Surfactant Protein A Binds to the Fusion Glycoprotein of Respiratory Syncytial Virus and Neutralizes Virion Infectivity

Reena Ghildyal,1,a Carol Hartley,1,a,b Annalisa Varrasso,1,a
Jayesh Meanger,1 Dennis R. Voelker,3
E. Margot Anders,2 and John Mills1

Collectins are a family of calcium-dependent collagenous lectins that appear to be important in innate host defense. We investigated the ability of three human collectins, namely, lung surfactant proteins A (SP-A) and D (SP-D) and the serum mannose-binding protein (MBP), to bind to the surface glycoproteins of respiratory syncytial virus (RSV). SP-A was shown to bind to the F (fusion) glycoprotein but not to the viral G (attachment) glycoprotein, and binding was completely abrogated in the presence of EDTA. Neither SP-D nor MBP bound to either glycoprotein. SP-A also neutralized RSV in a calcium dependent fashion. These results support a role for SP-A in the defense of infants against infection with RSV and indicate a possible mechanism for its protective activity.

There has been increasing interest in the role of collectins in innate immunity to infection in recent years [1]. Collectins are a family of structurally related proteins containing collagenous sequences linked to calcium ion (Ca2+)–dependent (C-type) lectin domains and include mannose-binding protein (MBP) and conglutinin in serum, and the lung surfactant-associated proteins SP-A and SP-D. These molecules have been shown to bind to surface oligosaccharides on a wide range of bacterial and fungal pathogens and to mediate a number of activities that may contribute to host defense, including opsonization and complement activation. Antiviral activity against human immunodeficiency virus type 1 [2], influenza virus [3, 4, 5], and herpes simplex virus [6] has also been reported.

Respiratory syncytial virus (RSV) causes about half of pneumonia admissions in children <2 years of age in developed countries [7]. Infants (3–36 months old), an age group in which maternally derived protective antibody titers are declining and the infant’s own antibody repertoire is immature, are most commonly and severely affected by RSV. It is during this interval that nonclonal or innate immunity could play a critical role in defense against RSV infection. The lung surfactant proteins SP-A and SP-D may be especially important, as RSV exclusively infects the respiratory tract. Vos et al. [8] have reported on 2 infants suffering from pneumonia caused by RSV whose symptoms were alleviated by instillation of natural surfactant, and another study showed that levels of surfactant proteins in bronchoalveolar lavage (BAL) of infants with RSV disease are decreased [9]. It was suggested that this reduction might contribute to the pathogenesis of respiratory failure in this condition. The role of SP-A in host defense against RSV has also been strongly supported by studies (published subsequent to submission of this paper) showing that SP-A knockout mice have increased susceptibility to RSV infection [10].

RSV has 2 integral envelope glycoproteins: G, which mediates cell attachment, and F, which mediates fusion of the virion and cell membranes, permitting entry of the ribonucleoprotein into the cytoplasm and initiation of virus transcription and translation [11]. The F glycoprotein (Mr 68–70 kDa, with 2 subunits, F1 and F2, 48 and 21 kDa, respectively) has 5–6 potential N-glycosylation sites. The G glycoprotein (Mr 85–90 kDa) has 3–8 potential N-linked and >60 potential O-linked glycosylation sites, and over half of its molecular weight is attributable to carbohydrate residues. Both F and G glycoproteins are targets for RSV neutralizing antibody and, given their high level of glycosylation, might also serve as potential ligands for collectin binding and subsequent virus neutralization. In this study we show that SP-A bound to the F glycoprotein of RSV and neutralized virus infectivity; both activities were abrogated in the presence of EDTA consistent with lectin activity.
Neither MBP nor SP-D could be shown to bind to the F or G glycoproteins of RSV.

Methods

**Virus strains.** RSV strain A2 (ATCC number CCL-23; obtained from Dr. Paul Young, Sir Albert Sakzewski Virus Research Centre, Brisbane, Australia) was propagated in HEp-2 cells (human breast carcinoma cell line) maintained in medium 199 supplemented with 5% fetal calf serum and antibiotics (Victorian Infectious Diseases Reference Laboratory, Fairfield, Australia). Semiconfluent (80%) monolayers were infected with RSV at an MOI of 1. Supernatants from RSV infected cultures were collected 4–5 days after infection, frozen, and thawed twice to disrupt cells, vortexed for 1 min, and clarified by centrifugation at 1200 g for 15 min. Virus was titrated prior to use in neutralization assays.

**Collectins and antibodies.** SP-A was purified from the BAL fluid of patients with alveolar proteinosis as described elsewhere [12]. Polyclonal antibody to SP-A was raised in rabbits against the purified protein and affinity purified. Recombinant human SP-D and polyclonal rabbit antibody to SP-D were gifts from Dr. E. Crouch (Department of Pathology, Washington University School of Medicine, St. Louis). Recombinant human MBP and the polyclonal rabbit antibody to MBP were gifts from Dr. R.A.B. Ezekowitz (Department of Paediatrics, Massachusetts General Hospital, Boston). All antisera used were specific for their respective antigens by ELISA and immunoblotting.

**Binding of collectins to RSV glycoproteins.** Microtiter wells (Maxisorp, Nunc, Denmark) were incubated overnight at 4°C with 100 µL coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6) containing 5 µg of mannan (Sigma, St. Louis) or 200 ng of purified RSV G or F glycoproteins (purified from virions, a generous gift from Dr. Dan Speelman, Lederle-Praxis Biologicals, Rochester, NY). The rest of the procedure was performed at room temperature. Wells were blocked for 2 h with 150 µL/well of bovine serum albumin, (BSA), 10 mg/mL in Tris-buffered saline (TBS; 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl). Wells were washed twice with TBS containing 0.05% Tween 20 (TBS) before adding varying concentrations of SP-A, SP-D, or MBP in 100 µL of 5 µg BSA/mL TBS (BSA,TBST) containing either 50 mM CaCl₂, or 10mM EDTA. After overnight incubation, wells were washed 4 times in TBST and reacted for 2 h with the appropriate polyclonal rabbit serum diluted in BSA,TBST/50 mM CaCl₂ (1/800 for anti-SP-A or -SP-D, 1/600 for anti-MBP). After a further 4 washes in TBST, bound antisera was reacted with swine anti-rabbit immunoglobulins conjugated to horseradish peroxidase (DAKO, Carpinteria, CA) for 1 h. After washing, plates were developed using 100 µL/well of sodium tetramethylbenzidine substrate in tablet form (Sigma) dissolved in 0.05 M phosphate-citrate buffer containing 0.006% H₂O₂. The reaction was stopped after 6 min with 50 µL/well of 0.1 M HCl, and absorbance at 450 nm was measured in a Titertek Multiskan plate reader (Flow Laboratories, Colorado). Effective coating of G glycoprotein to the microtiter wells was confirmed by titration of specific monoclonal antibody (MAb; data not shown).

**Neutralization assays.** The neutralizing activity of surfactant proteins toward RSV was determined by microtiter plate based neutralization assays. Dilutions of purified SP-A in Hanks’ balanced salt solution, containing either 25 mM CaCl₂ or 10 mM sodium citrate were prepared and an equal volume of suitably diluted virus added; the mixture was then incubated at 37°C for 30 min. A positive control was also included, comprising a neutralizing F glycoprotein MAb (SB209763; a gift from SmithKline Beecham Pharmaceuticals, UK), which was substituted for collectins. Each mixture was then transferred to washed HEp-2 cell monolayers and allowed to adsorb for 1 h at 37°C in a humidified atmosphere of 5% CO₂ in air. After adsorption, the virus-collectin mixture was aspirated and cells overlaid with medium 199 containing 2% fetal calf serum, and incubated overnight at 37°C in a humidified atmosphere of 5% CO₂ in air. The overlay was subsequently aspirated from the monolayers and the cells fixed with 80% acetone before washing with PBS, pH 7.4 (Oxoid, Hampshire, England). Infected cells were detected by a 30-min incubation of infected monolayers with fluorescein-conjugated goat RSV antisera (ViroStat, Portland, ME) diluted in PBS. After a final wash with PBS, the number of fluorescent cells in 4 fields at ×40 magnification were expressed as a percentage of the average count from wells incubated with virus alone.

**Results**

To investigate the possible interaction of RSV with pulmonary or plasma collectins, we assayed SP-A, SP-D, and MBP for binding to purified F and G glycoproteins prepared from RSV virions. As shown in figure 1, SP-D and MBP did not bind to either the F or G glycoproteins, whereas, as expected, these lectins bound strongly to yeast mannan. In contrast, SP-A bound to the F glycoprotein of RSV, and indeed binding of SP-A appeared to be stronger to F than to mannan. The binding of SP-A to the F glycoprotein was completely abrogated in the presence of EDTA, indicating that binding was calcium dependent and therefore likely mediated through the lectin domain of SP-A. SP-A did not bind significantly to the G glycoprotein.

To investigate the biologic significance of binding of SP-A to the F glycoprotein, we examined the ability of this collectin to neutralize the infectivity of RSV and compared it with neutralization by the F-glycoprotein–specific MAb SB209673. As shown in table 1, SP-A at 40 µg/mL reduced the infectivity of RSV by 50%, a level of neutralization similar to that obtained
Figure 1. Binding of lung surfactant proteins A (SP-A) and D (SP-D) and mannose binding protein (MBP) to respiratory syncytial virus (RSV) envelope glycoproteins by ELISA. Mannan (■, □), RSV F (●, ○), or RSV G (▲, △) glycoproteins were coated overnight onto microtiter plates. Wells were blocked for 2 h with 10 mg/mL bovine serum albumin in Tris-buffered saline, followed by overnight incubation with SP-A, SP-D, or MBP in the presence of 50 mM CaCl$_2$ (closed symbols) or 10 mM EDTA (open symbols). Binding of SP-A, SP-D, and MBP was detected with specific rabbit antisera, followed by horseradish peroxidase-conjugated swine anti-rabbit immunoglobulins and color development by sodium tetramethylbenzidine substrate. Absorbance was measured at 450 nm.

with the MAb at 5 μg/mL. SP-A failed to neutralize in the presence of sodium citrate, consistent with the calcium dependence of SP-A binding to the F glycoprotein noted earlier. MBP did not neutralize RSV, and neutralization of RSV by an adult human BAL fraction enriched in SP-D was not calcium dependent (data not shown).

Discussion

These data show that neutralization of RSV via binding to the F envelope glycoprotein is a likely mechanism for recently documented in vivo protective effects of SP-A [9, 10]. SP-A bound only to the F glycoprotein of RSV and not to the G
glycoprotein and neutralized RSV in a dose-dependent manner. Two distinct modes of binding in the interaction of SP-A with other microorganisms have been described elsewhere [13]. The first involves binding as a Ca\(^{2+}\)-dependent lectin through the carbohydrate-recognition domain (CRD) of SP-A to carbohydrate moieties on the surface of microorganisms. Alternatively, binding may occur independently of the CRD, as described for influenza virus, which binds to the sialic acid residues on SP-A [5] and herpes simplex virus, which binds carbohydrate moieties on SP-A in a heparin-inhibitable fashion [6]. Binding of SP-A to the F glycoprotein of RSV was completely abrogated in the presence of EDTA, indicating exclusive involvement of the calcium-dependent lectin domain in the binding event.

Although all 3 collectins, SP-A, SP-D, and MBP, bind to mannose and other mannose-containing glycans, distinct differences in their fine specificity for particular monosaccharides and oligosaccharides are well documented [1]. Thus, the failure of SP-D and MBP to bind to the F glycoprotein despite strong binding of SP-A presumably reflects the composition of the particular N-linked oligosaccharide structures present on the viral glycoprotein. Likewise, the failure of all 3 collectins to bind to the G glycoprotein of RSV indicates an absence of appropriate sugar ligands for the collectins on this molecule, despite its high level of glycosylation. Lectins that bind galactose and N-acetyl galactosamine bind well to the G glycoprotein [7], and these sugars are known not to represent high affinity ligands for these collectins [1].

SP-A is present in BAL fluid of normal adults at concentrations of 5.8–38.0 μg/mL; however, the actual concentration of SP-A in the epithelial lining fluid of alveolar compartments was estimated to be 50–100 times higher [14]. Such concentrations are clearly sufficient to mediate neutralization at this site, because we have shown that 40 μg/mL of SP-A can reduce the infectivity of RSV by 50%. SP-A may neutralize RSV by directly inhibiting the fusion function of the F glycoprotein, thereby blocking viral entry and subsequent syncytium formation. Viral aggregation by SP-A may also contribute to neutralization of RSV by reducing the number of infective units and perhaps by interfering physically with viral entry. SP-A is known to cause strong aggregation of influenza virus [4]. Furthermore, SP-A can act as an opsonin for the uptake of influenza virus [15] and herpes simplex virus [6] by rat alveolar macrophages. A recent study [10] has shown that SP-A knockout mice have more severe pulmonary infiltration after RSV infection when compared to control mice. This increased infiltration was abrogated when SP-A was administered at the time of RSV infection. Thus, binding of SP-A to the F glycoprotein of RSV in vivo may bring about not only a reduction in virus infectivity but also may opsonize the virus for uptake by phagocytic cells.

RSV infections are the most important cause of viral lower respiratory tract disease in human infants, particularly those <1 year of age. It is during this stage of immunologic development when the innate immune system would be of greatest significance, because maternal antibody is declining and the infant’s own antibody repertoire is immature. Because SP-A is present at the site of RSV infection and is able to bind to and neutralize the virus, this collectin may be of importance for defending the immunologically naive human infant against infection with RSV.

### References


