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 HA₁ 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]. Using a mouse model of IAV infection, Reading et al. [6] have shown that collectins present in pulmonary secretions are important in limiting the extent of replication of IAV in the lung *in vivo*. Given the probable role played by collectins in innate host defence, and the possibility that recombinant collectins could be developed as therapeutic agents against IAV, it is important to develop a precise understanding of how collectins attach to IAV and inhibit viral infectivity.

Collectins have a mannose-type carbohydrate-recognition domain (CRD) and are therefore expected to bind preferentially to high-mannose (type II) oligosaccharides, as opposed to complex oligosaccharides. Influenza viruses have two envelope glycoproteins, haemagglutinin (HA) and neuraminidase, that are involved in viral interactions with cells and/or other viral particles. The HA is heavily glycosylated in most IAV strains and is responsible for viral binding to cell-surface sialoglycoproteins [7,8]. The viral neuraminidase, which is one-tenth as abundant,

is believed to be involved in viral budding from infected cells 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 HA₁ 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 HA₁ 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

Abbreviations used: CRD, carbohydrate-recognition domain; DTT, dithiothreitol; HA, haemagglutinin; IAV, influenza A virus; mAb, monoclonal antibody; MBL, mannose-binding lectin; N-glycanase, peptide N-glycosidase F; RbConglutinin, bovine recombinant conglutinin; RhSP-D, human recombinant SP-D; RrSP-D, rat recombinant SP-D; SP-D, surfactant protein D.

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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_1 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^{Ala72}) 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^{Ala72} 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} hybrid 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 Jensenius (University of Aarhus, Aarhus, Denmark). All of the collectin preparations used in these studies were free of other contaminating proteins as

Table 2 Influenza A viral strains used in this study

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].

Strain	Subtype	Glycosylation of HA
Mem71H-BelN	H3N1	HA has seven potential glycosylation sites
Mem71H-BelN/BS	H3N1	Loss of single high-mannose oligosaccharide chain overlying sialic-acid-binding pocket of HA
Texas 77	H3N2	HA has eight potential glycosylation sites
Bangkok 79	H3N2	HA has eight potential glycosylation sites
Phil82	H3N2	HA has ten potential glycosylation sites
Phil82/BS	H3N2	Loss of single high-mannose oligosaccharide chain overlying sialic-acid-binding pocket of HA
PR-8(1934)	H1N1	Lacks high mannose carbohydrate attachments on HA; lacks carbohydrate attachments on globular domain of HA
Brazil 78	H1N1	
Brazil 78/BS	H1N1	Loss of single high-mannose oligosaccharide chain overlying sialic-acid-binding pocket of HA

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

'Multimerized' refers to the formation of large multimeric structures beyond the basic trimer (i.e. dodecamers or higher). Abbreviation: CHO, Chinese hamster ovary.

SP-D preparation	Source	Description	Reference
RhSP-D		Wild type; dodecamer or multimerized	[27]
RrSP-D	CHO-K1 cells	Wild type; dodecamer	[16,25]
RrSP-D ^{E321Q, N323D}	CHO-K1 cells	Altered carbohydrate preference; dodecamer	[24]
Conglutinin	Bovine serum	Dodecamer	
RbConglutinin	CHO-K1 cells	Wild type; dodecamer	[18]
SP-D/Cong _{neck+CRD}	CHO-K1 cells	Hybrid containing N-terminus and collagen domain or RrSP-D and neck and CRD of conglutinin; dodecamer	[18]
MBL	Human serum	Multimerized	

Abramson (Bowman-Gray School of Medicine, Winston-Salem, NC, U.S.A.). A/Mem71_H-Bel_N, 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 (Hofer 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 Ca²⁺ 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% (v/v) heat-inactivated human serum and 0.02% NaN₃] 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/Cong_{neck+CRD} (which contained the RrSP-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.

Figure 1 RhSP-D binds to HA and neuraminidase of Bangkok 79 (H3N2) IAV

Non-reducing (left lanes) or reducing (right lanes) gels of Bangkok 79 IAV were transferred to PVDF membranes and treated with either biotinylated anti-neuraminidase mAb (left panel) or RhSP-D (right panel). Results shown are chemiluminescence autoradiographs. These results and those in other Western blots in later figures are representative of three or more similar experiments. The positions of HA₀, HA₁ and the dimeric and trimeric forms of HA (above and below the 220 kDa marker) are indicated. These bands were confirmed to be HA by using the Hb66 mAb directed against the HA of Bangkok 79 IAV. The positions of the unreduced neuraminidase dimer and tetramer, and of the reduced neuraminidase monomer, are indicated by N. Although some binding to the neuraminidase monomer was evident, the neuraminidase mAb bound most intensely to the neuraminidase tetramer. The neuraminidase antibody also exhibited non-specific binding to the viral M protein (abundant protein of approx. 30 kDa). The positions of molecular mass markers are indicated (in kDa) between the panels.

SP-D and conglutinin bind to N-linked oligosaccharides on the HA₁ 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 HA₀ (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 HA₁ component of the HA molecule after disulphide reduction but did not bind to HA₂ (approx. 32 kDa molecular mass on the blot containing reduced viral proteins) (Figure 1). SP-D bound similarly to proteins of Texas 77 IAV (results not shown). Binding of SP-D to HA₀ and HA₁ (and not to HA₂) was confirmed with purified bromelain-solubilized HA (results not shown). A preparation consisting only of the isolated trimeric CRD domain of SP-D also bound faintly to HA₀ and HA₁ of Bangkok 79 IAV (results not shown). In addition, SP-D bound to additional bands of approx. 150 kDa on the non-reducing gel, and 75 kDa on the reducing gel, which was consistent with binding to dimeric and monomeric forms of neuraminidase (see neuraminidase mAb binding; Figure 1, left panel). The locations of the neuraminidase tetramer and the HA trimer were almost identical on the non-reducing gel shown in Figure 1.

Figure 2 Binding of RbConglutinin or SP-D/Cong_{neck+CRD} to proteins of Bangkok 79 IAV

Western blots of reduced or unreduced proteins of Bangkok 79 IAV were treated with RbConglutinin (left panel) or SP-D/Cong_{neck+CRD} (middle panel). Gradient gels were used to obtain a greater separation of higher-molecular-mass bands. Reduced proteins are on the left in each panel. The left and middle panels are chemiluminescence autoradiographs. Right panel: stain with indian ink of the blot that had been treated with SP-D/Cong_{neck+CRD}. Indian ink did not stain the areas of the blot where SP-D/Cong_{neck+CRD} bound. Frequently, as shown here, areas to which the collectins bound strongly did not stain with indian ink and appeared white after staining. The clarity of these white bands in this case actually exceeded that of the chemiluminescence autoradiograph. Note that it is possible to discern the binding of two discrete bands at approx. 240 kDa, which is consistent with binding to the HA trimer and neuraminidase tetramer (slightly below the more abundant HA trimer band). The positions of molecular mass markers are indicated (in kDa) between the middle and right panels.

As shown in Figure 2, similar findings were obtained with RbConglutinin and the chimaeric protein SP-D/Cong_{neck+CRD}, which contained the RrSP-D N-terminus and collagen domain linked to the neck and CRD domains of conglutinin [18]. Gradient gels were used to achieve greater separation between 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_{neck+CRD} was greater than that of RbConglutinin. On the reducing gels, binding to HA₁ 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 HA₀. These results indicate that RbConglutinin and SP-D/Cong_{neck+CRD} both bound to neuraminidase and HA₁ 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 HA₀ 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-

Figure 3 Binding of RhSP-D to proteins of Mem71_H-Bel_N or Bangkok 79 strains of IAV: effect of treatment with N-glycanase

Mem71_H-Bel_N 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 HA₀ of Mem71_H-Bel_N (recombinant H3N1) strain of IAV, but rather bound predominantly to a band below HA₀ (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_H-Bel_N with N-glycanase eliminated the binding of SP-D. The major bands bound by SP-D were less abundant on the original gel than HA₀ (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 X31 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 HA₁ 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

Figure 4 Binding of SP-D/Cong_{neck+CRD} or RbConglutinin to proteins of Phil82 or Phil82/BS strains of IAV

Results shown are chemiluminescence of Western blots of unreduced or reduced (+DTT) proteins of Phil82 (P) or Phil82/BS (PB), which were treated with SP-D/Cong_{neck+CRD} (left panel) or RbConglutinin (right panel). Note the absence of binding of RbConglutinin to Phil82/BS. The positions of HA₀ and HA₁ are indicated; the positions of molecular mass markers are indicated (in kDa) between the panels.

Figure 5 Binding of SP-D/Cong_{neck+CRD} or RbConglutinin to proteins of Brazil 78 or Brazil 78/BS

Experiments were performed as in Figure 4, except that Brazil 78 (lanes 1) or Brazil 78/BS (lanes 2) were used instead of Phil82 strains. Left panel: gel used to prepare the blot for the binding of SP-D/Cong_{neck+CRD}. Middle and right panels: chemiluminescence of blots treated with SP-D/Cong_{neck+CRD} (middle panel) or RbConglutinin (right panel). Note the lack of binding of RbConglutinin to Brazil 78/BS.

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

Figure 6 Binding of RhSP-D or RrSP-D to proteins of Phil82 or Phil82/BS strains of IAV

Results shown are chemiluminescence of Western blots of unreduced and reduced (+DTT) proteins of Phil82 (P) and Phil82/BS (PB) treated with RhSP-D (left panel) or RrSP-D (right panel). The positions of molecular mass markers are indicated (in kDa) between the panels.

and sensitive strains of IAV and mutant collectins to answer these questions.

Evidence from β -inhibitor-resistant strains: SP-Ds and conglutinin have different patterns of binding to glycoproteins of Phil82 and Brazil 78 parent strains and their serum β -inhibitor-resistant variants

Anders and colleagues [10,11] have developed the Phil82/BS, Brazil 78/BS and Mem71_H-Bel_N/BS variants of the Phil82 (H3N2), Brazil 78 (H1N1) and Mem71_H-Bel_N (H3N1) strains of IAV, which are resistant to growth inhibition by the bovine serum β inhibitor and conglutinin. As shown in Figures 4 and 5, RbConglutinin bound to the HA₀ and HA₁ proteins respectively in unreduced and reduced blots of Phil82 and Brazil 78, but did not bind at all to proteins of Phil82/BS or Brazil 78/BS. In

contrast with results obtained with Bangkok 79 IAV, RbConglutinin did not seem to bind to neuraminidase of either the Phil82 or Brazil 79 strains. RhSP-D and RrSP-D had a different pattern of binding to Phil82 and Phil82/BS from that of RbConglutinin (Figure 6). The SP-Ds bound intensely to bands that were consistent with the both the HA and neuraminidase of Phil82, and also bound intensely to HA₀ (or, on the reduced blot, HA₁) of Phil82/BS (Figure 6). The SP-Ds also bound to an additional protein bands on unreduced and reduced blots of Phil82/BS in the expected positions for neuraminidase. ELISAs were performed to assess the binding of collectins to whole virus preparations. As shown in Table 3, ELISA results paralleled results of binding to solubilized proteins. RbConglutinin showed markedly decreased binding to Phil82/BS and Brazil 78/BS strains in comparison with binding to the parent strains. Ratios of collectin binding to BS strains, in comparison with parent strains, are shown. Although there was also a decrease in ability of SP-Ds to bind to serum-resistant strains in comparison with parent strains, the SP-Ds were able to bind to the serum-resistant strains to a considerably greater extent than did conglutinin.

We hypothesized that the differences in binding of SP-D and conglutinin to serum-resistant strains of IAV resulted from differences in the binding properties of their CRDs. SP-Ds have the highest affinity for glucose and a relatively low affinity for GlcNAc, whereas conglutinin and MBL have a high affinity for GlcNAc and a low affinity for glucose. We tested this hypothesis with the SP-D/Cong_{neck+CRD} chimaera. In preliminary experiments we determined that SP-D/Cong_{neck+CRD} had monosaccharide binding properties similar to conglutinin and distinct from those of RrSP-D (results not shown). However, on a Western blot, SP-D/Cong_{neck+CRD} had a pattern of binding to glycoproteins of Phil82 and Brazil 78 strains that differed from that of RbConglutinin. Most notably, SP-D/Cong_{neck+CRD} was able to bind to the HA of both serum-resistant strains. In addition, binding of SP-D/Cong_{neck+CRD} to the parent strains was more intense than that of RbConglutinin, and unlike that of conglutinin it was not restricted to binding to HA₀ and HA₁. ELISA results were consistent with results of Western blots (Table 3). Although the binding of SP-D/Cong_{neck+CRD} to Phil82/BS and Brazil 78/BS was lower than those of RrSP-D or RhSP-D, it was clearly greater than that of RbConglutinin. These results suggest that the N-terminal and collagen-domain regions of SP-D contribute to the ability of SP-Ds to bind to serum-resistant strains. Further research is planned to help to clarify which aspects of the N-terminal and collagen domains

Table 3 Binding of collectins to serum inhibitor-sensitive and -resistant strains of IAV

Results are means $\pm$ S.E.M. for three or more experiments (except for RrSP-D^{E321Q,N323D}, for which $n = 2$). Initial dose-response experiments were performed and determined that maximal binding occurred at 0.5 μ g/ml (although significant binding occurred at 30 ng/ml). Therefore the concentrations of collectins used were 0.5 μ g/ml (except for RrSP-D^{E321Q,N323D}, for which 1 μ g/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^{E321Q,N323D} 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.

Collectin	Binding to strains of IAV as measured by ELISA					
	Phil (D_{450})	Phil/BS (D_{450})	Phil/BS to Phil (%)	Braz (D_{450})	Braz/BS (D_{450})	Braz/BS To Braz (%)
RbConglutinin	1.5 $\pm$ 0.3	0.06 $\pm$ 0.06	4	1.3 $\pm$ 0.16	0.16 $\pm$ 0.1	3
RrSP-D	1.3 $\pm$ 0.19	0.71 $\pm$ 0.3*	55	1.2 $\pm$ 0.15	0.67 $\pm$ 0.14*	57
RhSP-D	1.5 $\pm$ 0.2	1.09 $\pm$ 0.26*	74	1.3 $\pm$ 0.2	1.05 $\pm$ 0.03*	79
SP-D/Cong _{neck+CRD}	1.3 $\pm$ 0.1	0.45 $\pm$ 0.08*	34	1.2 $\pm$ 0.05	0.62 $\pm$ 0.14*	44
RrSP-D ^{E321Q,N323D}	0.36 $\pm$ 0.1	0.12 $\pm$ 0.06	33	0.2 $\pm$ 0.05	0.08 $\pm$ 0.04	47

Table 4 Inhibition of HA activity of wild-type and serum β -inhibitor-resistant strains of IAV by collectins

HA inhibition was measured as described [3]. Results are concentrations of collectins (in ng/ml) found to inhibit fully the HA activity of 40 haemagglutination units/ml of the indicated strains of IAV (means $\pm$ SEM; $n \geq 3$). Experiments were performed in TBS with 2 mM Ca^{2+} . No detectable HA inhibition of any of the viral strains was induced by RbConglutinin, RrSP-D, RhSP-D dodecamers or SP-D/Cong_{neck+CRD} when the assays were conducted in TBS with 10 mM EDTA (results not shown). * $P < 0.05$ compared with RbConglutinin or RrSP-D.

Collectin	Collectin concentration (ng/ml) causing HA inhibition of IAV strains					
	Brazil 78	Brazil 78/BS	Ratio	Phil82	Phil82/BS	Ratio
RbConglutinin	4 $\pm$ 1	29 $\pm$ 10	7.2	2.7 $\pm$ 0.3	> 6000	> 222
RhSP-D dodecamers	10 $\pm$ 2	66 $\pm$ 29	6.6	16 $\pm$ 8	157 $\pm$ 10	9.8
RrSP-D	20 $\pm$ 4	40 $\pm$ 8	2	17 $\pm$ 5	449 $\pm$ 61	26
SP-D/Cong _{neck+CRD}	2 $\pm$ 0.05	11 $\pm$ 6.6	5.5	4.3 $\pm$ 0.8	224 $\pm$ 45*	52

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

Infectious viral particles were quantified with a fluorescent focus assay (see the Materials and methods section). Results are means $\pm$ S.E.M. for three or more experiments. The degrees of inhibition of infectivity of Phil82 and Phil82/BS by RbConglutinin (Δ), RrSP-D ($\diamond$) and SP-D/Cong_{neck+CRD} ($\square$) are compared. These Phil82 results were reported previously [18] and are included for comparison. SP-D/Cong_{neck+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/Cong_{neck+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/Cong_{neck+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/Cong_{neck+CRD} are markedly more potent at neutralizing the infectivity of wild-type Phil82 IAV than are SP-Ds. Because RbConglutinin bound only to the HA₁ of these strains, whereas SP-D bound intensely to both the HA and neuraminidase bands (Figures 4–6), these results suggest that binding to HA₁ 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/Cong_{neck+CRD} than they were to RbConglutinin. In fact, SP-D/Cong_{neck+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/Cong_{neck+CRD} inhibited the infectivity of Phil82/BS to a significantly greater extent than either RbConglutinin or RrSP-D. Because SP-D/Cong_{neck+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 Mem71_H-Bel_N and Mem71_H-Bel_N/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

Figure 8 Binding of MBL and conglutinin to proteins of the Mem71_H-Bel_N and Mem71_H-Bel_N/BS strains of IAV

The binding of biotinylated MBL and conglutinin (as indicated) to proteins of the Mem71_H-Bel_N strain of IAV is shown in lanes A and E (non-reducing) and C and G (reducing), and to Mem71_H-Bel_N/BS in lanes B and F (non-reducing) and D and H (reducing). Results are again representative of several experiments. Experiments were performed as in Figure 2 with the exception that in the blot prepared for conglutinin binding (lanes E–H) TBS/BSA was used in place of TBS/milk as a blocking agent. This led to a higher degree of non-specific binding but also permitted the assessment of the binding of conglutinin to the viral HA. Note that on the non-reducing gel conglutinin bound much more extensively to HA₀ (large band located above the 87 kDa marker) of Mem71_H-Bel_N than that of Mem71_H-Bel_N/BS. The positions of neuraminidase monomer (N), HA₀ and HA₁ are indicated; the positions of molecular mass markers are indicated (in kDa) between the middle and left panels.

Mem71_H-Bel_N. SP-D bound most prominently to bands consistent with neuraminidase of the Mem71_H-Bel_N 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_H-Bel_N/BS strain. There was minimal apparent binding of SP-D or MBL to HA₀ or HA₁ bands of these strains. Similar results were obtained with conglutinin (results not shown). In further testing of the binding of conglutinin to Mem71_H-Bel_N and Mem71_H-Bel_N/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 HA₀ of Mem71_H-Bel_N in comparison with that of Mem71_H-Bel_N/BS (Figure 8). Because Mem71_H-Bel_N/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

RrSP-D^{E321Q,N323D} is a mutant form of RrSP-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, RrSP-D^{E321Q,N323D} bound to proteins of Bangkok 79 IAV in a distinctive manner compared with the binding of wild-type RrSP-D. RrSP-D^{E321Q,N323D} bound with less intensity to HA₀ and HA₁ bands but with greater intensity to some higher-molecular-mass bands on the unreduced blot (including one corresponding to neuraminidase tetramer).

Figure 9 Binding of RrSP-D (left panel) and RrSP-D^{E321Q,N323D} (right panel) to proteins of Bangkok 79 IAV

Western blots of unreduced and reduced proteins of Bangkok 79 IAV were treated with biotinylated RrSP-D or RrSP-D^{E321Q,N323D}. Unreduced proteins are in the left lane of each panel. The positions of molecular mass markers are indicated (in kDa) at the left.

Table 5 Comparison of the ability of wild-type RrSP-D and RrSP-D^{E321Q,N323D} to bind to inhibit HA activity of Bangkok 79 strain of IAV

Binding and HA inhibition were assessed as in Tables 3 and 4. The concentration of SP-Ds used for the ELISA assay was 0.25 µg/ml. ***Significantly ($P < 0.005$) less binding than with RrSP-D. †Significantly ($P < 0.05$) higher concentration needed to inhibit HA activity than RrSP-D.

SP-D	Binding (ELISA) (D_{450})	HA inhibition (ng/ml)
RrSP-D	2.15 ± 0.18	46 ± 12
RrSP-D ^{E321Q,N323D}	$0.56 \pm 0.05^{***}$	$286 \pm 14^{\dagger}$

Despite avid binding to these viral proteins on a Western blot, RrSP-D^{E321Q,N323D} had a markedly decreased ability to bind to whole viral particles compared with the binding of RrSP-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 RrSP-D^{E321Q,N323D} might not show decreased binding to the β -inhibitor-resistant strains. RrSP-D^{E321Q,N323D} bound significantly less to the resistant IAV strains than to the parent strains (Table 3). The degree of decrease in binding of RrSP-D^{E321Q,N323D} to the resistant strains was similar to the decrease obtained for RrSP-D. These results seem to indicate that RrSP-D^{E321Q,N323D} binds to Phil82 or Brazil 79 mainly through an attachment to high-mannose oligosaccharides.

Substantially higher concentrations of RrSP-D^{E321Q,N323D} than of wild-type RrSP-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 RrSP-D and RrSP-D^{E321Q,N323D} 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 RrSP-D^{E321Q,N323D} 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 HA₀ 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 in a Ca²⁺-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. RrSP-D^{E321Q,N323D}), concanavalin A and EDTA. SP-D, conglutinin and MBL bound to HA₀ and, more specifically, to the HA₁ 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 bound 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 chimaera SP-D/Cong^{neck+CRD} bound only to the HA of the serum β -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_H-Bel_N and showed equally intense

binding to the neuraminidase of the Mem71_H-Bel_N/BS serum β -inhibitor-resistant variant strain. However, the collectins have significantly less ability to inhibit the HA activity and infectivity of Mem71_H-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_H-Bel_N on a Western blot and thereby confirm the decreased binding of conglutinin to the HA of Mem71_H-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_H-Bel_N and Mem71_H-Bel_N/BS strains.

It is possible that solubilization of the Mem71_H-Bel_N virus makes the neuraminidase more accessible to binding than occurs with the intact virus. The HA of Mem71_H-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_H-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 RrSP-D^{E321Q,N323D} 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 HA₀ 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/Cong^{neck+CRD} 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 chimaera 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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