Mechanism of binding of surfactant protein D to influenza A viruses: importance of binding to haemagglutinin to antiviral activity

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Collectins are important in the initial containment of a variety of pathogens, including influenza A virus (IAV). We provide the first systematic evaluation of the oligosaccharide-binding sites for pulmonary surfactant protein D (SP-D) on specific IAV coat glycoproteins and define the relationship between this binding and antiviral activity. With the use of several techniques, SP-D was found to bind via its carbohydrate-recognition domain (CRD) to mannosylated, N-linked carbohydrates on the HA domain of the haemagglutinin (HA) and on the neuraminidase of IAV. Using a set of IAV strains that differed in the level and site of glycosylation, and a panel of recombinant collectins, we found that binding of SP-D to the globular domain of the HA was critical in mediating the inhibition of viral haemagglutination activity and infectivity. We also demonstrated that the pattern of binding of a collectin to IAV glycoproteins can be modified by altering the monosaccharide-binding affinity of its CRD or by linking the CRD to a different N-terminal/collagen domain. These studies clarify the mechanisms of viral neutralization by collectins and might be useful in engineering collectins for enhanced antiviral activity.

Key words: collectins, conglutinin, mannose-binding lectin, neuraminidase.

INTRODUCTION

The collectins are a group of collagenous lectins present in pulmonary secretions and mammalian serum that have a role in first-line host defence against a variety of pathogens by binding to specific microbial carbohydrate determinants [1,2]. We have demonstrated that lung collectins, surfactant protein D (SP-D) and surfactant protein A (SP-A), and the serum collectins mannose-binding lectin (MBL) and bovine serum conglutinin, inhibit haemagglutination activity and infectivity of influenza A viruses (IAVs) and also act as opsonins, increasing respiratory-burst responses of neutrophils treated with IAV [3–5]. In contrast, SP-A has a mannose-type carbohydrate-recognition domain (CRD) and is important in the proliferation of the IAV in vivo. Thus the binding of collectins to either HA or neuraminidase could be important for the inhibition of infectivity.

HA exists as a trimer, and neuraminidase as a tetramer, on the viral surface. The HA monomer (termed HA0) is composed of two domains, HA1 and HA2, which are connected by interchain disulphide bonds. HA1 contains the region of the HA that forms the globular domain most distal from the viral surface containing the sialic-acid-binding pocket. IAV strains are categorized numerically by the subtype of HA and neuraminidase (indicated numerically). The most common recently circulating strains of IAV are of the H1 and H3 subtype. The HA domain of recent H3 strains of IAV have been shown to contain type II oligosaccharides [9]. It is likely that type II oligosaccharides are also present on HA1 of recent H1 strains. However, the older H1 strain, PR-8 (isolated in 1934), lacks such attachments. The HA2 domains of recent H1 and H3 IAV strains also do not have type II oligosaccharide attachments. There is evidence that high-mannose type II oligosaccharide attachments on HA2 of recent H1 and H3 strains might be crucial for the binding of the serum collectins (i.e. MBL and bovine conglutinin) [10,11]. However, type II oligosaccharides are also present on the viral neuraminidase. Previous studies have demonstrated the binding of MBL either predominantly to influenza neuraminidase [12] or to both HA and neuraminidase [6,13]. SP-D, conglutinin and MBL inhibit the haemagglutination activity of several strains of IAV through a mechanism that is Ca2+-dependent and inhibitable by specific monosaccharides [3–5]. In contrast, SP-A has a
distinctive mode of inhibiting infectivity and haemagglutination activity of IAV, which involves the Ca^{2+}-independent binding of IAV to sialylated carbohydrates associated with the CRD of SP-A [14,15]. Here we demonstrate, by several independent lines of evidence, that the binding of SP-D, MBL and conglutinin to high-mannose carbohydrates on the viral HA is crucial for the inhibition of IAV infectivity.

MATERIALS AND METHODS

Reagents

Dulbecco’s PBS was purchased from Flow Laboratories (Costa Mesa, CA, U.S.A.). Biotinylated concanavalin A was obtained from Sigma Chemical Co. (St Louis, MO, U.S.A.). Endoglycosidase H and recombinant peptide N-glycosidase F (N-glycanase) were obtained from Oxford Glycosystems (Rosedale, NY, U.S.A.).

Collectin preparations

Table 1 provides an outline of native and recombinant collectin preparations used in this paper. Methods of production have been outlined elsewhere, as referenced in Table 1. In brief, a variety of rat recombinant SP-D (RrSP-D) forms were employed, including a wild-type form that exists predominantly as a dodecamer [16], a mutant form (RrSP-D_{E321Q,N323D}) that lacks the single N-linked carbohydrate attachment present on wild-type RrSP-D, and another form (RrSP-D_{E321Q,N323D}) that was mutated in specific residues in the CRD to change the carbohydrate preference of SP-D such that the affinity for galactose was increased relative to that for mannose. Both RrSP-D_{E321Q,N323D} and RrSP-D_{E321Q,N323D} are secreted as dodecamers, like wild-type RrSP-D.

Using the cDNA of bovine conglutinin [17] we prepared bovine recombinant conglutinin (RbConglutinin) and a hybrid collectin containing the neck domain and CRD of conglutinin coupled to the collagen domain and N-terminus of RrSP-D (termed SP-D/Cong_{neck+CRD}) by similar methods. The method used for constructing the SP-D/Cong_{neck+CRD} hybrids is described elsewhere [18]. RbConglutinin and SP-D/Cong_{neck+CRD} hybrids were eluted in the expected position for authentic rat SP-D dodecamers by gel filtration.

Human native MBL was purified on mannan Sepharose and anti-IgG columns [19]. Bovine serum conglutinin was purified from bovine serum as described [4,20]. Human serum MBL and bovine conglutinin were provided by Jens Jensensius (University of Aarhus, Aarhus, Denmark). All of the collectin preparations used in these studies were free of other contaminating proteins as judged by SDS/PAGE analysis. Collectins or antibodies (see below) were biotinylated by incubation at a 2:1 ratio (w/v) with NHS-LC-biotin (N-hydroxysuccinimide-long chain-biotin; Pierce, Rockford, IL, U.S.A.) for 2 h at room temperature, followed by dialysis overnight against PBS.

Collectin preparations and buffers were tested for the presence of endotoxin. In samples diluted to the same extent as those used in the assays reported in this paper, final concentrations of between 20 and 200 pg/ml of endotoxin were detected with a semiquantitative assay (Limulus Amebocyte Lysate; Bio-Whittaker, Walkersville, MD, U.S.A.). Purified *Escherichia coli* lipopolysaccharide at 50 pg/ml to 50 ng/ml did not significantly alter the results of assays for HA inhibition or virus neutralization.

Virus preparation

Table 2 outlines the various IAV preparations used in this study. IAVs were grown in the chorioallantoic fluid of 10-day-old embryonated hen eggs, then purified on a discontinuous sucrose-density gradient as described previously [21]. Virus stocks were dialysed against PBS, aliquoted and stored at 70°C until use. HA titres were determined by the titration of virus samples in PBS followed by the addition of thoroughly washed human type O erythrocytes. The A/Texas 77/H3N2 (Texas 77) and A/PR/8/34/H1N1 (PR8) strains of IAV were gifts from Dr Jon

Table 1 Summary of native and recombinant collectin preparations used in this study

<table>
<thead>
<tr>
<th>Sp-D preparation</th>
<th>Source</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RhSP-D</td>
<td>CHO-K1 cells</td>
<td>Wild type; dodecamer or multimerized</td>
<td>[27]</td>
</tr>
<tr>
<td>RiSP-D</td>
<td>CHO-K1 cells</td>
<td>Wild type; dodecamer</td>
<td>[16,25]</td>
</tr>
<tr>
<td>RhSP-D&lt;sub&gt;E321Q,N323D&lt;/sub&gt;</td>
<td>CHO-K1 cells</td>
<td>Altered carbohydrate preference; dodecamer</td>
<td>[24]</td>
</tr>
<tr>
<td>Conglutinin</td>
<td>Bovine serum</td>
<td>Dodecamer</td>
<td></td>
</tr>
<tr>
<td>RhConglutinin</td>
<td>CHO-K1 cells</td>
<td>Wild type; dodecamer</td>
<td>[18]</td>
</tr>
<tr>
<td>SP-D/Cong&lt;sub&gt;neck+CRD&lt;/sub&gt;</td>
<td>CHO-K1 cells</td>
<td>Hybrid containing N-terminus and collagen domain or RhSP-D and neck and CRD of conglutinin; dodecamer</td>
<td>[18]</td>
</tr>
<tr>
<td>MBL</td>
<td>Human serum</td>
<td>Multimerized</td>
<td></td>
</tr>
</tbody>
</table>

Table 2 Influenza A viral strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Subtype</th>
<th>Glycosylation of HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mem71H-BelN</td>
<td>H3N1</td>
<td>HA has seven potential glycosylation sites</td>
</tr>
<tr>
<td>Mem71H-BelN/BS</td>
<td>H3N1</td>
<td>Loss of single high-mannose oligosaccharide chain overlying sialic-acid-binding pocket of HA</td>
</tr>
<tr>
<td>Texas 77</td>
<td>H3N2</td>
<td>HA has eight potential glycosylation sites</td>
</tr>
<tr>
<td>Bangkok 79</td>
<td>H3N2</td>
<td>HA has eight potential glycosylation sites</td>
</tr>
<tr>
<td>Phi82</td>
<td>H3N2</td>
<td>HA has ten potential glycosylation sites</td>
</tr>
<tr>
<td>Phi82/BS</td>
<td>H3N2</td>
<td>Loss of single high-mannose oligosaccharide chain overlying sialic-acid-binding pocket of HA</td>
</tr>
<tr>
<td>PR-8(1934)</td>
<td>H1N1</td>
<td>Lacks high mannose carbohydrate attachments on HA; lacks carbohydrate attachments on globular domain of HA</td>
</tr>
<tr>
<td>Brazil 78</td>
<td>H1N1</td>
<td></td>
</tr>
<tr>
<td>Brazil 78/BS</td>
<td>H1N1</td>
<td>Loss of single high-mannose oligosaccharide chain overlying sialic-acid-binding pocket of HA</td>
</tr>
</tbody>
</table>

The glycosylation information is summarized in [6,8,9]. All strains designated with BS at the end were selected in culture (in eggs) for resistance to the bovine serum inhibitor [11].
Abramson (Bowman-Gray School of Medicine, Winston-Salem, NC, U.S.A.), A/Mem71/77, B/Philippines 82 (Phil82) and Brazil 78 strains were kindly provided by Dr E. Margot Anders (University of Melbourne, Melbourne, Australia). The A/Bangkok 79/H3N2 (Bangkok 79) strain was a gift from Robert Webster (St Jude’s Hospital, Memphis, TN, U.S.A.). Haemagglutination assays were performed with human type O erythrocytes as described [3].

Bromelain-solubilized HA was prepared with bromelain (Sigma, St Louis, MO, U.S.A.) and 5–20% sucrose gradients as described [22]. Monoclonal antibodies (mAbs) directed against the HA molecule of the Texas 77 and Bangkok 79 strains of IAV were incubated with the virus in various experiments. The Texas 77 mAb (designation 81/4) was a gift from Dr R. G. Webster and was provided in affinity-purified form in PBS. The hybridoma for Bangkok 79 mAb (designation 73/1; also originally produced by Dr Webster) was obtained from the American Tissue Type Collection (Manassas, VA, U.S.A.). The mAb was purified by Staphylococcus Protein A affinity chromatography and stored in PBS. Both of these antibodies were strongly inhibitory of HA activity of the IAV strains against which they were raised. mAbs directed against the Bangkok 79 and Texas 77 neuraminidase were kindly provided by Dr Alan Douglas (WHO Collaborative Centre for Reference and Research on Influenza, Mill Hill, London, UK).

Assessment of binding of collectins to IAV strains

Binding of collectins to IAV strains was tested with an ELISA in which suspensions (approx. 1 μg/ml) of virus were allowed to dry on 96-well plates, followed by washing and incubation with biotinylated SP-D [23]. Before the addition of collectins, plates were blocked with BSA and gelatin. Biotinylated collectins were then added and allowed to incubate with virus for 30 min at 37 °C, followed by the washing off of unbound lectin (three times in PBS). The presence of bound collectin was detected with streptavidin conjugated to horseradish peroxidase and tetramethylbenzidine (‘TMB’) substrate (Bio-Rad Labs, Hercules, CA, U.S.A.). The reaction was stopped with 0.5 M H2SO4. Attenuance was measured with an ELISA reader. Each individual data point was performed in triplicate. There was minimal background binding of the biotinylated collectin to wells not containing virus.

Assessment of binding of collectins to IAV glycoproteins

Influenza virus preparations were solubilized by boiling for 4 min in SDS buffer [62 mM Tris/HCl (pH 6.8)/2% (w/v) SDS/10% (v/v) glycerol]. Reduction of viral proteins was accomplished by the addition of 100 mM dithiothreitol (DTT). Samples were then subjected to SDS/PAGE on 10% (w/v) or 10–15% (w/v) gradient gels (as indicated). Molecular mass standards (Bio-Rad) were run with all gels. Some gels were then stained directly with Coomassie Blue. For Western blots, gels were transferred to PVDF transfer membrane (Immobilon-P; Millipore Corp., Bedford, MA, U.S.A.) with a semi-dry transfer unit (Hoefer Scientific). Transfer was performed for 2 h at 48 mA. After transfer the membranes were washed twice with 0.3% (v/v) Tween/TBS and then blocked for 1 h with Carnation non-fat dried milk [5% (w/v) in TBS]. On the basis of Coomassie Blue staining of the gels after transfer, it was evident that lower-molecular-mass proteins (less than 40 kDa) transferred more thoroughly to blots. Hence if any bias was present in final results it favoured the detection of proteins smaller than 40 kDa during blotting with lectin or mAb (see below). Membranes were then washed again and incubated with biotinylated collectins or mAbs either for 1 h at room temperature or overnight at 4 °C. The membranes were then washed twice and maintained in TBS until chemiluminescence assays were performed. Chemiluminescence assays were conducted by incubating the membranes with streptavidin-peroxidase (Kierkegaard and Perry, Gaithersburg, MD, U.S.A.) in 5% (w/v) milk/TBS for 1 h at room temperature, washed four times and exposed to LumiGLO chemiluminescence substrates (Kierkegaard and Perry) for 1 min, followed immediately by a brief (15 s to 3 min) exposure of X-OMAT film (Kodak Corp.) to the membranes. In some cases, membranes were repeatedly tested for chemiluminescence over several days after prolonged washing to decrease background chemiluminescence. After completion of chemiluminescence reactions, membranes were stained with Indian ink [50 μl in 50 ml of 0.3% (v/v) Tween/TBS] to confirm the presence and location of all proteins on the membrane.

Assay of IAV infectivity

Madin-Darby canine kidney monolayers were prepared in 96-well plates and grown to confluence. These layers were then infected with IAV preparations diluted in PBS with 2 mM CaCl2 for 45 min at 37 °C, followed by washing of the monolayer three times in serum and glucose-free DMEM (Dulbecco’s modified Eagle’s medium) containing 1% (w/v) penicillin and streptomycin. The monolayers were then incubated for 7 h at 37 °C under air/CO2 (19:1) in DMEM. The monolayers were subsequently washed three times with PBS and fixed with 80% (v/v) acetone for 10 min at 4 °C. The monolayers were then labelled by incubation with mAb A-3 directed against the IAV nucleoprotein (provided by Dr Nancy Cox, Centers for Disease Control, Influenza Branch, Atlanta, GA, U.S.A.) in reagent A [PBS containing 0.1% BSA, 1% (w/v) heat-inactivated human serum and 0.02% NaCl] for 30 min at 4 °C. The monolayers were washed three times in PBS with 2 mM calcium (PBS++) and incubated with FITC-labelled goat anti-mouse IgG. The fluorescent foci were counted directly under fluorescent microscopy. Initially, various dilutions of virus were used to find the dose yielding approx. 500 fluorescent foci per well. In general these foci seemed to be single infected cells. Subsequently, this dose of virus was preincubated with collectins followed by incubation of these collectin-treated virus samples with Madin-Darby canine kidney monolayers and counting of infected foci.

Statistics

Statistical comparisons were made with Student’s paired t test.

RESULTS

Collectins bind to solubilized IAV glycoproteins in solid-phase binding assays

Our initial goals were to clarify which IAV proteins are recognized by SP-D and to determine the mechanism of binding. We have previously shown that conglutinin and a chimaeric protein, SP-D/CongresCRD (which contained the RSP-D N-terminus and collagen domain linked to the neck and CRD domains of conglutinin), have greater IAV-neutralizing activity than SP-D [18]. Our next goal was to determine whether these collectins bind to different IAV proteins from SP-D, to account for this difference in neutralizing activity. Finally, we wished to determine whether the pattern of binding of SP-D to IAV glycoproteins differs between different IAV strains.
SP-D and conglutinin bind to N-linked oligosaccharides on the HA, of the HA and on the neuraminidase of Bangkok 79 H3N2 IAV

As shown in Figure 1, human recombinant SP-D (RhSP-D) bound to both HA and neuraminidase on Western blots of non-reducing SDS/PAGE gels of the Bangkok 79 strain of IAV. Binding to HA$_a$ (abundant band at approx. 80 kDa) and to the dimeric and trimeric forms of HA (approx. 160 and 240 kDa) was evident. SP-D bound specifically to the HA molecule after disulphide reduction but did not bind to HA$_b$ (approx. 32 kDa molecular mass on the blot containing approx. 220 kDa marker) and to the higher-molecular-mass bands so that it was possible to distinguish between the binding to the neuraminidase tetramer and HA trimer. Overall the intensity of binding of SP-D/Cong$_\text{neck+CRD}$ was greater than that of RBConglutinin. On the reducing gels, binding to HA$_a$ was most pronounced but additionally the collectins bound to two discrete bands of slightly higher molecular mass, which were compatible with the neuraminidase monomer and residual unreduced HA$_b$. These results indicate that RBConglutinin and SP-D/Cong$_\text{neck+CRD}$, both bound to neuraminidase and HA$_a$ of Bangkok 79 IAV, although the latter bound with greater intensity.

Binding of SP-D to solubilized glycoproteins of Bangkok 79 IAV was abolished fully by pre-treatment of the virus with N-glycanase (Figure 3). The apparent molecular mass of HA$_a$ was approx. 70 kDa after treatment with N-glycanase. Similarly, no binding was seen when the virus was pretreated with endoglycosidase H (specific for oligosaccharides terminating in mannose) or when the incubation of collectins with blots was performed in EDTA-containing buffer (results not shown). Concanavalin A bound to the same viral protein bands as SP-D on blots of reduced and unreduced Bangkok 79 IAV; the binding of concanavalin A was also largely eliminated by treatment of the virus with endoglycosidase H (results not shown). Taken together, these results indicate that SP-D binds preferentially via its CRD to mannosylated carbohydrates on the viral glyco-
Binding of surfactant protein D to influenza A viruses

Mem71, Bel, and Bangkok 79 IAV were solubilized by being boiled for 5 min in denaturing buffer, followed by incubation for 18 h at 37 °C with 0.3 unit of N-glycanase (right lanes) or not (left lanes). The samples were then subjected to non-reducing SDS/PAGE and transferred to PVDF membranes, which were then treated with biotinylated RhSP-D multimers. Bound RhSP-D was detected by chemiluminescence. Results shown are Coomassie Blue stains of SDS/PAGE gels [on the left of the indicated positions of molecular mass markers (in kDa)] or autoradiographs taken after chemiluminescence of the corresponding blots (on the right of the marker positions). Note the absence of RhSP-D binding to N-glycanase-treated blots.

Proteins and that these interactions do not involve interactions of HA or neuraminidase with sialylated carbohydrates on SP-D.

SP-D shows distinctive patterns of binding to glycoproteins of different IAV strains

Unexpectedly, SP-D did not seem to bind to the HA\textsubscript{n} of Mem71, Bel, (recombinant H3N1) strain of IAV, but rather bound predominantly to a band below HA\textsubscript{n} (see Figure 3) that was consistent with the neuraminidase of this strain (approx. 70 kDa). SP-D also bound intensely to a band of approx. 140 kDa that was consistent with neuraminidase dimer. Treatment of Mem71, Bel with N-glycanase eliminated the binding of SP-D. The major bands bound by SP-D were less abundant on the original gel than HA\textsubscript{n} (as expected for neuraminidase). This finding is similar to that obtained by Malhotra et al. [12] with the X31 (H3N2; 1968) strain. The HA of Memphis 71 and X-31 are similar in having only two glycosylation sites on the globular head region, whereas Bangkok 79 and other more recent strains have three or more sites [6]. In accordance with this, Memphis 71 and X-31 were less sensitive to neutralization by SP-D [6]. Therefore a relatively decreased binding of SP-D to the HA of these strains on a Western blot could reflect decreased glycosylation of the HA.

The PR-8 strain of IAV lacks high-mannose oligosaccharide attachments on the HA [8] and is highly resistant to neutralization by SP-D and other collectins [5]. SP-D, conglutinin and concanavalin A did not bind to any proteins of PR-8 in multiple experiments (results not shown).

Binding to HA\textsubscript{n} is crucial in mediating the inhibition of HA activity and infectivity

Given that SP-D was able to bind to HA and neuraminidase to different extents on different strains of IAV, we next sought to determine the relative importance of binding to HA or neuraminidase in mediating the attachment of collectins to whole viral preparations and/or antiviral activities of the collectins. We made use of paired bovine serum β-inhibitor-resistant strains...
Table 3 Binding of collectins to serum inhibitor-sensitive and -resistant strains of IAV

Results are means ± S.E.M. for three or more experiments (except for RrSP-D<sup>270/280N320D</sup>, for which n = 2). Initial dose–response experiments were performed and determined that maximal binding occurred at 0.5 μg/ml (although significant binding occurred at 30 ng/ml). Therefore the concentrations of collectins used were 0.5 μg/ml (except for RrSP-D<sup>270/280N320D</sup>, for which 1 μg/ml was used). The mean background $D_{450}$ was 0.07 in these experiments. The $D_{450}$ for all collectins was significantly greater than background. Phil and Braz are abbreviations for the Phil82 and Brazil 78 strains of IAV. For the percentage of Phil/BS to Phil and the percentage of Braz/BS to Braz, the $D_{450}$ for the resistant strain was divided by the $D_{450}$ of the parent strain and multiplied by 100. $D_{450}$ values for RrSP-D<sup>270/280N320D</sup> were significantly lower than for the other collectins. For all collectins, binding to the resistant IAV strains was significantly less than binding to the parent strain. *Significantly (P < 0.05) greater binding of collectin than RbConglutinin to the resistant viral strains.
Table 4  Inhibition of HA activity of wild-type and serum β-inhibitor-resistant strains of IAV by collectins

<table>
<thead>
<tr>
<th>Collectin</th>
<th>Brazil 78</th>
<th>Brazil 78/BS</th>
<th>Ratio</th>
<th>Phil82</th>
<th>Phil82/BS</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>RbConglutinin</td>
<td>4 ± 1</td>
<td>29 ± 10</td>
<td>7.2</td>
<td>2.7 ± 0.3</td>
<td>&gt; 6000</td>
<td>&gt; 222</td>
</tr>
<tr>
<td>RhSP-D dodecamers</td>
<td>16 ± 2</td>
<td>66 ± 29</td>
<td>6.6</td>
<td>16 ± 8</td>
<td>157 ± 10</td>
<td>9.8</td>
</tr>
<tr>
<td>RrSP-D</td>
<td>20 ± 4</td>
<td>40 ± 8</td>
<td>2</td>
<td>17 ± 5</td>
<td>449 ± 61</td>
<td>26</td>
</tr>
<tr>
<td>SP-D/Congneck+CRD</td>
<td>2 ± 0.05</td>
<td>11 ± 6.6</td>
<td>5.5</td>
<td>4.3 ± 0.8</td>
<td>224 ± 45</td>
<td>52</td>
</tr>
</tbody>
</table>

Figure 7  Inhibition of infectivity of Phil82 (upper panel) and Phil82/BS (lower panel) strains of IAV by RrSP-D, RbConglutinin and SP-D/Congneck+CRD

Infectious viral particles were quantified with a fluorescent focus assay (see the Materials and methods section). Results are means ± S.E.M. for three or more experiments. The degrees of inhibition of infectivity of Phil82 and Phil82/BS by RbConglutinin (△), RrSP-D (○) and SP-D/Congneck+CRD (□) are compared. These Phil82 results were reported previously [18] and are included for comparison. SP-D/Congneck+CRD caused a significantly greater inhibition of infectivity of either strain of virus than RbConglutinin or RrSP-D. RbConglutinin caused a significantly greater inhibition of Phil82 than RrSP-D.

might account for this difference. Nevertheless, the distinctive patterns of binding of SP-D, RbConglutinin and SP-D/Congneck+CRD to glycoproteins of these IAV strains were useful in elucidating the relative importance of binding to HA and/or neuraminidase in mediating the inhibition of haemagglutination activity and infectivity.

Evidence from β-inhibitor-resistant strains: inhibition of HA activity and infectivity of Phil82 and Brazil 78 parent and bovine serum β-inhibitor-resistant strains is correlated with the ability to bind viral HA

Table 4 presents data on the ability of various collectins to inhibit the HA activity of various IAV strains. Some previously reported results are included for ease of comparison. RbConglutinin and SP-D/Congneck+CRD were significantly more potent than SP-Ds at inhibiting the HA activity of wild-type Phil82 and Brazil 79. We have reported previously [18] that conglutinin and SP-D/Congneck+CRD are markedly more potent at neutralizing the infectivity of wild-type Phil82 IAV than are SP-Ds. Because RbConglutinin bound only to the HA of these strains, whereas SP-D bound intensely to both the HA and neuraminidase bands (Figures 4–6), these results suggest that binding to HA is most important in mediating the haemagglutination inhibition and neutralization of viral infectivity.

As expected, the Brazil 79/BS and Phil82/BS strains were highly resistant to the inhibitory effects of RbConglutinin. This resistance was particularly marked in the Phil82/BS strain. This finding is consistent with the failure of RbConglutinin to bind to these viral strains on Western blotting and ELISA. Although Phil82/BS and Brazil 79/BS were also relatively resistant (in comparison with the parent strains) to HA inhibition by SP-D, the degree of resistance was much less marked than for RbConglutinin. This is consistent with the finding that SP-Ds bound more avidly than RbConglutinin to these strains. It is noteworthy that the Brazil 78/BS and Phil82/BS strains were significantly less resistant to SP-D/Congneck+CRD than they were to RbConglutinin. In fact, SP-D/Congneck+CRD inhibited the HA activity of Brazil 78/BS and Phil82/BS to similar or greater extents than SP-Ds. In addition, as shown in Figure 7, SP-D/Congneck+CRD inhibited the infectivity of Phil82/BS to a significantly greater extent than either RbConglutinin or RrSP-D. Because SP-D/Congneck+CRD bound only to the HA of Phil82/BS (see Figure 4), these results again indicate that binding to viral HA is critical in mediating the HA inhibition and neutralization of viral infectivity.

Evidence from β-inhibitor-resistant strains: binding of collectins to neuraminidase of Mem71H-BelN and Mem71H-BelN/BS was not correlated with antiviral activities

Similar conclusions were reached from studies of the binding of collectins to wild-type and β-inhibitor-resistant strains of
Mem71<sub>H</sub>-Bel<sub>N</sub>. SP-D bound most prominently to bands consistent with neuraminidase of the Mem71<sub>H</sub>-Bel<sub>N</sub> strain (Figure 3); and similar findings were obtained with MBL (Figure 8). Furthermore, SP-D (results not shown) and MBL (Figure 8) bound with equal intensity to neuraminidase bands of the Mem71<sub>H</sub>-Bel<sub>N</sub>/BS strain. There was minimal apparent binding of SP-D or MBL to HA<sub>N</sub> or HA<sub>H</sub> bands of these strains. Similar results were obtained with conglutinin (results not shown). In further testing of the binding of conglutinin to Mem71<sub>H</sub>-Bel<sub>N</sub> and Mem71<sub>H</sub>-Bel<sub>N</sub>/BS, we adjusted the assay to permit a higher degree of non-specific binding. By doing this it was possible to show a substantial difference in the ability of conglutinin to bind to HA<sub>N</sub> of Mem71<sub>H</sub>-Bel<sub>N</sub> in comparison with that of Mem71<sub>H</sub>-Bel<sub>N</sub>/BS (Figure 8). Because Mem71<sub>H</sub>-Bel<sub>N</sub>/BS is highly resistant to haemagglutination inhibition and inhibition of infectivity by MBL and conglutinin [3,4], these results reinforce our conclusion that the binding of collectins to the viral HA is a more important determinant of neutralization of viral infectivity than binding to neuraminidase. This is true even when binding to neuraminidase is more pronounced than binding to HA on Western blots of solubilized proteins.

SP-D mutant with decreased ability to bind to mannose-containing oligosaccharides shows decreased binding to solubilized HA and decreased haemagglutination-inhibition activity

R<sub>r</sub>SP-D<sub>ΔE321Q,N323D</sub> is a mutant form of R<sub>r</sub>SP-D containing alterations in the CRD that result in an increased affinity for galactose relative to its affinity for mannose or glucose [24]. As shown in Figure 9, R<sub>r</sub>SP-D<sub>ΔE321Q,N323D</sub> bound to proteins of Bangkok 79 IAV in a distinctive manner compared with the binding of wild-type R<sub>r</sub>SP-D. R<sub>r</sub>SP-D<sub>ΔE321Q,N323D</sub> bound with less intensity to HA<sub>N</sub> and HA<sub>H</sub> bands but with greater intensity to some higher-molecular-mass bands on the unreduced blot (including one corresponding to neuraminidase tetramer). Despite avid binding to these viral proteins on a Western blot, R<sub>r</sub>SP-D<sub>ΔE321Q,N323D</sub> had a markedly decreased ability to bind to whole viral particles compared with the binding of R<sub>r</sub>SP-D as assessed by ELISA (see Table 5).

Because the major difference between the Phil82 and Phil82/BS strains is the loss of a high-mannose oligosaccharide on the viral HA, we thought that R<sub>r</sub>SP-D<sub>ΔE321Q,N323D</sub> might not show decreased binding to the β-inhibitor-resistant strains. R<sub>r</sub>SP-D<sub>ΔE321Q,N323D</sub> bound significantly less to the resistant IAV strains than to the parent strains (Table 3). The degree of decrease in binding of R<sub>r</sub>SP-D<sub>ΔE321Q,N323D</sub> to the resistant strains was similar to the decrease obtained for R<sub>r</sub>SP-D. These results seem to indicate that R<sub>r</sub>SP-D<sub>ΔE321Q,N323D</sub> binds to Phil82 or Brazil 79 mainly through an attachment to high-mannose oligosaccharides.

Substantially higher concentrations of R<sub>r</sub>SP-D<sub>ΔE321Q,N323D</sub> than of wild-type R<sub>r</sub>SP-D were required to inhibit the haemagglutination activity of the Bangkok 79 and Brazil 78 strains of IAV (Table 5, and results not shown). In addition, consistent with our prior results obtained with SP-D, MBL and conglutinin was our observation that R<sub>r</sub>SP-D and R<sub>r</sub>SP-D<sub>ΔE321Q,N323D</sub> showed no detectable binding to the PR-8 strain of IAV on Western blot.
and no inhibition of the haemagglutination activity of this strain (results not shown). Although PR-8 lacks high-mannose oligosaccharides on the HA molecule, it does contain complex oligosaccharides (as do the other strains of IAV) [9]. Complex oligosaccharides on IAV terminate in galactose owing to the action of the viral neuraminidase. Taken together, the results obtained with \( \text{RrSp-D}_{\text{BS}}^{\text{Q,N,3N2D}} \) are consistent with the interpretation that binding to intact viral particles and HA inhibition by the collectins are mediated largely by attachment to mannose-containing oligosaccharides on the HA. In addition, these results again indicate that the binding of collectins to HA on Western blots is the most reliable predictor of the ability of a collectin to inhibit viral HA activity.

**DISCUSSION**

In these studies, we have shown that SP-D binds to a Ca\(^{2+}\)-dependent manner to N-linked high-mannose oligosaccharides on specific subdomains of glycoproteins of recent strains of IAV. This conclusion was supported by the use of glycosidases, differentially glycosylated strains of IAV, mutant forms of SP-D (e.g. \( \text{RrSp-D}_{\text{BS}}^{\text{Q,N,3N2D}} \), concanavalin A and EDTA, SP-D, conglutinin and MBL bound to HA, and, more specifically, to the HA domain of the HA of more recent wild-type viral strains. Because this domain of the HA is furthest from the viral surface and contains the sialic-acid-binding site, it is probably the most accessible on intact viral particles. In addition, binding to this domain of the HA would be likely to result in the inhibition of viral haemagglutination activity and infectivity by interference with the sialic-acid-binding pocket.

As noted above, SP-A inhibits IAV infectivity by a distinctive mechanism involving binding of the IAV HA to carbohydrates on the CRD of SP-A. Native and recombinant SP-Ds have a single N-linked carbohydrate attachment located in the collagen domain of the molecule [25]. Deletion of this carbohydrate attachment did not decrease the ability of SP-D to inhibit the haemagglutination activity of IAV (results not shown and [14]). However, further studies are under way to determine whether this oligosaccharide contributes in some way to the interactions of SP-D with IAV or other pathogens. It should also be noted that the extent or identity of glycosylation of recombinant conglutinin or MBL have not been studied extensively. Although recombinant and serum-derived MBL and conglutinin have shown similar antiviral activities in previous studies [3–5, 14, 18], it is possible that N-linked oligosaccharides present on the serum collectins contribute to interactions with IAV.

Because SP-D also binds to the neuraminidase of recent strains of IAV, we next sought to determine the relative importance of binding to HA and neuraminidase in mediating antiviral effects. Several lines of evidence indicated that binding to the viral HA is most important in mediating the HA inhibition and neutralization of infectivity. On Western blots, conglutinin bound only to the HA of wild-type Phil82 and Brazil 79 (relatively recent representatives of the two major circulating IAV types, H3N2 and H1N1), whereas SP-Ds bound to both the HA and neuraminidase of these strains. However, conglutinin was significantly more potent at inhibiting HA activity and infectivity of these strains than was SP-D. Similarly, the chimera SP-D/Con\(_{\text{BS}}^{\text{Q,N,3N2D}}\) bound only to the HA of the serum \( \beta \)-inhibitor-resistant strain Phil82/BS but neutralized the infectivity of this viral strain to a significantly greater degree than SP-D, which bound to both HA and neuraminidase.

Conglutinin, MBL and SP-D bound preferentially to the neuraminidase of Mem71\(_{n}\)-Bel\(_{n}\) and showed equally intense binding to the neuraminidase of the Mem71\(_{n}\)-Bel\(_{n}\)/BS serum \( \beta \)-inhibitor-resistant variant strain. However, the collectins have significantly less ability to inhibit the HA activity and infectivity of Mem71\(_{n}\)-Bel\(_{n}\)/BS. Only through decreasing the stringency of binding conditions was it possible to demonstrate the binding of conglutinin to the HA of Mem71\(_{n}\)-Bel\(_{n}\) on a Western blot and thereby confirm the decreased binding of conglutinin to the HA of Mem71\(_{n}\)-Bel\(_{n}\)/BS. Clearly, binding to neuraminidase on Western blots of solubilized proteins was not correlated with the antiviral activities of collectins against the Mem71\(_{n}\)-Bel\(_{n}\) and Mem71\(_{n}\)-Bel\(_{n}\)/BS strains.

It is possible that solubilization of the Mem71\(_{n}\)-Bel\(_{n}\) virus makes the neuraminidase more accessible to binding than occurs with the intact virus. The HA of Mem71\(_{n}\)-Bel\(_{n}\) is less glycosylated than those of more recent strains (see Table 2), which could contribute to the decreased binding of collectins to the HA of this strain on a Western blot. This might also account for the finding of Malhotra et al. [12] that MBL bound to the neuraminidase of the X31 strain (H3N2, 1968) without any apparent binding to the viral HA. In any case, results obtained with the Mem71\(_{n}\)-Bel\(_{n}\) strains also indicate that binding to the viral HA was a more important determinant of neutralization of viral infectivity than binding to the neuraminidase. This conclusion was supported by results obtained with the \( \text{RrSp-D}_{\text{BS}}^{\text{Q,N,3N2D}} \) mutant form of SP-D.

Overall, in these studies the intensity of binding to solubilized viral proteins (especially the neuraminidase) on a Western blot did not correlate consistently with antiviral activity. However, there was a consistent association between the ability of specific collectins to bind HA, with that collectin’s ability to inhibit viral haemagglutination activity and the infectivity of a given IAV strain. These results do not exclude the possibility that the binding of collectins to neuraminidase might inhibit neuraminidase activity and that this action contributes to viral containment in vivo.

These studies confirm that SP-D/Con\(_{\text{BS}}^{\text{Q,N,3N2D}}\) has enhanced anti-influenza activity in comparison with either of the wild-type proteins from which it was derived. We have obtained similar results with a chimera containing the human SP-D N-terminus and collagen domain, and the neck region and CRD of MBL [26]. These findings indicate that modifications of the collectin CRD domain through recombinant techniques will be a useful strategy for enhancing the antiviral activity of collectins.

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**REFERENCES**


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