenza activity, namely of SP-D^{22, 23}, mannan-binding lectin²⁴ and galectins^{25, 26}, have focused on complexes with isolated carbohydrate ligands rather than with viral glycoproteins. Similarly, computational studies

on hemagglutinins²⁷⁻²⁹ have not addressed binding to lectins; a major barrier has been the large size of the system, necessitating advanced computational approaches.

In the present studies, we have used x-ray crystallography, molecular modeling and large-scale molecular dynamics to probe the interactions between SP-D and HA at the atomic level. Our studies target HA from the A/Aichi/68/H3N2 strain of IAV (also known as X31). This HA has only one N-linked glycan on the head domain, at residue 165, an important glycosylation site location for H3N2 strains⁷, as lack of this residue is associated with collectin-resistance⁹. We demonstrate that native SP-D possesses antiviral activity against the Aichi strain, indicating that the 165 N-glycan is sufficient for SP-D recognition of IAV. We have shown previously that SP-D does not inhibit PR-8 (A/Puerto Rico/8/1934/H1N1), an IAV strain that has no HA head glycans, and that SP-D strongly inhibits Phil82 (A/Philippines/1982/H3N2), which has over 90% sequence identity with Aichi and contains the 165 N-linked glycan among other head glycans⁹. The present studies offer the first computational investigation involving a complex of a full hemagglutinin trimer and a trimeric NCRD of SP-D. Additionally, the binding affinity between the HA glycan and SP-D has been studied and compared with experimental data. Comparisons between WT and D325A+R343V SP-D NCRDs allow the linking of viral neutralization to molecular properties underlying HA/SP-D binding, thereby promoting a greater understanding of the recognition of IAV by innate host defense molecules.

EXPERIMENTAL PROCEDURES

Preparation of materials

Recombinant human SP-D NCRD was prepared as described previously^{30, 31} with an N-terminal S tag for the neutralization assays, or as a tagless molecule with an additional truncation of 7 residues at the amino terminus of the neck region to facilitate crystallization³⁰. The Aichi (A/Aichi/68/H3N2) strain was obtained from American Type Culture Collection, propagated in hens' eggs, and purified as described⁸. The synthetic nonamannoside (Man9) with a methyl group rather than Asn at the reducing end was prepared as described³², and the analytical data are in agreement with previous reports³³.

Fluorescent focus assay of IAV infectivity

MDCK cell monolayers were prepared in 96 well plates and grown to confluency. These layers were then infected with diluted IAV preparations for 45 min at 37°C in PBS with 2mM calcium and magnesium. After the 45-min incubation, the cells are washed with MEM media containing 10% FBS and 1% P/S. They are then incubated for an additional 6 hrs and 15 min in this media. MDCK cells were tested for presence of IAV infected cells after 7 hrs of virus addition using a monoclonal antibody directed against the influenza A viral nucleoprotein (provided by Dr. Nancy Cox, CDC, Atlanta, GA) as previously described (22). IAV was pre-incubated for 30 min at 37°C with various concentrations of collectins or control buffer, followed by addition of these viral samples to the MDCK cells. Similar assays were also performed using undifferentiated primary human tracheo-bronchial epithelial cells obtained from ATCC and cultured as per manufacturer's recommendations.

Crystal Structure Determination

Crystals of D325A+R343V were grown in hanging drops by mixing equal amounts of purified D325A+R343V (12-15 mg/ml in 20 mM HEPES buffer, pH 7.5, 150 mM NaCl, and 10 mM calcium acetate) and reservoir solution (0.1 M HEPES buffer, pH 7.5, 0.25 M NaCl, and 20-22% w/v PEG 3350) on a siliconized coverslip and equilibrating over a sealed well containing 0.5 ml reservoir solution at 17°C. Large crystals grew within a few days. For ligand soaking, crystals were transferred to reservoir solution in which solid Man9 was

dissolved at a concentration of 0.5 mM or 10 mM. Crystals were soaked for either 2 hours (0.5 mM Man9) or overnight (10mM). Prior to freezing in a cold nitrogen stream, the crystals were soaked in the Man9 solution with either 1 M 1,6-hexanediol (0.5 mM Man9) or 15% v/v ethylene glycol (10mM) for about 20 seconds as a cryoprotectant. X-ray diffraction data were collected on an RAXIS-IV image plate detector with an RU-300 rotating anode x-ray generator. Data integration, indexing, merging and scaling were performed using the programs DENZO and Scalepack³⁴. The structure was solved by molecular replacement using the previously solved structure of R343V SP-D complexed with α -1,2-dimannose, less the waters and ligands and incorporating the expected D325A mutation as well as the truncation of the construct, as a search model using the AutoMR program in Phenix³⁵. The solved structure contains four trimers. The low resolution structure was solved by difference Fourier transformation using the high resolution structure as a starting model. Iterative cycles of manual rebuilding and refinement were performed using Coot³⁶ and Phenix. In both cases, metal restraints were used throughout refinement as defined by ReadySet in Phenix. For the low resolution structure, non-crystallographic symmetry restraints on the protein torsion angles were used throughout. In the final steps of refinement for the liganded structure, TLS parameters were refined for both structures with two zones per monomer used – one for the neck (residues 214-232) and one for the CRD (residues 233-355).

Molecular modeling

To create the starting model for molecular dynamics calculations, coordinates from three crystal structures were assembled to form an initial HA-Man9-SPD complex. The model employed the HA crystal structure from the A/Aichi/68/H3N2 strain (PDB code 1HGE)³⁷. This structure was selected because it has only one glycan on the HA head at N165. The first three sugars of this N-linked glycan (residues NAG500, NAG501, and BMA502 in Figure 1) are visible in the crystal structure, and the remainder of the complex was built onto this glycan. To attach the SPD trimer, the mannose chain present in the D325A+R343V crystal structure (comprising MAN505A, MAN504A, and MAN503A) was extended by one mannose (equivalent to residue BMA 502) using the computational tools in Maestro (Schrodinger, LLC, New York, NY, USA). This added mannose was then superimposed onto the BMA 502 residue from chain A of the HA crystal structure. Removal of the added mannose from the SPD model resulted in a HA-Man4-SPD complex. To complete the remaining 5 mannoses, the BMA502 residue of the visible Man8 chain from an Fab-Man9 crystallographic complex (PDB code 3TV3³⁸) was superimposed onto the BMA 502 of the HA model, and MAN503B, MAN504B, MAN504C, and MAN505C from the superimposed coordinates were added to the model. To complete the HA-Man9-SPD model, MAN505B was added with Maestro. The glycosidic bonds of sugars added in Maestro were set to initial torsion angles of -60° and 0° for ϕ and ψ respectively, values which are in the favored region of the conformational energy map for the dimannose linkages created³⁹ and which introduce no major steric clashes with other atoms in the model. Figure 1 shows the resulting model of the Man9 component of the D325A+R343V/HA complex. This model was the only one consistent with the crystallographic data and favorable glycosidic linkages.

Since the objective of the molecular dynamics (MD) simulations is to compare the WT/HA and D325A+R343V/HA complexes, the starting coordinates of both models need to be chosen as similar as possible. In wild-type or mutant SP-D crystal structures, residues at positions 325 and 343 do not interact in any way, nor is there any evidence that the side chains contribute to the overall conformation of the lectin site^{15, 16, 40}. Therefore, we used the coordinates of the D325A+R343V/HA complex as a template to obtain the starting model for WT. We restored the WT structure by mutating Ala325 and Val343 back to Asp325 and Arg343, respectively. These mutations were made using VMD⁴¹. Additionally,

the Man9 chain had to be remodeled because WT binds to the reducing end of the mannose-9 (Man9) chain (circle in vertical stripes), while D325A+R343V binds to the non-reducing end (circle in horizontal stripes) as shown in the schematic diagram of Figure 1.

To model the Man9 chain in the WT/HA model, two mannose residues (MAN504A and MAN505A), which interact with the lectin site of SP-D, were replaced by two corresponding mannose residues found in the WT/dimannose complex (PDB ID 3G83,¹⁵) The latter two residues were manually connected to the remaining 7 mannose residues, resulting in a long bond connecting MAN503A and MAN504A. To correct this long bond, energy minimization was performed for 10,000 steps on the Man9 chain while restraining the coordinates of MAN504A, MAN505A and the protein complex. All mannose residues in the minimized complex were found in our calculations to be in the stable chair conformation, indicating that the rearrangement of the Man9 chain did not introduce large steric and torsional strains onto the chain.

Molecular Dynamics (MD) Simulations

Figure 2 shows the protein system that was simulated for the present study: the HA trimer glycosylated with one Man9 and complexed with one trimer of SP-D (either WT or D325A+R343V) with bound calcium ions. Table 1 lists size and timescale of the simulations carried out in the present study. Each complex was solvated in a water box chosen large enough, so that the minimum distance between the complex and the boundary of the water box is 15 Å along all three axes. The system was neutralized with 150 mM NaCl to match the optimum salt concentration present in *in vitro* experiments⁴². The final simulated systems contain approximately 320,000 atoms, including proteins, water molecules and ions.

The systems described were subjected to conjugate gradient minimization for 50,000 steps and subsequently heated to 310 K in a time less than 4 ps. The procedure was followed by 0.5 ns equilibration with all protein and sugar atoms fixed under an NPT ensemble, and 2 ns equilibration under an NVT ensemble. Finally, 100 ns production simulations under an NVT ensemble were performed. To ensure that the starting structures retain the key interactions, the hydrogen bond network and other non-covalent interactions between O3, O4 of the calcium-bound mannose residue and the SP-D protein were maintained by imposing virtual springs to constrain their distances. The constants of these virtual springs were set to 50 kcal/mol/Å² during minimization, to 20 kcal/mol/Å² during NPT ensemble equilibration and to 10 kcal/mol/Å² during NVT ensemble equilibration. The constraints were removed in the final 100 ns simulation.

All coordinated ligands of the calcium ion were restrained to circumvent potential force field limitations and preserve the bidentate property of calcium-bound mannose (see Supporting Information). All 8 coordinated ligands were constrained with a harmonic potential of the form $U(x) = k(x - x_{\text{ref}})^2$ where k was 1000 kcal/mol/Å and x_{ref} was set to 2.41 Å⁴³ throughout minimization, heating, equilibration and final 100 ns simulations. The constraint is a reasonable approach given the sub-microsecond timescale of our simulations; unbinding rates of calcium-bound ligands are measured experimentally to be on the order of seconds to minutes⁴⁴ such that unbinding, impossible with the constraints imposed, is expected to be very rare..

Simulations were performed using the program NAMD 2.9⁴⁵ assuming the CHARMM27 force field with CMAP corrections⁴⁶ for the protein and assuming the TIP3P model for water molecules⁴⁷. The CHARMM36 force field was used for the sugars^{48, 49}. Periodic boundary conditions were assumed, and the particle-mesh-Ewald summation method was employed for the evaluation of Coulomb forces. The van der Waals energy was calculated using a cutoff of 12 Å. Temperature and pressure were maintained at 310K and 1 atm using

a Langevin thermostat with a damping constant of 1 ps^{-1} and Nosé-Hoover Langevin piston methods⁵⁰. The integration time step was 1 fs.

Residue-based interaction energies between SP-D and HA were calculated using a modified generalized-Born (GB) model⁵¹. This model considers van der Waals interaction, electrostatics, and a solvation energy component that takes solvent-polarization into account. The solvent model did not include hydrophobic interactions, unlike the GB-molecular surface area approach⁵². However, the GB model is a sufficiently good approach to quantify the interaction energies since the interactions between SP-D with HA and man9 involve mostly polar residues.

RESULTS

Fluorescent Focus Assay

We used a fluorescent focus assay for viral neutralization to compare the ability of full length SP-D and SP-A and the various NCRD preparations to inhibit infectivity of the H3N2 pandemic IAV strain Aichi68 in vitro. We previously reported that D325A+R343V had equivalent neutralizing activity as native SP-D for a seasonal H3N2 strain¹⁶. As shown in Figure 3A, D325A+R343V strongly inhibited the Aichi68 strain in MDCK cells. The strong activity of D325A+R343V was confirmed in primary HTBE cells as well (Figure 3B). The wild type hNCRD had no inhibitory activity in either assay. We note that D325A+R343V also had stronger neutralizing activity than either R343V or native SP-D or SP-A. The 50% neutralizing concentration for D325A+R343V was $\sim 0.25 \text{ }\mu\text{g/ml}$, whereas the corresponding concentration for native SP-D, the next best inhibitor, was $\sim 0.50 \text{ }\mu\text{g/ml}$.

D325A+R343V Crystal Structures

The crystal structure of D325A+R343V was solved unliganded at $2.1 \text{ }\text{\AA}$ resolution and in complex with Man9 at $3.2 \text{ }\text{\AA}$ resolution. The final structures show good geometry and agreement to the diffraction data (Table 2). The D325A+R343V crystals are in a different space group than R343V or WT¹⁵ and have more monomers in the asymmetric unit (12 versus 3). In the D325A+R343V complex, only three mannose sugars are visible in the electron density map, all linked with α 1-2 glycosidic bonds and therefore these sugars have been assigned as Man505A, Man504A, and Man503A of Man9 based on the connectivity. The third sugar (Man503A) is less well defined in the electron density maps (and absent in two of the monomers in the asymmetric unit) and occupies space made available by the Asp325 to Ala mutation. Ligands are oriented similarly in all twelve subunits.

The unliganded and complexed D325A+R343V structures are similar, with a root-mean-square-deviation (r.m.s.d.) of $0.2\text{--}0.6 \text{ }\text{\AA}$. The protein portion of the D325A+R343V complex is structurally similar to those in the WT and R343V single mutant dimannose complexes¹⁵. The superimposed monomers have root-mean-square-deviation (r.m.s.d.) values of $0.4 \text{ }\text{\AA}$ – $0.7 \text{ }\text{\AA}$, calculated using all protein alpha carbons in the NCRD for the two available structures (PDB identifiers 3G83 and 3G84).

In the D325A+R343V complex with Man9, the ligand is bound in a similar conformation and location as in the α 1,2-dimannose complex with R343V. In D325A+R343V, as for R343V, one sugar (Man504A) is bound in the lectin site, making coordination interactions to the calcium ion through the 3 and 4 hydroxyl groups of the mannose sugar. The non-reducing end sugar (Man505A) is bound above Val343. Overall, the Asp325 to Ala mutation shows little effect on the binding of dimannose: the sugars have r.m.s.d. values between 0.4 – $1.0 \text{ }\text{\AA}$ calculated using all non-hydrogen dimannose atoms in the superimposed single and double mutant protein structures.

MD Simulations

In simulations WTS and DMS (Table 1), both WT and D325A+R343V are found to be stably bound to HA. Furthermore, both SP-D proteins are seen to block the sialic acid binding site of HA (Figure 4). To quantify the inhibition of the sialic acid binding site by SP-D, the sialic acid (SA) accessible surface area (ASA) was calculated using the rolling ball algorithm⁵³. Instead of using the radius of a water molecule, i.e., 1.4 Å for the rolling probe, we used the radius of gyration of sialic acid, i.e. 3.0 Å, to evaluate the ASA. As shown in Figure 5, both WT and D325A+R343V resulted in similar SA ASA values of approximately 150 Å² at the end of either simulation, carrying out in each case an average over three independent trajectories. At the beginning of the simulation, the SA binding site was only partially blocked by SP-D as reflected in an SA ASA value of 200 Å². The decrease of SA ASA over time, implying that the SA binding site becomes less accessible to SA in the course of the simulation. Given that the SA ASA for a fully exposed SA binding site is approximately 450 Å², it is apparent that both WT and D325A+R343V SP-D fully block the SA binding site at the end of the simulations.

The interaction energies between SP-D and HA residues were also monitored. The average total interaction energies between SP-D with HA and the Man9 chain are -123 ± 8 kcal/mol (WT) and -142 ± 14 kcal/mol (D325A+R343V); the interaction energies between SP-D and the Man9 chain alone are -87 ± 7 kcal/mol and -97 ± 5 kcal/mol for WT and D325A+R343V, respectively. Table 3 shows that the mannose residue, which is directly bound to the SP-D lectin calcium ion, accounts for about half of the total interaction energy of the protein complex. Such a significant contribution to this interaction underscores that the sugar-calcium ion interaction at the lectin site is essential for SP-D binding to saccharide ligands.

Two key loop regions were seen in the simulations to inhibit the sialic acid binding site of HA. Table 4 lists interaction energies for different residues of SP-D. Residues 300 to 304 in the short loop (residues 297-304) show lower interaction energies for D325A+R343V, while residues 324 to 329 in the long loop (residues 307-331) indicate lower energies for WT. Residues 300 to 304 of D325A+R343V are observed to inhibit the SA binding site while residues 324 to 329 of WT inhibit this site. Residues 300 to 304 and 324 to 329 are highlighted in blue and tan, respectively, in Figure 4.

In the simulations the binding of HA with D325A+R343V, but not with WT, reveals involvement of hydrophobic interactions. Figure 6A illustrates the difference in average hydrophobic interaction between SP-D and HA for WT and D325A+R343V. As shown in Figure 6B, Ala325 of D325A+R343V is observed to interact with another hydrophobic residue of HA, Trp222. Additionally, Pro319 of D325A+R343V interacts with Ala138 of HA throughout the entire trajectory. It is evident that the Asp-to-Ala mutation of residue 325 increases the hydrophobic interaction between D325A+R343V and HA, and provides additional stabilization to the complex. This hydrophobic interaction may provide a basis for the enhanced HA binding by the D325A+R343V mutant compared with the HA binding by the R343V mutant.

As shown in Figure 8, the charged residues Arg343 and Asp325 in WT stabilize Man9 chain binding through formation of hydrogen bonds. As a result of the two mutations, the Man9 chain becomes engaged in a strong hydrogen bond formed between Glu333 and MAN505A.

Despite the stability of the SP-D/HA complexes in the presence of Man9, SP-D is seen to diffuse away from the SA binding site within the first 40ns in the absence of the glycan as shown in Figure 7, though D325A+R343V without glycan is observed to diffuse back to its starting location. These results justify the timescale of the simulations, i.e., if an SP-D/HA

complex is not stably bound together, the SP-D has sufficient time in our simulations to leave the SA binding site by diffusion.

The 7 ns-long simulation DMNC showed that SP-D with its calcium ions removed became dissociated from the Man9 chain in 7 ns (see Supporting Information), illustrating the importance of the calcium ion for the function of SP-D.

DISCUSSION

We have undertaken a crystallographically-informed molecular dynamics approach to investigating SP-D interaction with HA glycans and its influence on antiviral activity. NMR and molecular dynamics approaches^{54, 55}, together with statistical analyses⁵⁶⁻⁵⁸, show that despite the inherent flexibility of glycosidic linkages, branched high-mannose glycans such as those found on viral surfaces have a limited number of overall conformations. These structures can be recognized by pattern recognition receptors such as C-type lectins of the innate immune system. Binding of mannosyl oligosaccharides has been explored structurally for several C-type lectins, including SP-D^{15, 40}, mannose binding protein⁵⁹⁻⁶³, dendritic cell surface receptor DC-SIGN and endothelial cell receptor DC-SIGNR⁶⁴⁻⁶⁸, all of which recognize N-linked glycans on viral pathogens such as influenza and HIV. The crystallographic complexes are similar overall in that the major point of contact between sugar and protein is at the lectin site; significant contacts with other lectin residues are sparse. Nonetheless, nearby residues, e.g. Asp325 and Arg343 in SP-D^{15, 40}, influence spatial accessibility for portions of the branched oligosaccharide chains, thereby imposing a degree of selectivity. Our previous studies of SP-D have shown that mutating these residues greatly influences antiviral activities, with D325A+R343V gaining significant functional capabilities compared with WT. Therefore our studies focus on the dynamic properties of both WT and mutant SP-D with respect to N-glycan binding and HA inhibition.

Our computational studies of SP-D binding to Aichi HA show excellent agreement with experimental data for SP-D binding to HA from Phil82, another H3N2 strain containing glycan 165. The critical importance of the lectin activity in the interaction is underscored in two sets of simulations, WTNS and DMNS, in which both WT/HA and D325A+R343V/HA complexes exhibit significant fluctuation and drifting in 100ns when the Man9 chain is absent (Figure 7). Similar results were obtained in simulations with porcine SP-D²². In prior SPR experiments with Phil82 HA, D325A+R343V was found to bind more tightly than WT, with mean apparent K_d values of 0.04 nM versus 0.231 nM, respectively¹⁶. The computational results with Aichi HA are consistent with the experimental Phil82 HA data in that while WT and D325A+R343V both bind to the viral glycoprotein, D325A+R343V binds with higher affinity (Table 4). The computational results further suggest that the greater HA binding by D325A+R343V may be due, in part, to higher glycan binding affinity. Table 3 shows that compared with WT, D325A+R343V binds more strongly to the Man9 chain by approximately 10 kcal/mol. These results are consistent with carbohydrate microarray experiments in which R343V, which shares the same arginine mutation as D325A+R343V, binds more strongly to branched mannose oligosaccharides than does WT¹⁵.

The nature of the mutations appears to play a role in the differences between WT and D325A+R343V in binding Man9. Table 3 shows a difference in interaction energies for two neighboring mannose residues, namely MAN505A/MAN503A for D325A+R343V, and MAN504A/MAN503A for WT. These data suggest that an increase in interaction energy at MAN505A is due to the Arg343 to Val mutation, consistent with structural evidence¹⁵. Detailed hydrogen bond searching allows determination of the key residue that interacts most strongly with the extended glycan chain. Formation of hydrogen bonds between

Glu333/Arg349 and MAN505 (labeled in Figure 8) is potentiated by the Arg343 to Val mutation, which removes the steric hindrance caused by Arg343. Molecular dynamics simulations are able to reproduce this feature and suggest that a hydrogen bond between MAN505 and Glu333 is more likely to form than between MAN505 and Arg349. A decrease in interaction energy at MAN503A suggests that the Asp325 to Ala mutation stabilizes the binding of the Man9 chain on SP-D. Ala325 is in close proximity to MAN503A suggesting loss of a steric constraint due to the Asp325 to Ala mutation. However, simulation SMS with the single mutant R343V does not support this hypothesis as it shows that Asp325 interacts strongly with MAN503A due to the formation of hydrogen bonds. Further, crystallographic data regarding the role of Asp325 are obscured by this residue's involvement in crystal contacts⁴⁰. Therefore, the underlying molecular mechanism of the effect of the Asp325 to Ala mutation on Man9 binding remains unclear.

Mannose interactions are not the only component of D325A+R343V binding to HA. From the interaction energies calculated from simulations WTS and DMS, D325A+R343V binds more strongly than WT to HA by approximately 19 kcal/mol. After subtracting the interactions between SP-D and the Man9 chain, it is observed that D325A+R343V still interacts more strongly with HA by an average of 9 kcal/mol. This difference may be explained by protein-protein interactions, particularly hydrogen bonds that form between the short and long loops of SP-D and the HA surface. It is important to note that the interaction energies do not account for hydrophobic contributions and that D325A+R343V/HA has higher interaction energy than WT/HA if one takes only electrostatic interactions into account. As shown in Figure 5, D325A+R343V forms hydrophobic contacts with HA, but WT does not. As a result, D325A+R343V binds more strongly to HA than does WT by more than 19kcal/mol. The observation of hydrophobic interactions in the DMS simulation is novel in that protein-protein interactions in mammalian lectins have not been widely described.

The key loop interactions with the SA binding site of HA are different for WT and D325A+R343V. As seen in Figure 4, Table 4, and the interaction energies between SP-D and HA calculated for simulations WTS and DMS and shown in Figure 5, residues 324-329 form the primary, long loop for WT that interacts with HA and inhibits its SA binding site. Interestingly, for pig SP-D, there is an insertion of three amino acid residues between residues 328 and 329⁶⁹. The elongation increases the flexibility of the loop and may give rise to the enhanced inhibition of pig SP-D activity against IAV^{22, 30, 70}. However, in contrast to WT and pig SP-D, the long loop of D325A+R343V contributes less to the overall stabilization of the complex; instead, in the D325A+R343V complex, short loop residues 300-304 interact primarily with HA at the SA binding site. Although the interaction energies for residues on the short loop fluctuate, the importance of the short loop for D325A+R343V could still be harnessed by averaging the energies over three independent trajectories.

In addition to using different loops, WT and D325A+R343V exhibit different modes of glycan binding that impact their interactions with the HA trimer. In crystallographic studies of α -1,2-dimannose complexes with WT or R343V, a reversed mode of dimannose binding is observed between the two proteins¹⁵. In WT, the non-reducing end of mannose (MAN505A) is bound to the lectin site. In the mutant, MAN505A occupies space vacated by the mutation and the reducing end mannose (MAN504A) is bound in the lectin site, where it makes similar interactions as does MAN505A in the WT complex. MAN504A in R343V is rotated 180° such that its O3 and O4 atoms are swapped compared to MAN505A in WT (Figure 8). In the present liganded D325A+R343V crystal structure, the double mutant binds to the dimannose moiety of Man9 in a manner similar to the binding of dimannose to R343V, providing further evidence that the Arg343 to Val mutation is responsible for the

altered binding mode. Figure 9 shows how these different binding modes alter the overall orientation of the modeled HA-SP-D complexes.

We have proposed previously that in D325A+R343V, viral aggregation can be achieved by SP-D-mediated cross-linking of HA on neighboring viral particles. Experimental evidence from that study shows that trimeric D325A+R343V NCRD is nearly as effective at aggregating IAV as is native dodecameric SP-D, whereas the WT NCRD trimer does not aggregate or neutralize the virus¹⁶. These observations suggest that D325A+R343V is able to utilize a mechanism that circumvents the need for an extended oligomer to aggregate viral particles. Significant differences in spatial orientation adopted by WT and D325A+R343V, such as those seen in Figure 9, could provide such a mechanism by altering the ability to cross-link HA and promoting viral aggregation and neutralization.

In summary, simulation of entire SP-D/HA complexes has allowed us to investigate the detailed molecular interactions between SP-D and IAV HA. Using modeling and molecular dynamics approaches, we have determined that SP-D not only binds to the glycan chains on HA, but additionally blocks the SA binding site upon binding to the glycan chains for the H3N2 strain that we studied. To test our hypotheses, site-directed mutants will be made in the short and long loops to evaluate the *in vitro* and *in silico* properties of individual residues, e.g., systematic mutation of Ala325 to residues that vary in charge and size should alter interactions with Trp222 from HA. We also plan to investigate HA from other strains to test the generality of our conclusions. For example, HA glycan 104, a functionally important site found in most H1N1 strains, is also located near the SA site and may similarly block it. We additionally have suggested means through which binding of HA glycans by D325A+R343V could lead to increased viral neutralization. Based on experimental and computational evidence, we speculate that altered binding orientation and increased viral aggregation, perhaps coupled with the increased binding to HA by the mutant, enhance the ability of D325A+R343V to neutralize IAV. Viral aggregation studies of novel mutants should address this speculation. These studies thus shed light on how factors associated with HA binding correlate with antiviral activities of SP-D.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Aichi	A/Aichi/68/H3N2
ASA	accessible surface area
CMA	correction maps
CRD	carbohydrate recognition domain
HA	hemagglutinin
HTBE	human respiratory epithelial cells
IAV	influenza A virus
Man	D-mannose

Man9	methyl α -D-Mannopyranosyl-(1R2)- α -D-mannopyranosyl-(1R2)- α -D-mannopyranosyl-(1R3)-[α -D-mannopyranosyl-(1R2)- α -D-mannopyranosyl-(1R3)-[α -D-mannopyranosyl-(1R2)- α -D-mannopyranosyl-(1R6)]- α -D-mannopyranosyl-(1R6)]- α -D-mannopyranoside
MDCK	Madin-Darby canine kidney
MD	molecular dynamics
NAG	N-acetylglucosamine
NCRD	neck and carbohydrate recognition domain
NPT	constant number of particle, pressure and temperature
NVT	constant number of particle, volume and temperature
Phil82	A/Philippines/1982/H3N2
PR8	A/Puerto Rico/8/1934/H1N1
r.m.s.d.	root-mean-square-deviation
SA	sialic acid
SP-D	surfactant protein D
TLS	translation libration screw
VMD	Visual Molecular Dynamics
WT	wild-type human SP-D NCRD.

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Figure 1. Schematic diagram (left) and structure (right) of the Man9 glycan of HA

Left: Mannose (MAN) residues are represented as circles and N-Acetylglucosamine (NAG) as squares. MAN504 and MAN505 interact with calcium ions in D325A+R343V and WT and are differentiated in order to specify in the text which sugar residue actually contacts the ions directly. NAG residues connect Man9 to Asn165 of HA. Right: MAN505A (conformation as used in this work) binds to the lectin calcium ion of WT, shown as an orange sphere. Man9 and NAG are represented in licorice with oxygen red, carbon orange, nitrogen blue, and hydrogen white.

Figure 2. View of the entire simulated system

Hemagglutinin (HA) is shown in cartoon and transparent surface representation in cyan and lung surfactant protein-D (SP-D) is shown in cartoon and transparent surface representation in green. HA and SP-D are connected non-covalently through a Man9 chain, shown enlarged in the insert with the sialic acid binding site shown in surface representation in pink, and the calcium ions of SP-D as orange spheres. Water molecules and ions are not shown.

Figure 3. Viral neutralizing activity of SP-D NCRDs (WT NCRD, R343V, and D325A+R343V) and native SP-D and SP-A against Aichi68 H3N2

Aliquots of the Aichi68 viral strain were incubated with the indicated concentrations of various native collectin or NCRD preparations. The samples were then used to infect MDCK cell monolayers (panel A) or HBTE cells (panel B) and tested for infectious foci 7 hrs later using anti-nucleoprotein antibodies and fluorescence detection. WT did not inhibit Aichi68 in either cell type. R343V or native SP-D and SP-A were inhibitory (* indicates $p < 0.05$ vs. control). D325A+R343V caused significantly greater inhibition than any of the other NCRDs tested (** indicates $p < 0.02$ compared with other NCRDs and control). The results are mean $\pm$ SEM of 3 (panel A) or 4 (panel B) experiments.

Figure 4. Inhibition of the sialic acid binding site

The binding site, located on HA, is shown as a pink surface; the remainder of HA is shown in cartoon and transparent surface representation in cyan; the Man-9 chain linked to HA is shown in licorice representation in red-orange. (A) HA bound to WT, the latter shown in cartoon and transparent surface representation in green. (B) HA bound to D325A+R343V, the latter shown in cartoon and transparent surface representation in purple. Two loop regions of both WT and D325A+R343V, colored in tan and blue, are seen to cover and thereby inhibit the sialic acid binding site.

Figure 5. Accessible surface area of the HA sialic acid binding site

Shown is the time evolution of the average sialic acid binding site accessible surface area for WT (black) and D325A+R343V (grey) bound to HA.

Figure 6. Hydrophobic interaction formed between SP-D and HA

(A) Shown is the time evolution of the average hydrophobic contact area between HA and WT (black) or D325A+R343V (grey) in the simulations WTS and DMS, respectively (B) HA and D325A+R343V are shown in cartoon and transparent surface representation in cyan and purple, respectively. Hydrophobic interactions arise mainly between Ala325 and Pro319 of D325A+R343V (labeled green) with Trp222 and Ala138 of HA (labeled dark orange).

Figure 7. Role of mannose-9 chain in stabilizing the complex between SP-D and HA

A comparison of simulations DMS, DMNS, and WTNS demonstrates the role of the sugar in stabilizing the complex between SP-D and HA. Shown is the time evolution of the RMSD of SP-D relative to HA for WT (black) and D325A+R343V (grey) without the mannose-9 chain and for D325A+R343V with Man9 chain.

Figure 8. R343V mutation alters the Man9 binding mode

Figure shows the difference in hydrogen bond networks for Man9 chain binding for WT (left) and D325A+R343V (right). The red arrows point to the hydrogen bonds, which are represented as black solid lines. WT and D325A+R343V are shown in transparent cartoon representation in green and purple, respectively; D325A+R343V calcium ions are shown as orange spheres; mannose residues are shown in licorice representation with oxygen red, carbon orange and hydrogen white; protein residues are shown in licorice representation with oxygen red, carbon cyan, nitrogen blue and hydrogen white.

Figure 9. Differences in orientation in HA complexes with WT (A) or D325A+R343V (B)
The end points of three repeated simulations are overlapped for simulations WTS and DMS, respectively. Calcium ions are represented in VDW representation in orange, red and white; WT and D325A+R343V are in transparent cartoon representation in green and purple, respectively; HA is shown in cartoon representation in cyan.

Table 1
Simulations performed in this study

Simulation	Description	Time (ns)	Number of runs
WTS	Wild type, equilibration of WT/HA complex with Man9 chain	100	3
DMS	Double mutant, equilibration of D325A+R343V/HA complex with Man9 chain	100	3
WTNS	Wild type no sugar, equilibration of WT/HA complex without Man9 chain	100	1
DMNS	Double mutant no sugar, equilibration of D325A+R343V/HA complex without Man9 chain	100	1
SMS	Single mutant, equilibration of R343V/HA with Man9 chain	100	1
DMNC	Double mutant no calcium, equilibration of D325A+R343V/HA complex with Man9 chain and without calcium ions	7	1

Table 2
Crystallographic Data Collection and Refinement Statistics

	Unliganded D325A+R343V	Man9/D325A+R343V complex
Data Collection		
Space group	P 2 ₁ 2 ₁ 2 ₁	P 2 ₁ 2 ₁ 2 ₁
Unit cell dimensions (Å, °)	a = 107.524, b = 160.108, c = 160.073, α = β = γ = 90	a = 107.833, b = 159.454, c = 159.894, α = β = γ = 90
Resolution (Å)	15-2.1 (2.17-2.10)	15-3.2 (3.3-3.2)
No. of unique reflections	154671 (12482)	45301 (4406)
Completeness (%)	95.8 (78.3)	99.6 (98.1)
Mosaicity (°)	0.653	0.778
Redundancy	5.8 (4.2)	4.2 (3.5)
I / σI	13.9 (4.8)	8.2 (2.8)
Data cutoff	I > -3σ	I > -3σ
R _{merge}	0.073 (0.324)	0.169 (0.431)
Refinement		
R _{work} / R _{free}	0.1627/0.1878	0.1868 / 0.2222
No. of reflections	154598	45242
Twin fraction (operator)	0.490 (-h, 1, k)	0 (none)
No. of atoms total	14057	13487
Protein atoms	12789	13049
Calcium and Ligands	36	424
Waters	1232	14
Mean B values (Å ²)	29.9	49.2
Protein atoms	29.9	48.7
Calcium and Ligands	34.5	67.1
Waters	30.0	20.2
Deviations from ideal		
Bonds lengths (Å)	0.011	0.014
Bond angles (°)	0.572	0.753
Ramachandran plot		
Favored (%)	97.11	97.82
Allowed (%)	2.59	2.18
Outliers (%)	0	0

Table 3

Average interaction energies by residues between SP-D and mannose-9 chain only in simulations WTS and DMS. The three mannose residues nearest to the calcium ion interact strongly with SP-D. The mannose residue that directly interacts with the calcium ion of the SP-D is labeled with *.

Residue Name (WT / D325A+R343V)	Average Energy for WT (kcal/mol)	Average Energy for D325A+R343V (kcal/mol)	Difference (kcal/mol)
MAN503A	-1.9 ± 1.4	-5.1 ± 0.7	3.2 ± 2.0
MAN505A /MAN504A*	-69.0 ± 0.5	-69.6 ± 0.4	0.5 ± 0.9
MAN504A / MAN505A	-11.2 ± 5.1	-14.8 ± 1.8	3.7 ± 6.9
Remaining residues in Man9 chain	-4.9 ± 1.9	-7.7 ± 5.5	2.8 ± 7.4

Table 4

Average interaction energies by residues between SP-D and HA + Man9 chain in simulations WTS and DMS. Positive values in the difference column indicate that the corresponding residues of D325A+R343V contribute more towards the overall stability than they do for WT. The interaction energies are averages over three independent simulations.

Residue Name (WT / D325A+R343V)	Average Energy for WT (kcal/mol)	Average Energy for D325A+R343V (kcal/mol)	Difference (kcal/mol)	Label
THR300	-1.2 ± 0.3	-2.9 ± 2.1	1.7 ± 2.4	Short loop
GLU301	-5.1 ± 1.8	-6.8 ± 4.0	1.7 ± 5.8	
GLY302	-1.6 ± 1.2	-4.0 ± 0.7	2.7 ± 1.9	
LYS303	-1.6 ± 1.1	-3.8 ± 0.2	2.2 ± 1.3	
PHE304	-0.3 ± 0.1	-1.8 ± 1.1	1.5 ± 1.2	
Residue 300 to 304	-9.8 ± 4.0	-19.3 ± 5.1	9.5 ± 9.1	
ASP32	-4.5 ± 3.4	-2.4 ± 1.2	-2.0 ± 4.6	Long loop
ASP325/ALA325	-9.4 ± 2.5	-4.2 ± 0.3	-5.2 ± 2.8	
GLY326	-4.1 ± 0.8	-1.5 ± 0.1	-2.6 ± 0.9	
GLY327	-4.2 ± 2.1	-0.5 ± 0.1	-3.7 ± 2.2	
SER328	-1.6 ± 0.8	-0.3 ± 0.0	-1.3 ± 0.8	
GLU329	-12.4 ± 0.9	-7.6 ± 0.2	-4.8 ± 1.1	
Residue 324 to 329	-36.1 ± 4.8	-16.5 ± 1.5	-19.6 ± 6.3	
GLU333	0.4 ± 1.3	-14.6 ± 0.4	15.0 ± 1.7	
ARG343/VAL343	-10.7 ± 4.5	-0.8 ± 0.1	-9.9 ± 4.6	

Table 5

Occupancy percentage of hydrogen bonds between SP-D and Man9 chain during simulations WTS and DMS. During these simulations a strong hydrogen bond is formed between GLU333 and the extended mannose. The first four rows are for the main hydrogen bonds that stabilize the binding of Man9 chain at the lectin site. The last four rows highlight the difference in glycan binding between WT and D325A+R343V. All hydrogen bonds are selected among polar atoms (N, O, S, and F) with a cutoff distance of 3.5 Å and an angle tolerance of 50°.

WT / Man9 chain		
Donor	Acceptor	Occupancy
MAN505A	GLU329	100%
MAN505A	GLU321	100%
ASN341	MAN505A	88%
ASN323	MAN505A	86%
ARG343	MAN504A	24%
MAN505A	ASP325	54%
MAN504A	GLU333	0%
ARG349	MAN504A	0%

D325A+R343V / Man9 chain		
Donor	Acceptor	Occupancy
MAN504A	GLU329	100%
MAN504A	GLU321	100%
ASN341	MAN504A	92%
ASN323	MAN504A	52%
VAL343	MAN505A	0%
MAN504A	ALA325	0%
MAN505A	GLU333	100%
ARG349	MAN505A	14%