Site-directed Mutagenesis of Cys-15 and Cys-20 of Pulmonary Surfactant Protein D

EXPRESSION OF A TRIMERIC PROTEIN WITH ALTERED ANTI-VIRAL PROPERTIES*

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Surfactant protein D (SP-D) molecules are preferentially assembled as dodecamers consisting of trimeric subunits associated at their amino termini. The NH₂-terminal sequence of each monomer contains two conserved cysteine residues, which participate in interchain disulfide bonds. In order to study the roles of these residues in SP-D assembly and function, we employed site-directed mutagenesis to substitute serine for cysteine 15 and 20 in recombinant rat SP-D (RrSP-D), and have expressed the mutant (RrSP-Dser15/20) in Chinese hamster ovary (CHO-K1) cells. The mutant, which was efficiently secreted, bound to maltosyl-agarose, but unlike RrSP-D, was assembled exclusively as trimers. The constituent monomers showed a decreased mobility on SDS-polyacrylamide gel electrophoresis resulting from an increase in the size and sialylation of the N-linked oligosaccharide at Asn-70. Although RrSP-Dser15/20 contained a peptidase-resistant triple helical domain, it showed a decreased Tm, and acquired susceptibility to proteolytic degradation. Like RrSP-D, RrSP-Dser15/20 bound to the hemagglutinin of influenza A. However, it showed no viral aggregation and did not enhance the binding of influenza A to neutrophils (PMN), augment PMN respiratory burst, or protect PMNs from deactivation. These studies indicate that amino-terminal disulfides are required to stabilize dodecamers, and support our hypothesis that the oligomerization of trimeric subunits contributes to the anti-microbial properties of SP-D.

Surfactant protein D (SP-D) is a collagen C-type lectin (collectin) that is secreted into the distal airways and alveoli of the lung (1, 2). Recent studies have demonstrated binding of SP-D to a variety of bacterial, viral, and fungal pathogens (3–5) and effects of SP-D on leukocyte function in vitro (4, 6, 7). For example, SP-D has been shown to enhance the binding of influenza A to neutrophils, to inhibit the viral hemagglutinin and inhibit infectivity in vitro, to potentiate the neutrophil respiratory burst in response to bound virus, and to protect neutrophils from viral deactivation (4).

Cloning and sequence analysis of SP-D has shown that the 43-kDa monomer has four major structural domains (8–11). The short, non-collagenous amino-terminal domain contains two absolutely conserved cysteine residues (Cys-15 and Cys-20) involved in interchain disulfide bonding. The uninterrupted collagenous domain contains 59 Gly-X-Y repeats (in rat and human), and is connected to the carboxyl-terminal carbohydrate recognition domain (CRD) by a short linking sequence (L). A single N-linked glycosylation site at Asn-70 is found within the collagenous domain, and studies of SP-D from natural and recombinant sources have shown that this site is utilized and contains a sialylated sugar moiety (12, 13).

Other collagenous C-type lectins include: pulmonary surfactant protein A (SP-A), serum mannose-binding protein (MBP), bovine serum conglutinin, and bovine serum lectin 43 (CL-43) (14, 15). SP-D is predominantly assembled as a dodecamer consisting of four homotrimeric subunits associated at their amino termini (12, 16). Analysis of proteolytic fragments and limited reduction and alkylation of SP-D dodecamers suggests that the amino-terminal cysteine residues form intra- and intertrimeric disulfide bonds, which stabilize the fully assembled dodecamer (12).

Trimeric CRDs (but not monomers) show high affinity binding to various saccharide ligands in vitro (17). In addition, ultrastructural studies suggest that the rigid collagenous arms restrict the spatial distribution of the lectin domains and allow for bridging interactions between ligands separated by up to 100 nm (8, 12). Accordingly, we hypothesized that amino-terminal cross-linking of trimeric subunits is critical for SP-D-mediated microbial agglutination. We further hypothesized that trimeric subunits of SP-D (i.e. single arms) will bind to microorganisms but show decreased agglutinating activity. Although SP-D "arms" can be generated by reduction and alkylation, the intrachain bonds required for lectin activity are also disrupted (12). We therefore sought to test these hypotheses by mutating the amino-terminal cysteine residues in rat SP-D and utilizing CHO K1 expression system. Previous work in our laboratory has shown that wild type recombinant rat SP-D (RrSP-D) produced by this system is chemically, morphologically, and functionally indistinguishable from natural rat SP-D (13).

Using this approach, we have produced a full-length mutant SP-D molecule, RrSP-Dser15/20, that is secreted as a trimer. These studies indicate that amino-terminal disulfide bonds are...
required to form or stabilize dodecamers, and provide support for our hypothesis that the oligomerization of trimeric subunits to form dodecamers is required for some of the previously observed interactions of SP-D with influenza A. Our results also show that the cysteine substitutions decrease the thermal stability of the collagen hexamer and increase the protein's susceptibility to proteolytic degradation.

MATERIALS AND METHODS

Site-directed Mutagenesis of rSP-D—Site-directed mutagenesis was performed on a full-length rat SP-D cDNA done provided by Drs. J. H. Fisher and D. R. Voelker, Denver, CO (20). The cDNA was isolated using the PCR overlap extension method (18). Primers for substituting serine for Cys-15 and Cys-20 were designed with the orientation of the subclones determined by restriction map.

Expression of Rat SP-D cDNA Mutants in CHO K1—Mutated rSP-D cDNAs were excised from pGEM-3Z with EcoRI and subcloned into pGEM-3Z. Clones were sequenced up to the corresponding site in wild type rSP-D. The reaction buffer contained 2 mM MgCl₂, 2 mM dithiothreitol, and 5 unit of Taq polymerase. Separate reactions containing the forward and T7 primers or reverse and Sp6 primers were performed for 15 cycles with a 45°C annealing temperature. The resulting products were gel-purified and combined in a second PCR reaction for 10 cycles at 52°C annealing, followed by 20 cycles at 45°C annealing using Sp6 and T7 as primers. The PCR products were filtered, gel-purified, digested with EcoRI, and subcloned into pGEM-3Z. Clones were sequenced up to the Apal site within rSP-D to verify the presence of the desired mutations and the absence of any additional point mutations. An EcoRI/Apal fragment containing the mutated region was then subcloned into the corresponding site in wild type rSP-D-GEM-3Z. Subcloning of the mutated rSP-D cDNA was performed in order to avoid introduction of extraneous Tag generated mutations. Expression of Rat SP-D cDNA Mutants in CHO K1—Mutated rSP-D cDNAs were excised from pGEM-3Z with EcoRI and subcloned into the corresponding site within the multiple cloning site of pEE14 (13, 19, 20). Orientation of the subclones was determined by restriction mapping. cDNA containing the desired Cys mutant (1.5 μl each) were incubated with 1 × 10⁶ m of each of enzyme in a total mixture volume of 50 μl (buffer = 0.05 mM Tris, pH 7.5, 0.1 mM CaCl₂, 0.15 mM NaCl, 0.02% Brij) at 37°C overnight. The reaction mixture was heated to 95°C for 5 min to inactivate the enzyme and the supernatant was used for SDS-PAGE and visualized by fluorography.

Assessment of Viral Aggregation—Aggregation was assessed by measuring changes in light transmission through suspensions of IAV after addition of various concentrations of SP-D (28). After aggregation of the virus, aliquots were removed and infection was assessed by plaque assay on MDCK cells.

Measurement of Neutrophil Activation and Protection—Neutralophils (PMN) from healthy volunteer donors were isolated by 80% purity using dextran sedimentation, followed by a Ficol-Hypaque gradient centrifugation for removal of mononuclear cells, and hypotonic lysis to remove contaminating erythrocytes. H₂O₂ production was measured by the oxidation of scopoletin, and O₂⁻ detection was performed using the superoxide dismutase inhibitable reduction of cytochrome c (30). PMN protection assays measured the superoxide response to FMLP (5 × 10⁻⁷ m) after exposure to IAV preincubated in the absence or presence of SP-D (4, 31, 32).

RESULTS

Construction and Expression of RrSP-D Site-directed Mutants—Sequence analysis of the constructs showed that we had generated the expected Cys-15/Cys-20 double substitution mutant, designated RrSP-Dser15/20. RrSP-Dser15/20 was efficiently secreted into the culture medium and bound quantitatively to maltsyl-agarose (Figs. 1 and 2). The recovery was comparable to that obtained for wild type SP-D under compa-
attributed to maturation and terminal sialylation of the mutant proteins relative to the major intracellular species (see below). The lower mobility of the secreted wild type and attributable to differences in post-translational modification of RrSP-Dser15/20 with purified bacterial collagenase liberated F (see below). As observed for wild type SP-D, incubation of components migrated with approximately the same apparent absence of other protein components in the non-bound fraction reflects enrichment of proline in the collagen domain of SP-D. B, affinity-purified [14C]RrSP-Dser15/20 was fractionated on 4% agarose in TBS containing 2 mM EDTA and 0.2% Triton X-100. Eluted proteins were quantified by liquid scintillation spectrometry. The elution volumes of SP-D dodecamers and trimers were determined using recombinant human SP-D as a standard.

Assembly of RrSP-Dser15/20—Oligomerization of affinity-purified RrSP-Dser15/20 was assessed by gel filtration chromatography (Fig. 2B). RrSP-Dser15/20 eluted at approximately the same position as natural hSP-D trimers (single arms) or components liberated by reduction and alkylation of rSP-D under non-denaturing conditions (12). The presence of a collagenous triple helix of approximately normal length was verified by overnight incubation with 100 μg/ml porcine pepsin A (Worthington, 3352 units/mg) at 27 °C. Pepsin liberated two fragments, a minor component (~29 kDa, globular standards, reduced) that co-migrated with the major pepsin-resistant fragment of RrSP-D, and a major fragment of ~25 kDa.

To identify the sites of peptic cleavage, fragments of RrSP-Dser15/20 were resolved by SDS-PAGE, transferred to Immobilon membranes, and submitted for gas-phase amino-terminal microsequencing as described previously (22). The 29-kDa fragment gave sequence beginning within the amino-terminal peptide domain (Val18, Gly-Ser-Pro-Thr-Glu-Asn-Gly-Leu) and including the substituted serine at position 20 (10). However, the major fragment gave sequence beginning within the 10th Gly-Arg-Gly) (9). Post-translational Processing of RrSP-Dser15/20—To determine if the size differential of RrSP-Dser15/20 versus reduced wild type RSP-D was due to overhydroxylation of proline or lysine residues in the collagen domain and/or overglycosylation of hydroxylysine, transfected cells were metabolically labeled in the presence or absence of 2,2'-dipyridyl, an inhibitor of prolyl and lysyl hydroxylase. Comparison of mutant and wild type proteins synthesized under these conditions suggested that there was little if any overmodification of the helical domain of RrSP-Dser15/20 (data not shown).

To determine if the increased mass of double cysteine mutant reflected differences in the size or structure of the N-linked oligosaccharide at Asn-70, we compared the mobility of pro-
teins synthesized in the absence or presence of up to 6 μg/ml tunicamycin (TM), and after glycosidase digestion. Consistent with the possibility, wild type and mutant proteins synthesized in the presence of tunicamycin showed approximately the same molecular mass (data not shown). Furthermore, incubation of the wild type and mutant proteins with Endoglycosidase F generated cleavage products with the same mobility by SDS-PAGE (Fig. 3). Neuraminidase digestion of RrSP-Dser15/20 resulted in a greater size shift than digestion of RrSP-D. However, differences in sialylation did not completely account for the size differential (Fig. 3).

Thermal Stability Analysis—Although intra-trimer disulfide bonds were not required for trimerization and formation of a pepsin-resistant triple helix, we hypothesized that they might contribute to stabilization of the collagenous triple helix at physiologic temperatures. To address this possibility, we examined the temperature-dependent acquisition of sensitivity to degradation by trypsin, a technique commonly used to characterize the effects of mutations on collagen helix stability in inherited connective tissue disorders (23). As expected, the wild type protein was relatively insensitive to trypsin digestion following incubation at temperatures through 38 °C, but showed increasing degradation after incubation between 40 and 44 °C (Fig. 4). Even after heating to 44 °C, some intact RrSP-D remained. RrSP-Dser15/20 was similarly resistant to trypsin degradation following incubation at temperatures up to 34 °C. However, in contrast to wild type, RrSP-Dser15/20 exhibited an abrupt increase in susceptibility to degradation after heating to 36 °C and was completely degraded by trypsin after heating to 40 °C (Fig. 4). Based on densitometric measurements of bands corresponding to undigested protein, RrSP-D has a $T_m$ of approximately 40 °C, while the mutant RrSP-Dser15/20 has a $T_m$ of 37 °C (Fig. 4).

Susceptibility to Degradation by Mammalian Proteases—Unpublished experiments demonstrated that natural SP-Ds are highly resistant to degradation by a wide range of proteolytic enzymes that might be released at sites of pulmonary inflammation including: human and rat interstitial collagenases (MMP-1 and -13), 72- and 92-kDa gelatinases (MMP-2 and -9, respectively), stromelysin (MMP-3), matrilysin (MMP-7), human and mouse macrophage metalloelastase (MMP-12), and human leukocyte elastase. Given the differences in thermal stability, we explored the possibility that the mutant SP-D might be abnormally susceptible to proteolytic attack. Consistent with our previous results, wild type RrSP-D was not degraded during prolonged incubation with high concentrations (1 × 10^{-6} M each) of these enzymes at 37 °C (Fig. 5, top panel). By contrast, RrSP-Dser15/20 was degraded to varying degrees by all of the enzymes (Fig. 5, bottom panel) and appeared particularly susceptible to degradation by the gelatinases and human leukocyte elastase.

Viral Interactions—Interactions of the wild type and mutant proteins with the hemagglutinin of selected strains of influenza A virus (IAV) were initially compared in hemagglutination inhibition assays. For example, RrSP-Dser15/20 showed an identical hemagglutination inhibition titer for Mem71H. BelN/BS (Table I), a strain that is resistant to the serum collectin, conglutinin, and MBP. On the other hand, the mutant was significantly less effective at inhibiting the hemagglutinin of the Bangkok strain. The hemagglutination inhibition activity of RrSP-Dser15/20 was inhibited by maltose or EDTA, as documented previously for the wild type protein. Surprisingly, the mutant also inhibited the hemagglutination activity of PR-8, a viral mutant lacking oligosaccharides on the hemagglutinin head which does not bind to wild type RrSP-D. However, the hemagglutination inhibiting activity was not significantly inhibited by EDTA.

As predicted, RrSP-Dser15/20 failed to give detectable viral aggregation in light transmission assays (Fig. 6) and was similarly defective in its capacity to generate aggregates that could
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Fig. 5. Proteolytic degradation. Wild type (upper panel) and mutant (lower panel) SP-Ds were incubated with selected proteases as described under “Materials and Methods.” Lane 1, globular protein standards; lane 2, incubated control; lane 3, matrilysin; lane 4, leucocyte elastase; lane 5, 72-kDa gelatinase; lane 6, 92-kDa gelatinase; lane 7, human interstitial collagenase; lane 8, stromelysin; lane 9, murine macrophage elastase; lane 10, rat uterine collagenase; lane 11, human macrophage elastase. A major fragment (~45–50 kDa) generated by several of the enzymes comigrated with a degradation product identified in some preparations of purified RrSP-Dser15/20 (lane 2). Comparable susceptibility to proteolytic cleavage was observed for preparations lacking the endogenous degradation products. Lower and higher molecular weight bands observed in some of the lanes correspond to added protease.

Table I

|                  | RrSP-D | RrSP-Dser15/20 |
|------------------|--------|----------------|
| Bangkok 79       | 0.031 ± 0.008 | 0.208 ± 0.042* |
| Mem71-N-BelN     | 0.18 | 0.667 ± 0.167* |
| Mem71-N-BelN/BS  | 0.25 ± 0 | 0.25 ± 0 |
| PR-8             | 1.43 ± 0.36 | 0.812 ± 0.063 |
| + Maltose        | >2.0 | >2.0 |
| + EDTA           | >1.5 | >2.0 |
| + Maltose        | >2.0 | >2.0 |
| + EDTA           | 1.0 ± 0 | 1.0 ± 0 |

*p* Our previous studies (4) have demonstrated a very low hemagglutination inhibition titer (~2.0) for RrSP-D incubated with various IAV strains in the presence of maltose or EDTA.

be sedimented by low speed centrifugation (data not shown). The mutant also failed to enhance binding of IAV to neutrophils (Fig. 7, A and B), failed to enhance the H2O2 response of PMNs to virus (Fig. 8), and was unable to protect these cells against the deactivating effects of IAV on FMLP-stimulated respiratory burst response (Fig. 9).

**DISCUSSION**

The combined substitution of serine for cysteine at positions 15 and 20 of rat SP-D resulted in the production of a mutant SP-D consisting exclusively of trimers. The identification of pepsin-resistant fragments comparable in size to the major pepsin-resistant fragments of wild type SP-D establishes that the monomers within each trimer are aligned in parallel and can fold to form a triple helical domain of approximately normal length. Thus, the mutant assembles as a single “arm” of the “four-armed” rSP-D molecule (dodecamer). In this respect, RrSP-Dser15/20 is structurally similar to the bovine serum collectin CL-43 (14, 16) and to subpopulations of hSP-D trimers isolated from human amniotic fluid (33) and bovine bronchoalveolar lavage (8, 16). Because we did not detect dodecamers or higher order oligomers by gel filtration chromatography, we conclude that interchain disulfide bonds are necessary for stable oligomerization of the trimeric subunits.

The assembly of triple helical arms in the absence of the amino-terminal interchain disulfide bonds is consistent with studies of other collagenous proteins. In the fibrillar matrix collagens, helix formation proceeds toward the amino terminus by a “zipper-like” mechanism following association of the carboxyl-terminal propeptides and disulfide cross-linking of the COOH-terminal domains (34, 35). Although the collectins lack carboxyl-terminal interchain disulfide bonds, structural analysis of a peptide corresponding to the linking domain of bovine SP-D suggests that this region forms a stable α-helical coiled-coil, thereby associating collectin monomers and driving collagenous helix formation in vitro (36). Finally, deletion analysis of recombinant SP-A, MBP, and rat SP-D have shown that the linking and/or CRD domains are necessary and sufficient for the assembly of trimers (37–39).

Wild type SP-D displays a remarkable insensitivity to proteases,2 which we hypothesized is due to tight folding of the CRD and the collagenous domain, and steric hindrance of protease binding near the hub of SP-D dodecamer. The capacity of pepsin to deave RrSP-Dser15/20 within the 10th Gly-X-Y triplet of the collagen domain (at 27°C), and the decreased thermal stability and increased protease sensitivity of the mutant protein relative to wild type, together suggest that a major function of the conserved Cys residues is to stabilize the amino-terminal end of the triple helix. It is possible that the formation of dodecamers further enhances the stability of the collagen

2 C. Fliszar, H. G. Wéégus, and E. C. Crouch, unpublished observations.
domain or limits accessibility of proteases to cleavage sites near the amino terminus in RrSP-D. Although the measured \( T_m \) for the wild type protein is consistent with the formation of a stable triple helix in vivo, the melting temperature of the mutant (\( T_m = 37^\circ C \)) suggests that its helical domain is incompletely folded in the cell. To our knowledge, these studies provide the first demonstration of stabilization of a contiguous collagenous helix by interchain disulfide bonds. We speculate that the amino-terminal and intrahelical cysteine residues found in other collectins play a similar role.

To our surprise, the mutant showed a slower mobility on SDS-PAGE than wild type RrSP-D or the predominant forms of natural SP-D. Although some degree of overhydroxylation of the collagen domain cannot be excluded, our data indicate that lower mobility of mutant monomers primarily results from an increase in the apparent size and sialylation of the \( N \)-linked sugar at Asn-70. We speculate that the mutant offers a less restricted access of Golgi enzymes to the glycosylation site, which is close to the hub of cross-linked dodecamers.

**Fig. 7.** Effect of wild type RrSP-D and RrSP-Dser15/20 on IAV binding to neutrophils. A, neutrophils were allowed to adhere to plastic coverslips followed by incubation of the cells with either FITC-labeled Bangkok79 IAV alone (top panels), or the same amount of IAV, which had been preincubated with either 1.6 \( \mu \text{g/ml} \) RrSP-D (middle panels) or RrSP-Dser15/20 (bottom panels). Phase contrast images are shown on the left and fluorescence images of the same fields on the right (magnification, \( \times 100 \)). In the absence of SP-D, faint fluorescence is seen distributed over the surface indicating bound virus; negative controls were devoid of surface immunostaining. In the presence of RrSP-D, coarse aggregates of viral particles (e.g. white arrows) were observed in association with the cell surface. The identification of viral aggregates in association with the cell surface was confirmed by electron microscopy (40). In the presence of the mutant, there was a marked decrease in the binding of virus and no coarse viral aggregates were observed. B, FITC-labeled Bangkok79 IAV was preincubated with the indicated concentrations of either wild type RrSP-D (dark squares) or RrSP-Dser15/20 (open squares), followed by assessment of binding of these samples to human neutrophils by flow cytometry. Results shown are mean \( \pm \) S.E. of four experiments. Viral binding was significantly enhanced by wild type RrSP-D (* indicates \( p < 0.05 \) compared to binding of untreated IAV). In contrast, RrSP-Dser15/20 did not cause any enhancement of viral binding. In fact, significantly reduced IAV binding was seen using samples preincubated with the highest concentration of RrSP-Dser15/20 (indicated by **).

**Fig. 8.** Ability of wild type RrSP-D and RrSP-Dser15/20 on neutrophil \( \text{H}_2\text{O}_2 \) responses to IAV. Samples of Bangkok79 IAV were preincubated with the indicated concentrations of wild type RrSP-D (dark squares) or RrSP-Dser15/20 (open squares) as described in Fig. 7. Neutrophils were then added to these samples (final concentration \( 4 \times 10^6 \text{ cells/2 ml} \)) and \( \text{H}_2\text{O}_2 \) production measured using the scopolectin assay as described under "Materials and Methods." Results shown are mean \( \pm \) S.E. of three experiments. Preincubation with wild type RrSP-D caused significant enhancement of neutrophil \( \text{H}_2\text{O}_2 \) production (* indicates \( p < 0.05 \) compared to response elicited by unopsonized IAV), while preincubation with RrSP-Dser15/20 did not.

**Fig. 9.** Ability of wild type RrSP-D or RrSP-Dser15/20 to protect neutrophils against the deactivating effects of IAV on FMLP-stimulated respiratory burst responses. Neutrophils were treated with control buffer, Bangkok79 IAV, or Bangkok79 IV that had been preincubated with the indicated concentrations of wild type RrSP-D or RrSP-Dser15/20 (as reported previously (4)). Preincubation with wild type RrSP-D significantly protected neutrophils against IAV-induced depression of \( \text{O}_2 \) responses to FMLP (as indicated by *), while preincubation with RrSP-Dser15/20 (even at higher concentrations) did not.
These studies have also confirmed that important anti-viral properties of SP-D are entirely dependent on the oligomerization of trimeric subunits.

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