Mutagenesis of Surfactant Protein D Informed by Evolution and X-ray Crystallography Enhances Defenses against Influenza A Virus in Vivo*

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Background: SP-D plays important roles in the defense against influenza A.

Results: A recombinant SP-D with combinatorial mutations shows enhanced interactions with hemagglutinin-associated glycans and augmented antiviral activity in vitro and in vivo.

Conclusion: Exogenous forms of recombinant SP-D can rescue mice from a lethal challenge with influenza.

Significance: It may be possible to develop collectin-based interventions for influenza.

The recognition of influenza A virus (IAV) by surfactant protein D (SP-D) is mediated by interactions between the SP-D carbohydrate recognition domains (CRD) and glycans displayed on envelope glycoproteins. Although native human SP-D shows potent antiviral and aggregating activity, trimeric recombinant neck+CRDs (NCRDs) show little or no capacity to influence IAV infection. A mutant trimeric NCRD, D325A/R343V, showed marked hemagglutination inhibition and viral neutralization, with viral aggregation and aggregation-dependent viral uptake by neutrophils. D325A/R343V exhibited glucose-sensitive binding to Phil82 hemagglutinin trimer (HA) by surface plasmon resonance. By contrast, there was very low binding to the HA trimer from another virus (PR8) that lacks glycans on the HA head. Mass spectrometry demonstrated the presence of high mannose glycans on the Phil82 HA at positions known to contribute to IAV binding. Molecular modeling predicted an enhanced capacity for bridging interactions between HA glycans and D325A/R343V. Finally, the trimeric D325A/R343V NCRD decreased morbidity and increased viral clearance in a murine model of IAV infection using a reasortant A/WSN/33 virus with a more heavily glycosylated HA. The combined data support a model in which altered binding by a truncated mutant SP-D to IAV HA glycans facilitates viral aggregation, leading to significant viral neutralization in vitro and in vivo. These studies demonstrate the potential utility of homology modeling and protein structure analysis for engineering effective collectin antivirals as in vivo therapeutics.

Surfactant Protein D (SP-D) is a member of a family of collagenous C-type lectins, or collectins. This family includes mannose-binding protein (MBP), surfactant protein A, and certain bovine serum homologs, which are evolutionary descendents of SP-D. All collectins are characterized by an N-terminal collagen domain and a C-terminal lectin domain, and all appear to have roles in innate immunity.

There is substantial evidence that SP-D contributes to the neutralization and clearance of respiratory viruses (1–5). For example, SP-D-deficient mice show delayed clearance and heightened inflammatory responses to strains of respiratory syncytial virus and influenza A virus (IAV) (6–9). Significantly, SP-D-deficient mice show a selective impairment in clearance of strains reactive with SP-D in vitro (6, 9) and can be rescued by transgenic overexpression of trimeric rat SP-D subunits or with purified native human SP-D (6, 10).

Binding of SP-D to IAV involves interactions with asparagine-linked glycans located near the sialic acid receptor on the hemagglutinin (11–13). Furthermore, endoglycosidase H eliminates binding of SP-D to denatured HA in viral lysates, indicating preferential interactions with high mannose or hybrid oligosaccharides (11). Viral interactions require calcium and are specifically inhibited by monosaccharide ligands of SP-D, directly implicating the lectin activity of the SP-D carbohydrate recognition domain (CRD) (11). This is consistent with crystallographic studies of recombinant trimeric neck plus carbohydrate recognition domains (NCRDs) that have demonstrated binding of the equatorial hydroxyl groups of mannoses to calcium at the lectin site (14–16).

SP-D and the native serum collectins differ in their capacity to interact with respiratory viruses. For example, native bovine

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‡ The abbreviations used are: SP-D, surfactant protein D; CRD, carbohydrate recognition domain; IAV, influenza A virus; NCRD, neck+carbohydrate recognition domain; hNCRD, human NCRD; SPR, surface plasmon resonance; MBP, mannose-binding protein; HAI, hemagglutination inhibition; Ni-NTA, nickel-nitrilotriacetic acid; HBS, Heps-buffered saline.
SP-D Mutant Enhances Defenses against Influenza A Virus

Table 1

| Residues at positions 325 and 343 in SP-Ds and other collectins |
|---------------------------------|
| **Position 325** | **Position 343** |
| Surfactant protein D | | |
| Human | Asp | Arg |
| D325A | | |
| D325N | | |
| R343V | | |
| D325A/R343V | | |
| Rat/mouse | Asn | Lys |
| Cow | Asp | Lys |
| Pig | Asn | Lys |
| Bovine serum collectins | | |
| Conglutinin | Ser | Val |
| CL-43 | Arg | Ile |
| CL-46 | Asn | Val |
| Human MBP (MBP-C) | Ala | Val |
| Surfactant protein A | | |
| Human | Arg | Arg |
| Rat | Gln | Arg |

serum conglutinin and bovine serum CL-43 show greater binding to IAV than SP-D (17–19). Alignments of these collectins have revealed non-conservative substitutions at positions corresponding to 325 and 343 (Table 1) that are highly exposed on the opposing N-terminal and C-terminal ridges of the carbohydrate binding groove, respectively. Crystallographic studies of SP-D lectin domains have shown that neither of these residues coordinates with calcium; however, their side chains can participate in hydrogen bonds with various bound ligands (14–16, 20–23). Furthermore, differing interactions with these side chains contribute to known differences in saccharide selectivity. For example, the preference of human SP-D for d-N-acetylmannosamine over d-mannose can be attributed to hydrogen bonding of the Asp-325 carboxyl group with the N-acetyl substituent of the sugar (15, 24).

MBP and the bovine serum collectins are characterized by amino acids with short side chains at the 325 position, and CL-43 shows a distinctive insertion that begins with arginine at this position (Table 1). We have previously demonstrated small increases in anti-viral activity with similar insertions of three amino acids (RAK or AAA) in the context of the wild-type human NCRD (25, 26).

In addition, our previous studies have shown that residue 343 (arginine in humans or lysine in rodents and other species; Table 1) contributes to the binding of SP-D to phosphatidylglycerol, the major surfactant-associated ligand (14), and to the inner core oligosaccharide domain of rough bacterial lipopolysaccharides (LPS) (15). In fact, the greater capacity of rodent SP-D to recognize these particular ligands is largely determined by the structure of the 343 side chain (14, 15). More recently, we have shown that the substitution of Arg-343 with valine (R343V), as found in MBP or conglutinin (Table 1), substantially increases interactions with certain mannose-rich oligosaccharides, enhances viral binding and neutralization, and confers the capacity to mediate viral aggregation (21, 27). Similar but less marked effects were observed when Arg-343 was substituted with isoleucine (R343I), as found in CL-43 (Table 1).

In the present studies, we employed a novel surface plasmon resonance assay, viral binding and neutralization assays, mass spectrometry of HA glycans, and a new in vivo model of IAV infection to examine interactions between a recently developed combinatorial SP-D mutant and influenza A virus. The findings demonstrate the potential for collectin NCRD-based antiviral interventions.

EXPERIMENTAL PROCEDURES

The pET-30a(+) vector, S-protein horseradish peroxidase, and RosettaBlue competent cells were from Novagen (Madison, WI). Fatty acid free BSA (BAH66-0050) was from Equitech-Bio, Inc, Kerrville, TX. All mono- and disaccharides were the D-anomers and were of the highest purity available from Sigma. Gel filtration protein standards were from Bio-Rad, and the HisCap Biosensors were from ICX Technologies, Oklahoma City, OK.

Expression and Characterization of Trimeric NCRD Fusion Proteins—The expression and characterization of the N-terminal-tagged, trimeric human neck + CRD (hNCRD) fusion proteins has been previously described (14, 20, 21, 24, 25).

Site-directed mutagenesis was performed using a QuikChange II XL site-directed mutagenesis kit (200521; Stratagene, LA Jolla, CA). D325A was generated using the wild-type hSP-D neck + CRD DNA as template (25), whereas the D325A/R343V double mutant was generated using the cDNA for the previously characterized R343V mutant (14, 21). Sequences were verified by automated sequencing of the entire coding sequence of the fusion protein. Glycerol stocks of transformed bacteria were stored in single-use aliquots at −80 °C.

RosettaBlue competent cells were transformed with the wild-type or mutant construct in pET-30a(+) vector, and expressed proteins were isolated from inclusion bodies (25). After refolding and oligomerization, the D325A/R343V and D325A fusion proteins were purified by nickel-affinity chromatography, and trimers were isolated by gel filtration chromatography on an AKTA Purifier system. The purified proteins showed the expected mobility on SDS-PAGE (Fig. 1A, third and fourth lanes) with a slower migration in the presence of dithiothreitol, consistent with the formation of normal intrachain disulfide bonds, as illustrated for D325A/R343V (Fig. 1A, sixth lane).

Protein concentrations were determined using the bicinchoninic (BCA) assay (Pierce) with BSA as a standard. Endotoxin levels were less than ~3 pg/μg of protein, including preparations used for the in vivo experiments. Proteins were stored in small single-use aliquots at −80 °C. Analytical gel filtration confirmed the absence of aggregation of the purified trimers under our usual conditions of storage.

Other NCRD constructs used for these experiments included R343V (21), an expressed tagless wild-type human NCRD (hNCRD NoFT) (21), and E321K, a human NCRD mutant that is defective in calcium coordination at the primary sugar binding site and shows no detectable lectin activity (28).

Viral Interaction Assays—The Phil82 (H3N2) strain of IAV was grown and isolated as previously described (13). Binding of NCRD fusion proteins to solid-phase IAV, hemagglutination inhibition (HAI) assays, fluorescent focus assays of viral neutralization, and assays of viral aggregation and neutrophil uptake were performed as described in...
Isolation and Purification of IAV Hemagglutinins—HA molecules were released from Phil82 (H3N2), Phil82BS (H3N2), and PR8 (H1N1) virions using bromelain, a protease that cleaves the stalk of the trimeric hemagglutinin near the site of membrane insertion (30). The purified bromelain-solubilized HAs were examined by SDS-PAGE in the absence and presence of dithiothreitol (Fig. 1B). The unreduced PR8 protein migrated as a single major species of ~70 kDa relative to globular standards; after reduction, there were two species of ~61 and 25 kDa, consistent with the HA1 with HA2 subunits, respectively. Similar results were obtained for the Phil82BS HA (data not shown). As expected, the unreduced PR8 protein (61 kDa) and the PR8 HA1 subunit (53 kDa) migrated more rapidly than the corresponding Phil82 species, consistent with the absence of glycans on the HA1 subunit of the PR8 HA.

The assembly of the purified HAs was assessed by gel filtration under non-denaturing conditions using an AKTA Tricorn 10/300 GL Superose 12 column (GE Healthcare) and a series of globular standards (Bio-Rad, #151-1901) (25). The proteins eluted as single major peaks near the expected position of HA trimers, between thyroglobulin and γ-globulin standards (data not shown). The molecular mass was ~250 kDa as estimated using a plot of elution volume versus log molecular weight plot, with the Phil82 or Phil82BS HA eluting very slightly earlier than the PR8 HA.

Determination of N-Glycosylation Pattern of Phil82 HA Using Mass Spectrometry—Mass spectral data were acquired using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, San Jose, CA) interfaced with a nanoAcquity ultra performance liquid chromatograph (Waters Corp, Milford, MA) and a Triversa Nanomate robot (Advion Biosciences, Inc., Ithaca, NY). The protein (100 μg) was reduced using dithiothreitol and alkylated using iodoacetamide, desalted, and digested using trypsin using standard protocols.

Verification of HA Protein Sequence—Using a reversed phase chromatography column (Waters BEH C18, 150 μm × 15 cm), liquid chromatography/tandem mass spectrometry data (LC/MS) were acquired using conditions as described (31), and the data were analyzed using the MASCOT Server software (Matrix Science, Inc. Boston, MA) operating in-house. The results were used to generate a custom data base of H3 type HA sequences for subsequent glycoproteomics analysis.

Identification of Asn Residues Occupied by N-Glycans—An aliquot (10 μg) of the HA tryptic peptide mixture was digested with peptide N-glycosidase F (New England Biolabs, Andover, MA) in the presence of H218O and then analyzed by C18 reversed phase LC/MS as above with manual interpretations.

Identification of Glycosylated Peptides—For glycoproteomics, LC/MS was conducted using an in-house packed HILIC column (Amide-80, 3-μm beads, 250 μm × 15 cm, Tosoh Bioscience, Mongomeryville, PA) as described (32). Higher energy dissociation tandem MS scans containing oxonium ions corresponding to Hex and HexHexNAc triggered subsequent electron capture dissociation tandem mass spectra. Datasets were acquired using on-line LC/tandem MS. Additional data were acquired by collecting fractions from the HILIC column and analyzing off-line using the Triversa Nanomate robot.

recent publications (13, 25). In particular, binding of NCRD fusion protein to solid-phase viral particles was assessed using the S-protein horseradish peroxidase detection system; all assays were performed within the linear range of optical density. For some studies, findings for Phil82 were compared with SP-D resistant strains, such as Phil82BS and PR8 (H1N1) (13).

The A/WSN/33 HAnC-Asp225Gly (H1N1) hybrid strain (hereafter designated WSNHAnC-Asp225Gly) was kindly provided by Dr. Donald Smee, Utah State University (29). We also examined the wild-type, mouse-adapted A/WSN/33 (H1N1) strain (hereafter designated WSN).
TABLE 2

Glycosylation of Phil82 HA

Glycan structures were determined as described under “Experimental Procedures.” The N-glycan types for the two glycosylated asparagine residues on the head of the HA are shown. Glycan structures are identified as high mannose (M), hybrid/complex (H/C), and/or complex (C). The amount of each identified structure is expressed relative to the most abundant species. There was no glycosylation at another potential site, asparagine 126. However, there were 4 additional sites of N-glycosylation on the stalk, at asparagine 38 (M + H/C), 63 (M + H/C), 285 (M), and 483 (H/C) (data not shown).

| Glycosylation site | Hex | HexNAc | dHex | Type | Relative amount |
|-------------------|-----|--------|------|------|----------------|
| 144               | 8   | 2      | 1    | H/C  | 0.3–0.4       |
| 165               | 7   | 2      | 1    | H/C  | <0.05         |
|                   | 7   | 2      |      | H/C  | 0.1–0.2       |
|                   | 6   | 2      |      | M    | <0.01         |
|                   | 3   | 3      |      | H/C  | 0.10–0.20     |
|                   | 2   | 2      |      | M    | 0.30–0.40     |
|                   | 10  | 2      |      | M    | <0.01         |
|                   | 9   | 3      |      | H/C  | 0.40–0.50     |
|                   | 9   | 3      |      | H/C  | <0.05         |

Data Analysis—Candidate glycopeptides were identified based on the presence of 18O-Asp from manual interpretations. Glycopeptides were identified based on the presence of oxo-mannose from electrospray mass spectrometry. The peptide sequence was determined from the electron capture dissociation scans. Only those glycopeptides producing consistent proteomics and higher energy dissociation/electron capture dissociation analyses are reported (Table 2).

Surface Plasmon Resonance (SPR) Assays Using Trimeric NCRDs—SPR assays described in this publication were performed using a SensiQ Pioneer biosensing system and HisCap (Ni²⁺-nitrilotriacetic acid (NTA)) biosensors (ICx Technologies). However, certain preliminary experiments were performed using a BioCore 2000 and the corresponding BioCore Sensor Chip NTA (GE Healthcare).

For the SensiQ, the usual configuration was to attach NCRD fusion proteins to cells 1 and/or 3, leaving cell 2 as a reference. Briefly, all cells were charged with 500 μM nickel chloride in 10 mM Heps, 150 mM NaCl, pH 7.5 (HBS), resulting in a predictable change in refractive index. The trimeric hNCRDs were then coupled via their N-terminal hexahistidine tags to the desired flow cells, as recommended by the manufacturer, at a concentration of 1–10 μg/ml. For the experiments reported here protein couplings were performed in 10 mM sodium acetate, pH 5.0, to maximize attachment. Because all the proteins are stored at similar high concentrations in the same buffer, the coupling conditions were effectively identical. Preliminary experiments showed nearly equivalent changes in response units for equivalent amounts of several different trimeric NCRDs, indicating reproducible binding of the fusion proteins to the biosensor and confirming equivalence of the flow cells.

Before the assay, the bound trimeric NCRD fusion proteins were “activated” by priming the system three times for a total 15 min with HBS containing 5 mM calcium chloride (HBS + C). The cells were then “blocked” with 0.1% fatty acid free bovine serum albumin in HBS + C, which helped minimize non-specific binding to the reference cell. The initial rationale for calculating the bound NCRDs was to preclude lectin-dependent binding to dextran on the biosensor. Although this proved to be an unnecessary precaution, the approach facilitated experiments confirming the calcium-dependence of binding.

Analytics such as bromelain-solubilized HAs were injected into the mobile phase at the indicated concentration (0.02–20 μg/ml) at a controlled temperature of 25 °C. Flow rates were optimized to minimize mass transport effects; 25 μl/min was used for the current studies. For some preliminary experiments, competing sugars were co-injected with the analyte to confirm specificity. However, for the experiments shown here a competing sugar was added during the dissociation and/or regeneration phase.

The biosensors were routinely stripped with alternating injections of 350 mM EDTA in HBS, pH 8.0, and 1 mM imidazole in deionized water. The stripped biosensors could be recharged with nickel and protein for multiple assays without deleterious effects on performance.

Kinetic analyses and curve-fitting for the SensiQ were performed using Qdat (ICx Technologies). To facilitate formatting for figure preparation, data were exported to Sigmaplot (Systat Software).

Murine Models of IAV Infection—In vivo mortality studies were conducted with 8–12-week-old female DBA/2J mice (The Jackson Laboratory, Bar Harbor, ME). Mice were lightly anesthetized with isofluorane. 50 μl of WSN or WSNH232Ggly stock in DPBS with Ca²⁺ and Mg²⁺ ions was co-administered via intratracheal delivery with or without 5 μg/mouse D325A/R343V NCRD or wild-type NCRD. Viral titers of the inoculate were 1 × 10⁵ and 3.3 × 10⁵ viral particles/ml, respectively, as assessed by real-time quantitation (APM3600, EMD Millipore, Billerica, MA). Mice were weighed every other day and checked daily for viral induced mortality. All animals were maintained in a specific pathogen-free facility and were handled according to an institutional animal care and use committee (IACUC)-approved protocol and National Institutes of Health guidelines. In some experiments whole lungs were harvested 5 days post-viral infection (n = 3/group). The tissue was homogenized in DPBS without Ca²⁺ and Mg²⁺ and spun at 3000 × g for 10 min. The supernatant was collected and stored at −80 °C.

Assessment of Viral Clearance—Total RNA was isolated from infected mouse lung homogenate using the QiAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany), and viral RNA was purified by the Viral RNA Spin Protocol (Qiagen) and stored at −20 °C. Real-time fluorescence detection of viral RNA was performed in a single, closed-tube system (ABI Prism 7500, Applied Biosystems, Carlsbad, CA) using Bio-Rad One-Step RT-PCR enzyme mix (Bio-Rad) and the Amplifluor ID Influenza A Virus Detection kit (EMD Millipore, Darmstadt, Germany). The cycling parameters were 10 min at 10 °C, 5 min at 95 °C, and 45 cycles of 15 s at 95 °C and 60 s at 60 °C.

Modeling of HA/NCRD Interactions—The R343V/D325A mutant was built in Maestro (Schrodinger LLC, New York) from the previously published coordinates of the R343V-di-mannose complex (PDB code 3GB8e)(21). Oligomannose coordinates containing eight mannose and two N-acetylgluco-
samine residues were obtained from the crystal structure of the anti-HIV antibody 2G12 complexed to oligomannose (PDB code 1OP5) (33). The sugar coordinates were minimized in Macromodel (Schrödinger LLC) in an OPLS-2005 force field to regularize the geometry. Mannose residues 237 and 238 of the minimized oligomannose model were superposed onto the dimannose sugars in either the double mutant model or the wild-type SP-D NCRD structure (PDB code 3G83) (21). To position the hemagglutinin, the two N-acetylgalcosamine residues bound to Asn-165 of the A chain of X31 (H3N2) hemagglutinin (PDB code 1HGE) (34) were superposed on the two N-acetylgalcosamine residues of the minimized oligomannose model. Some torsions of the oligomannose sugar were performed in the case of the wild-type complex model to remove steric clashes between the SP-D and hemagglutinin.

**RESULTS**

**D325A Shows Enhanced Interactions with IAV in Vitro**—We initially confirmed lectin activity by examining the binding of trimeric D325A NCRD fusion protein to solid phase yeast mannan and compared saccharide affinities in competition assays (25). D325A showed calcium-dependent and maltose-sensitive binding to solid-phase mannan, with total binding increased by nearly 5-fold relative to wild-type (data not shown). D325A showed a selective decrease in affinity for N-acetyl-mannosamine, consistent with loss of H-bonding between Asp-325 and the N-acetyl substituent (15, 24). However, there was no effect on the affinity for mannose or other tested monosaccharides.

Using the Phil82 (H3N2) strain of IAV, D325A showed significantly increased HAI, i.e., lower protein concentrations were required to achieve inhibition of viral hemagglutination (Table 3). There was also a small but reproducible increase in viral binding compared with wild type (Fig. 2A). However, there was no detectable increase in viral neutralization using the fluorescent focus assay (Fig. 2B), and there was limited capacity to increase neutrophil uptake of IAV (Fig. 2D).

**TABLE 3**

| SP-D Preparation | HA inhibiting concentration for Phil82<sup>a</sup> | HA inhibiting concentration for Phil82BS<sup>a</sup> | HA inhibiting concentration for PR-8<sup>a</sup> | 50% Neutralizing concentration<sup>a</sup> |
|------------------|--------------------------------------------|--------------------------------------------|---------------------------------|------------------|
| SP-D dodecamer<sup>c</sup> | 0.02 ± 0.008 | 0.16 ± 0.01 | >10 | 0.04 |
| hNCRD (wild type) | >4 | >40 | >10 | >40 |
| D325A | 7.3 ± 0.41<sup>c</sup> | N.D.<sup>e</sup> | >10 | ~20 |
| R343V | 1.03 ± 0.28<sup>d</sup> | >2<sup>c</sup> | >10 | 0.12 |
| D325A/R343V | 0.31 ± 0.06<sup>d</sup> | 1 ± 0.06<sup>c</sup> | >10 | 0.05 |

<sup>a</sup>Values shown for HA inhibition are the mean (± S.E.) amounts of SP-D preparations that inhibited 40 hemagglutination units of the indicated strain of IAV (n ≥ 4).

<sup>b</sup>Values shown were obtained from dose-response curves on inhibition of fluorescent focus formation by the collectins (n > 4). Phil82 virus was used in experiments shown in this column.

<sup>c</sup>Not determined.

<sup>d</sup>Significantly increased inhibition compared with wild type NCRD (p < 0.01).

<sup>e</sup>Values shown were obtained from dose-response curves on inhibition of fluorescent focus formation by the collectins (n > 4). Phil82 virus was used in experiments shown in this column.

<sup>f</sup>Based on previously published data.

<sup>g</sup>Values shown were obtained from dose-response curves on inhibition of fluorescent focus formation by the collectins (n > 4). Phil82 virus was used in experiments shown in this column.

<sup>h</sup>Values shown were obtained from dose-response curves on inhibition of fluorescent focus formation by the collectins (n > 4). Phil82 virus was used in experiments shown in this column.

<sup>i</sup>Significantly decreased inhibition for Phil82BS as compared with Phil82 (p < 0.002).

<sup>j</sup>Indicates significantly increased inhibition compared with R343V (p < 0.02).

The capacity of SP-D to aggregate IAV is dependent on higher order multimeric structure, and trimeric NCRDs are devoid of aggregating activity (35). However, we recently observed that trimeric NCRDs with the R343V substitution can efficiently aggregate Phil82 but not SP-D resistant strains (27, 36). As shown in Fig. 2C, D325A/R343V trimers more efficiently aggregated IAV than trimeric R343V. In similar assays, D325A showed no significant viral aggregating activity (data not shown). The capacity of SP-D to augment viral uptake by neutrophils is dependent on aggregation (27). As predicted, D325A/R343V was also substantially more potent than R343V or D325A in enhancing uptake of Phil82 by human neutrophils (Fig. 2D).

**D325A/R343V NCRD Shows Enhanced Binding to Phil82 Hemagglutinin Trimmers**—SPR was employed to study the interactions of trimeric D325A/R343V with bromelain-solubilized trimeric HA (Fig. 1, Table 2). Trimeric wild-type and mutant fusion proteins were stably coupled via their N-terminal His...
tags to a Ni-NTA biosensor as described under “Experimental Procedures.” There was no detectable binding of a tagless wild-type hNCRD to the biosensor (Fig. 3A).

As illustrated in Fig. 3A, there was calcium-dependent binding of the wild-type protein to the Phil82 HA trimer. Although there was slow reversal during the dissociation phase, the biosensor could be rapidly and completely regenerated by the injection of D-glucose (data not shown). Notably, there was no significant binding to equivalent amounts of the trimeric E321K loss-of-function mutant (Fig. 3A), further confirming dependence on the lectin activity of the NCRD.

D325A/R343V showed much greater binding to Phil82 HA as compared with an equivalent amount of wild-type hNCRD (Fig. 3B). When averaged across all comparable experiments, total binding for D325A/R343V was increased by ~9-fold. By contrast, total binding was increased by only 3-fold for R343V and ~2.6-fold for D325A (data not shown). Apparent dissociation was slower for D325A/R343V than wild-type but still completely reversible in the presence of D-glucose (Fig. 3C).

There was ~6-fold lower binding of D325A/R343V to a comparable preparation of trimeric PR8 HA (Fig. 3D), which lacks glycans on the HA1 (13). Treatment of native Phil82 HA with endoglycosidase H reproducibly decreased binding (~15%); however, analysis of cleavage reactions by SDS-PAGE showed that only a small fraction of the total endoglycosidase H-sensitive carbohydrate was removed in the absence of prior protein denaturation (data not shown). Consistent with other studies using Phil82BS virus (Table 3), purified Phil82BS HA, which lacks the glycosylation site at Asn-165, showed a 61% reduction in binding (mean ± S.D., n = 4, p < 0.001). The residual binding can probably be attributed to interactions with the glycan at residue 144 (Table 2).

Our use of homologous fusion proteins, each with an identical mechanism of attachment remote from the site of ligand interaction, insures that direct comparisons of total binding can be made. Although the trimeric nature of both the NCRD and HA and associated rebinding effects preclude the calculation of true affinities, apparent affinities were calculated as illustrated in Table 4. The apparent affinity of D325A/R343V for the Phil82 HA was substantially increased for all NCRD and analyte conditions examined, primarily as a consequence of the decreased apparent dissociation rate constant (k_d). In addition,
the apparent affinity was significantly increased relative to R343V or D325A, which were significantly increased relative to wild type (Table 4).

**D325A/R343V Shows Enhanced Interactions with the WSNHAn Anc-Asp225Gly Strain of IAV, but Not the Parental WSN Strain, in Vitro**—The Phil82 (H3N2) strain has been used originally developed to cause illness in mice while retaining sensitivity to cyanovirin, a mannose binding lectin (29). The hybrid strain was derived from A/WSN/33 (H1N1) but expresses the glycosylated HA of a recent seasonal H1N1 virus (A/New Caledonia/20/99). The HA also has an Asp225Gly mutation that alters siaic acid receptor specificity and facilitates replication in mice.

| Trimeric NCRD | n  | Mean $k_a$ | Mean $k_d$ | Mean apparent $k_d$ |
|--------------|----|------------|------------|--------------------|
| Wild type    | 15 | 4.72E+06   | 1.09E-03   | 0.231              |
| D325A        | 4  | 6.85E+06   | 4.90E-04   | 0.073 (p < 0.0001) |
| R343V        | 4  | 3.14E+06   | 2.72E-04   | 0.088 (p < 0.0001) |
| D325A/R343V  | 7  | 3.48E+06   | 1.36E-04   | 0.04 (p < 0.0001)  |

*p* values for the difference between each mutant and wild type are shown. Differences between D325A/R343V and the single site mutants were also highly significant. However, there was no significant difference in the apparent affinity of D325A and R343V. Wild-type and mutant NCRDs were paired on different flow cells of the same chip. Data for wild type include two different preparations of protein.

**TABLE 4**

**Binding of trimeric wild type and mutant NCRDs to trimeric Phil82 HA**

All experiments used approximately 1000 response units of the indicated NCRD on the chip and a series of HA concentrations (0.01–0.3 μg/ml).

SPR assays were performed as described under “Experimental Procedures.” Briefly, trimeric NCRD fusion proteins were coupled to Ni-NTA biosensors via their N-terminal tags, and trimeric HA molecules were delivered in the mobile phase. A, binding of human NCRDs to Phil82 HA is shown. NCRDs (2.0 μg/ml) were affixed to different cells on the same Ni-NTA biosensor chip. The wild-type protein bound to the Phil82 HA (2.5 μg/ml) in the presence, but not the absence, of 5 mM calcium chloride (curves are labeled +Ca+ and -Ca+, respectively). The calcium-dependent binding was rapidly reversed in the presence of 100 mM D-glucose, as illustrated below for the double mutant. Predictably, a biosensor cell identically pretreated with the tagless trimeric NCRD showed no HA binding (unlabeled, but also shown near base line). Sensorgrams are shown without reference or blank subtraction. The beginning of the dissociation phase is identified by an arrow below the x axis. B, shown is a comparison of D325A/R343V and wild-type NCRDs. The NCRDs (2.0 μg/ml) were affixed to different cells on the same Ni-NTA biosensor chip, and binding to Phil82 HA (0.01–0.63 μg/ml) was examined. Conditions were selected to allow a comparison of total binding by wild-type and mutant NCRDs on the same run. For clarity, only the highest concentration of wild-type NCRD is shown (0.63 μg/ml). Sensorgrams for this figure and the subsequent kinetic analysis were blanked to a buffer control and reference cell subtracted. C, dissociation of D325A/R343V is shown. The double mutant (2.0 μg/ml) was bound to Phil82 HA (0.63 μg/ml) in the presence of 5 mM calcium as above, except a saccharide competitor, D-glucose, was injected during the dissociation phase. The signal in the reference cell was subtracted; the negative deflection results from incomplete correction of the massive bulk shift resulting from glucose in the mobile phase. Predictably, a biosensor cell identically pretreated with the tagless trimeric NCRD showed no HA binding (unlabeled, but also shown near base line). Sensorgrams are shown without reference or blank subtraction. The beginning of the dissociation phase is identified by an arrow below the x axis. D, binding of D325A/R343V to Phil82 versus PR8 HA is shown. Binding of double mutant (1.0 μg/ml) to purified bromelain-solubilized Phil82 and PR8 hemagglutinin trimers (5.0 μg/ml) was examined in the presence of calcium. A relatively high HA concentration was used to better demonstrate low binding to the PR8 HA. In as panel C, binding to both HAs was sensitive to glucose. Sensorgrams are shown without reference subtraction.

**FIGURE 3. Interactions of D325A/R343V with HA as assessed by SPR.** SPR assays were performed as described under “Experimental Procedures.” Briefly, trimeric NCRD fusion proteins were coupled to Ni-NTA biosensors via their N-terminal tags, and trimeric HA molecules were delivered in the mobile phase. A, binding of human NCRDs to Phil82 HA is shown. NCRDs (2.0 μg/ml) were affixed to different cells on the same Ni-NTA biosensor chip. The wild-type protein bound to the Phil82 HA (2.5 μg/ml) in the presence, but not the absence, of 5 mM calcium chloride (curves are labeled +Ca+ and -Ca+, respectively). The calcium-dependent binding was rapidly reversed in the presence of 100 mM D-glucose, as illustrated below for the double mutant. There was no binding of the E321K loss of function mutant in the presence of calcium (line at base line). Predictably, a biosensor cell identically pretreated with the tagless trimeric NCRD showed no HA binding (unlabeled, but also shown near base line). Sensorgrams are shown without reference or blank subtraction. The beginning of the dissociation phase is identified by an arrow below the x axis. B, shown is a comparison of D325A/R343V and wild-type NCRDs. The NCRDs (2.0 μg/ml) were affixed to different cells on the same Ni-NTA biosensor chip, and binding to Phil82 HA (0.01–0.63 μg/ml) was examined. Conditions were selected to allow a comparison of total binding by wild-type and mutant NCRDs on the same run. For clarity, only the highest concentration of wild-type NCRD is shown (0.63 μg/ml). Sensorgrams for this figure and the subsequent kinetic analysis were blanked to a buffer control and reference cell subtracted. C, dissociation of D325A/R343V is shown. The double mutant (2.0 μg/ml) was bound to Phil82 HA (0.63 μg/ml) in the presence of 5 mM calcium as above, except a saccharide competitor, D-glucose, was injected during the dissociation phase. The signal in the reference cell was subtracted; the negative deflection results from incomplete correction of the massive bulk shift resulting from glucose in the mobile phase. Note that the signal is at base line after termination of the glucose injection. D, binding of D325A/R343V to Phil82 versus PR8 HA is shown. Binding of double mutant (1.0 μg/ml) to purified bromelain-solubilized Phil82 and PR8 hemagglutinin trimers (5.0 μg/ml) was examined in the presence of calcium. A relatively high HA concentration was used to better demonstrate low binding to the PR8 HA. In as panel C, binding to both HAs was sensitive to glucose. Sensorgrams are shown without reference subtraction.

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Modifications to IAV that render them lethal in mice usually eliminate sites of glycosylation and collectin recognition. However, the WSNHAn Anc-Asp225Gly strain is a hybrid virus that was originally developed to cause illness in mice while retaining sensitivity to cyanovirin, a mannose binding lectin (29). The hybrid strain was derived from A/WSN/33 (H1N1) but expresses the glycosylated HA of a recent seasonal H1N1 virus (A/New Caledonia/20/99). The HA also has an Asp225Gly mutation that alters siaic acid receptor specificity and facilitates replication in mice.

D325A/R343V showed significant hemagglutination inhibition activity for WSNHAn Anc-Asp225Gly, indicating efficient viral bind-
neutralization by the wild-type NCRD at concentrations up to 100 μg/ml with R343V and native human SP-D dodecamers. Neutralizing activity was significantly greater (*) than observed for R343V or dodecamers. There was no neutralizing activity for WSN (H1N1) strain (Fig. 4A), wild-type NCRD and D325A were inactive in the fluorescent focus assay. Notably, D325A/R343V showed no hemagglutination inhibition activity for the glycan-deficient wild-type WSN (H1N1) strain (Table 5), and there was no detectable neutralizing activity (data not shown).

D325A/R343V NCRD Shows Antiviral Activity against WSNHAnc-Asp225Gly, but Not the Parental WSN Strain of IAV, in Vivo—We next compared the ability of wild-type NCRD and D325A/R343V to protect mice against challenge by the two mouse-adapted strains of IAV in vivo. Under the conditions of challenge, both the WSNHAnc-Asp225Gly and glycan-deficient WSN wild-type strain were lethal at an estimated 500 and 1650 viral particles per mouse, respectively (Fig. 5, A and B). As expected, the WSN wild-type strain was more virulent; animals challenged with WSN died 2–3 days earlier than animals challenged with WSNHAnc-Asp225Gly.

D325A/R343V NCRD was protective against challenge with WSNHAnc-Asp225Gly and substantially increased viral clearance, as measured by quantifying viral RNA at 5 days post-challenge (Fig. 5, A and C). D325A/R343V-treated animals surviving at the termination of the experiment had not yet regained weight, and we cannot exclude late deaths. Consistent with the in vitro findings, D325A/R343V administration did not increase survival or enhance clearance of the poorly glycosylated WSN wild-type strain (Fig. 5, B and D). The wild-type human trimeric NCRD showed no evidence of protection against mortality and no effect on viral clearance when co-administered with either strain of IAV.

### DISCUSSION

Our studies have shown that combinatorial mutagenesis of the residues that flank the calcium ion at the lectin site of human SP-D can greatly enhance interactions with specific strains of IAV both in vitro and in vivo. Previous studies have shown that exogenous native human SP-D can rescue SP-D null mice challenged with specific strains of IAV (6); however, this is the first study demonstrating antiviral effects of the more therapeutically practical trimeric NCRDs. In addition, although we have described other mutants with enhanced neutralizing activity in vitro, this is the first study demonstrating enhanced antiviral activity of an SP-D mutant in vivo. Thus, the studies provide the evidence that exogenous recombinant collectins with augmented interactions with viral glycans can increase antiviral activity in vivo.

Enhanced Interactions of D325A/R343V NCRD with IAV—Native SP-D shows efficient binding to specific strains of seasonal IAV, which is associated with efficient inhibition of HAI, viral neutralization, and viral aggregation. Aggregation of IAV by native collectins requires cooperative interactions among trimeric subunits, and a variety of observations indicate that viral aggregation is required for efficient viral neutralization and effects on the neutrophil response to IAV (27, 37). As for full-length trimeric human SP-D subunits, the wild-type trimeric human NCRD shows no significant binding to IAV in ELISA-type assays, negligible HAI, no significant viral aggregating or neutralizing activity, and no capacity to enhance neutrophil uptake of IAV despite efficient binding to various other ligands, including certain forms of LPS (38).

**FIGURE 4. Neutralization of WSNHAnc-Asp225Gly.** The WSNHAnc-Asp225Gly reassortant possesses the glycosylated HA1 of a seasonal strain of IAV and binds to SP-D. Viral neutralization was assessed using the fluorescent focus assay and Madin-Darby canine kidney cells. A, the activity of D325A/R343V is compared with R343V and native human SP-D dodecamers. Neutralizing activity was significantly greater (*) than observed for R343V or dodecamers. There was no neutralization by the wild-type NCRD at concentrations up to 100 μg/ml (data not shown). B, D325A/R343V (300 ng/ml) was combined with the indicated weight or SP-D dodecamers. Neutralizing activity was significantly increased (*) at each of the examined concentrations of SP-D.

### TABLE 5

**Antiviral activities of trimeric NCRDs for mouse-adapted strains in vitro**

| SP-D preparation | HA inhibiting concentration for WSN wild type | HA inhibiting concentration for WSN mutant |
|------------------|-----------------------------------------------|------------------------------------------|
| Wild type NCRD   | >46.25 μg/ml                                  | >46.25 μg/ml                             |
| D325A/R343V      | 6.5 ± 0.29 μg/ml                              |                                          |

* Values shown for HA inhibition are the mean (± S.E.) amount of SP-D preparations that inhibited 16 hemagglutination units of the indicated strain of IAV (n = 4).

* Values shown for HA inhibition are the mean (± S.E.) amount of SP-D preparations that inhibited five hemagglutination units of the indicated strain of IAV (n = 4).

* Significantly increased inhibition compared with wild type NCRD (p < 0.01).
As demonstrated here, the combinatorial mutation of Asp-325 and Arg-343 greatly enhances the binding of the trimeric human NCRD to Phil82 IAV and the purified trimeric Phil82 HA (Figs. 2 and 3). The interactions with purified Phil82 were completely inhibited by saccharide competitors, and there was very low binding to the PR8 HA (Fig. 3), implicating the HA1 glycans. Although D325A/R343V showed no detectable HAI for the PR8 virus, detectable binding suggests that the mutant can interact with other glycans associated with the stalk of the solubilized HA, which are unlikely to be accessible on the intact virions.

The double mutant also showed increased HAI, increased viral neutralizing and aggregating activities, and increased viral uptake by neutrophils (Fig. 2). The effects were not incremental. Notably, the viral neutralizing activity of the double mutant approached the high activity observed for the native SP-D dodecamers for Phil82 (Table 2), i.e. approximately half as effective as wild type based on the molar concentration. The approximate rank ordering of antiviral activity in vitro for Phil82 is NCRD < D325A < R343V < D325A+R343 < native SP-D. Although R343V appears to be the key modification, there were synergistic interactions between the D325A and R343V mutations with respect to viral aggregation and aggregation-dependent activities such as enhancement of neutrophil uptake of IAV.

The double mutant also appears to have particularly increased neutralizing activity for strains of IAV that are partially resistant to native SP-D. For example, it inhibits the WSNHAn-Asp225Gly, whereas native SP-D and R343V show low neutralization activity of this strain (Fig. 4). For reasons discussed below, we hypothesize that this reflects greatly increased access of HA glycans on the viral envelope to the lectin-site, further enhancing the potential for bridging interactions between virions. Given these observations, efforts are under way to characterize the effects of these mutations on recognition of SP-D-resistant pandemic strains.

Enhanced Interactions of D325A/R343V with Phil82 (H3N2) HA Glycans—Previous studies have shown that SP-D preferentially interacts with specific high mannose sugars on the viral hemagglutinin (13). Each chain of the three chains of a Phil82 hemagglutinin molecule contains three potential sites of N-linked glycosylation on the head of the HA (at positions 126, 144, and 165); however, mass spectrometry showed that Asn-126 is not glycosylated. Previous studies have demonstrated that the glycans on the HA head, those at Asn-165 and Asn-144 are the most important for binding to native SP-D (13). As shown in Table 2, our preparations of Phil82 HA have a mixture of high mannose and hybrid-complex glycans at both of these positions. We infer that the double mutant participates in altered interactions with one or more of these glycans. Nota-
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bly, there was significantly decreased HAI activity for Phil82BS, a strain that lacks the glycan at Asn-165 (Table 3). In addition, there were major reductions in binding to the purified Phil82BS and PR8 HAs as assessed by surface plasmon resonance (see Fig. 3).

Phil82 is propagated in eggs to permit isolation of sufficient amount for biochemical analysis, and differences in the host cell and viral strain can be associated with differences in patterns of HA glycosylation. However, similar mixtures of HA glycans, including abundant high mannose structures, have been demonstrated for H3N2 strains expressed in mammalian Madin-Darby canine kidney cells and Vero cells (39). To our knowledge it has not yet been possible to characterize HA glycans expressed by virus propagated during a productive infection in vivo. Thus, based on the available data, our preparation of Phil82 (H3N2) HA provides a biologically relevant model for studying HA/SP-D interactions.

The SPR assay showed an increase in the apparent affinity of D325A/R343V for the Phil82 HA (Table 3) and a conspicuous increase in total binding (e.g. Fig. 3B). These observations suggest that D325A/R343V (and R343V) has acquired the capacity to participate in an expanded range of interactions with one or more glycans on the trimeric HA.

Enhanced interactions of the double mutant and R343V with high mannose glycans seem likely based on enhanced binding of the mutant to mannan and other mannose-rich glycoconjugates. However, we cannot exclude qualitative alterations in carbohydrate recognition that allow enhanced interactions with hybrid or truncated complex glycans. In this regard glycomic array data obtained for our wild-type NCRD, D325A, and R343V, which are now available in the public domain on the Consortium for Functional Glycomics web site, indicate that the 325 and 343 substitutions are associated with qualitative changes in carbohydrate recognition. Although wild-type hNCRD and D325A exhibit binding that is largely limited to a subset of mannose-rich oligosaccharides, R343V shows a greatly expanded repertoire, including enhanced binding to oligosaccharides terminating with N-acetylgalactosamine or fucose. The potential of the mutants to interact with hybrid or complex glycans is the subject of active investigation.

Molecular Modeling Provides a Potential Mechanism for the Enhanced Viral Binding and Aggregating Activity of D325A/R343V—Our recent studies have shown that the R343V mutation confers the capacity to bind to the reducing mannose in α1,2-dimannose, predicting an alternative binding conformation and the capacity to interact with non-terminal mannoses of a high mannose glycan (21). Given this information, we have modeled potential binding conformations of the trimeric wild-type and R343V-containing NCRDs with the glycan at Asn-165 on the Phil82 (H3N2) HA, the glycan known to be absent in Phil82BS. As illustrated in Fig. 6, utilization of this alternative binding mechanism by D325A/R343V (and R343V) could confer the capacity to participate in bridging interactions between HA molecules, a prerequisite for lectin-dependent viral aggregation. The modeling further suggests that Asp-325 could place steric constraints on interactions with the glycan that are alleviated by the alanine substitution. This model is also consistent with additional interactions involving the glycan at Asn-144 (data not shown).

Immobilization of NCRD Fusion Proteins on Ni-NTA Biosensors—In this study, highly purified wild-type and mutant trimeric fusion proteins containing N-terminal hexahistidine tags were reversibly, but stably, immobilized on a nickel-activated 2-nitrotriacetic acid biosensor (Ni-NTA), and the ligands were passed over the flow cells in the aqueous phase. Use of Ni-NTA biosensors has not found widespread use for the study of His-tagged proteins in part because of the relative instability of the nickel complex (40). For example, investigators found that it was necessary to covalently couple monomeric His-tagged cyclosporine A to the biosensor, circumventing the potential advantages of the tag (41). It is likely that the unusually stable binding of trimeric NCRD proteins to the NiNTA biosensor results from cooperative interactions of the three hexahistidine tags of each trimer with the Ni2+-activated NTA surface.

The assay offers a number of potential advantages for studying the binding activities of trimeric NCRDs; 1) it avoids covalent modifications to the ligand or analyte, 2) it insures a predictable orientation of the fusion proteins with their binding surfaces exposed to the mobile phase, 3) the biosensor can be readily regenerated with EDTA, and 4) equivalent protein loading can be confirmed by monitoring the increase in response units when the NCRD is bound. The assay format also minimizes certain valency effects associated with the use of solid-phase carbohydrate ligands to study interactions with multivalent lectins (42). Last, binding to the nickel-charged biosensor provides a convenient quality control to assess the integrity of the N-terminal His tags, which are used for the detection of NCRD binding to cells by flow cytometry (43) or for screening of glycomic arrays (21, 28).

Implications of the D325A/R343V Phenotype for the Function of Native SP-D—The conserved hydrophilic residues at 325 and 343 in wild-type SP-D greatly decrease viral interactions, as evidenced by the enhanced antiviral activities of D325A and R343V and much greater antiviral activity of D325A/R343V. In particular, Arg-343 limits the type of binding interactions and may preclude efficient viral aggregation. The findings are consistent with our hypothesis that viral recognition has not been a major driving force in the evolution of the human SP-D CRD and that the unique flanking sequences evolved to enhance selectivity for endogenous ligands or other classes of microbial ligands, such as LPS or mycobacterial lipoglycans.

Although full-length human trimeric subunits resemble NCRDs in their low antiviral activity, dodecamers are very potent, and larger multimers show substantial increases in molar aggregating and neutralization activity; similar effects have been described for a variety of other microorganisms (44, 45). The current studies suggest that the structure of the wild-type CRD limits bridging interactions by individual trimeric lectin domains while favoring longer range interactions involving spatially separated trimeric lectin domains. The SPR studies further suggest that the structure of the wild-type CRD favors comparatively rapid dissociation of virions from individual trimeric lectin domains, increasing dependence on cooperative interactions between native subunits.
Planned studies examining the mutations in the context of recombinant dodecamers should be informative. In any case, activity of the NCRD has now been sufficiently enhanced to allow it to approach the anti-viral activity of the native protein in vitro. Recent studies suggest that these activities can be further enhanced through the generation of bivalent molecules using F(ab’)/H11032 fragments of specific monoclonal anti-CRDs or using multivalent S-protein conjugates (27, 36).

**Murine Model of IAV Infection**—The use of WSN HAnc-Asp225Gly reassortant strain of IAV provides a significant advantage for studies of the anti-IAV activity of SP-D and probably other collectins. The virus retains significant pathogenicity for the examined strains of mice while having the capacity to interact with SP-D in vitro and in vivo. The site and structure of the relevant HA glycan(s) have not yet been determined. However, the expression of the A/New Caledonia/20/99 (H1N1) HA1, which contains four sites of potential N-linked glycosylation, clearly confers the capacity of the WSN virus to bind to the mutant SP-D. Studies with the isolated HA and glycan characterization are planned.

Given the differential effects of the double mutant NCRD on the WSN wild-type and mutant strains in vitro and in vivo (Fig. 4 and 5), it is likely that the protective effects of D325A/R343V result from enhanced interactions of the mutant NCRD with the WSNHAnc-Asp225Gly hemagglutinin. However, SP-D and trimeric NCRDs can show a variety of effects on pulmonary homeostasis and can suppress inflammatory responses to microorganisms or biologically active glycoconjugates such as LPS. Regardless of mechanism, our studies suggest considerable therapeutic potential for mutant trimeric NCRDs and offer strategies for further enhancement of their antiviral activities.

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**REFERENCES**

1. Crouch, E. C. (2006) in Encyclopedia of Respiratory Medicine (Laurent, G., and Shapiro, S., eds) Vol. 4, pp. 152–158, Elsevier Ltd., Oxford, UK
2. Kishore, U., Greenhough, T. J., Waters, P., Shrive, A. K., Ghai, R., Kamran, M. F., Bernal, A. L., Reid, K. B., Madan, T., and Chakraborty, T. (2006) Mol. Immunol. 43, 1293–1315
3. Wright, J. R. (2005) Nat. Rev. Immunol. 5, 58–68
SP-D Mutant Enhances Defenses against Influenza A Virus

4. Whitsett, J. A. (2005) *Biol. Neonate* 88, 175–180
5. Holmskov, U., Thiel, S., and Jensenius, J. C. (2003) *Annu. Rev. Immunol.* 21, 547–578
6. LeVine, A. M., Whitsett, J. A., Hartshorn, K. L., Crouch, E. C., and Korfhagen, T. R. (2001) *J. Immunol.* 167, 5868–5873
7. LeVine, A. M., Elliott, J., Whitsett, J., Srikaitthachorn, A., Crouch, E., DeSilva, N., and Korfhagen, T. R. (2004) *Am. J. Respir. Crit. Care Med.* 31, 193–199
8. Vigerust, D. J., Ulett, K. B., Boyd, K. L., Madsen, J., Hawgood, S., and McCullers, J. A. (2007) *J. Virol.* 81, 8593–8600
9. Hawgood, S., Brown, C., Edmondson, J., Stumbough, A., Allen, L., Goerke, J., Clark, H., and Pouilan, F. (2004) *J. Virol.* 78, 8565–8572
10. Zhang, L., Hartshorn, K. L., Crouch, E. C., Ikegami, M., and Whitsett, J. A. (2002) *J. Biol. Chem.* 277, 22453–22459
11. Hartshorn, K. L., White, M. R., Voeller, D. R., Coburn, J., Zaner, K., and Crouch, E. C. (2000) *Biochem. J.* 351, 449–458
12. Reading, P. C., Holmskov, U., and Anders, E. M. (1998) *J. Gen. Virol.* 79, 2235–2263
13. Hartshorn, K. L., Webby, R., White, M. R., Teclc, T., Pan, C., Boucher, S., Moreland, R. J., Crouch, E. C., and Scheule, R. K. (2008) *Respir. Res.* 9, 55
14. Crouch, E., McDonald, B., Smith, K., Roberts, M., Mealy, T., Seaton, B., and Head, J. (2007) *Biochemistry* 46, 5160–5169
15. Wang, H., Head, J., Kosma, P., Brade, H., Müller-Loennies, S., Sheik, S., McDonald, B., Smith, K., Caferella, T., Seaton, B., and Crouch, E. (2008) *Biochemistry* 47, 710–720
16. Shrive, A. K., Tharia, H. A., Strong, P., Kishore, U., Zhang, P., Meschi, J., Mogues, T., White, M. R., and Crouch, E. C. (2002) *Biochem. J.* 366, 87–96
17. Hartshorn, K. L., Sastry, K., White, M. R., Okarma, T. B., Lee, Y. M., and Tauber, A. I. (1993) *J. Immunol.* 151, 6265–6273
18. Hartshorn, K. L., Sastry, K., White, M. R., Anders, E. M., Super, M., Eze-kowitz, R. A., and Tauber, A. I. (1993) *J. Clin. Invest.* 91, 1414–1420
19. Crouch, E., McDonald, B., Smith, K., Caferella, T., Seaton, B., and Head, J. (2006) *J. Biol. Chem.* 281, 18008–18014
20. Crouch, E., Hartshorn, K., Horalcher, T., McDonald, B., Smith, K., Caferella, T., Seaton, B., Seeberger, P. H., and Head, J. (2009) *Biochemistry* 48, 3335–3345
21. Håkansson, K., Lim, N. K., Hoppe, H. J., and Reid, K. B. (1999) *Structure Fold Des.* 7, 255–264
22. Shrive, A. K., Martin, C., Burns, J., Paterson, J. M., Martin, J. D., Townsend, J. P., Waters, P., Clark, H. W., Kishore, U., Reid, K. B., and Greenhough, T. J. (2009) *J. Mol. Biol.* 394, 776–788
23. Crouch, E. C., Smith, K., McDonald, B., Briner, D., Linders, B., McDonald, J., Holmskov, U., Head, J., and Hartshorn, K. (2006) *Am. J. Respir. Cell Mol. Biol.* 35, 84–94
24. Crouch, E., Tu, Y., Briner, D., McDonald, B., Smith, K., Holmskov, U., and Hartshorn, K. (2005) *J. Biol. Chem.* 280, 17046–17056
25. Hartshorn, K. L., White, M. R., Smith, K., Sorenson, G., Kuroki, Y., Holmskov, U., Head, J., and Crouch, E. C. (2010) *Scand. J. Immunol.* 72, 22–30
26. Hartshorn, K. L., White, M. R., Teclc, T., Sorenson, G., Holmskov, U., and Crouch, E. C. (2010) *Am. J. Physiol. Lung Cell Mol. Physiol.* 298, L79–L88
27. Carlson, T. K., Torrellas, I. B., Smith, K., Horlacher, T., Castelli, R., Seeberger, P. H., Crouch, E. C., and Schlesinger, L. S. (2009) *Glycobiology* 19, 1473–1484
28. Sme, D. F., Bailey, K. W., Wong, M. H., O’Keefe, B. R., Gustafson, K. R., Mishin, V. P., and Gubareva, L. V. (2008) *Antiviral Res.* 80, 266–271
29. Wilson, I. A., Skehel, J. J., and Wiley, D. C. (1981) *Nature* 289, 366–373
30. Perlman, D. H., Bauer, S. M., Ashrafian, H., Bryan, N. S., Garcia-Saura, M. F., Lim, C. C., Fernandez, B. O., Infusini, G., McGob, M. E., Costello, C. E., and Feidtsh, M. (2009) *Circ. Res.* 104, 796–804
31. Naimy, H., Buczko-Thomas, J. A., Nugent, M. A., Leymarie, N., and Zai, J. (2011) *J. Biol. Chem.* 286, 19311–19319
32. Calarase, D. A., Scanlon, C. N., Zwick, M. B., Deechongkit, S., Mimura, Y., Kunert, R., Zhu, P., Wormald, M. R., Stanfield, R. L., Roux, K. H., Kelly, J. W., Rudd, P. M., Dwek, R. A., Katinger, H., Burton, D. R., and Wilson, I. A. (2003) *Science* 300, 2065–2071
33. Sauter, N. K., Hansm, J. E., Glick, G. D., Brown, J. H., Crowther, R. L., Park, S. J., Skehel, J. J., and Wiley, D. C. (1992) *Biochemistry* 31, 9609–9621
34. White, M., Kingma, P., Teclc, T., Kacak, N., Linders, B., Heuser, J., Crouch, E., and Hartshorn, K. (2008) *J. Immunol.* 181, 7936–7943
35. White, M. R., Boland, P., Teclc, T., Gantz, D., Sorenson, G., Tornoe, I., Holmskov, U., McDonald, B., Crouch, E. C., and Hartshorn, K. L. (2010) *J. Innate Immun.* 2, 267–279
36. Teclc, T., White, M. R., Sorenson, G., Gantz, D., Kacak, N., Holmskov, U., Smith, K., Crouch, E. C., and Hartshorn, K. L. (2008) *Biochem. J.* 412, 323–329
37. Leth-Larsen, R., Garred, P., Jensenius, H., Meschi, J., Hartshorn, K. L., Madsen, J., Tornoe, I., Madsen, H. O., Sorenson, G., Crouch, E., and Holmskov, U. (2005) *J. Immunol.* 174, 1532–1538
38. Schwarzer, J., Rapp, E., Hennig, R., Genzel, Y., Jordan, I., Sandig, V., and Reichl, U. (2009) *Vaccine* 27, 4325–4336
39. Nieba, L., Nieba-Axmann, S. E., Persson, A., Hämäläinen, M., Edebratt, F., Hansson, A., Liidholm, J., Magnusson, K., Karlsson, A. F., and Plückthun, A. (1997) *Annu. Chem.* 282, 214–226
40. Hartshorn, K., Chang, D., Rust, K., White, M., Heuser, J., and Crouch, E. C. (1996) *Am. J. Physiol.* 271, L753–L762