Biosynthesis of Surfactant Protein D

CONTRIBUTIONS OF CONSERVED NH₂-TERM NAL CYSTEINE RESIDUES AND COLLAGEN HELIX FORMATION TO ASSEMBLY AND SECRETION*

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Patricia Brown-Augsburger, Donald Chang, Kevin Rust, and Edmond C. Crouch‡
From the Department of Pathology, Washington University School of Medicine, St. Louis, Missouri 63110

Surfactant protein D (SP-D) is preferentially secreted as dodecamers consisting of four collagenous trimers cross-linked by disulfide bonds. In these studies, we examined the biosynthesis of wild-type rat SP-D (rSP-D) and selected mutants by stably transfected CHO-K1 cells to determine the roles of a conserved N-linked oligosaccharide, the collagen helix, and interchain disulfide bonds in SP-D assembly and secretion. The major intracellular form of RrSP-D accumulated in the RER as complexes containing up to four trimeric subunits. Disulfide cross-link formation and RrSP-D secretion were selectively inhibited by 2,2'-dipyridyl, an inhibitor of prolyl and lysyl hydroxylase, and by 2 mM dithiothreitol, but unaffected by tunicamycin or elimination of the consensus sequence for glycosylation at Asn⁷⁰. Although mutants with serine substituted for Cys⁵⁰ and Cys⁸⁰ (RrSP-Dser15/20) are secreted as trimeric subunits, proteins with single cysteine substitutions were retained in the cell. Surprisingly, the secretion of RrSP-Dser15/20 was unaffected by 2,2'-dipyridyl. These studies strongly suggest that the most important and rate-limiting step for the secretion of SP-D involves the association of cross-linked trimeric subunits to form dodecamers stabilized by specific inter-subunit disulfide cross-links. Interference with collagen helix formation prevents secretion by interfering with efficient disulfide cross-linking of the NH₂-terminal domain.

Pulmonary surfactant protein D (SP-D) is a member of the collectin subfamily of mammalian C-type lectins (1, 2). It is secreted by alveolar type II and nonciliated bronchiolar epithelial cells (Clara cells) into the alveolar spaces and airways, where it is thought to participate in nonimmune host defense, in part through lectin-mediated agglutination of inhaled microorganisms and its interactions with pulmonary leukocytes (3–8).

The predominant molecular form of natural rat SP-D (rSP-D) consists of homotrimeric subunits that associate at their NH₂ termini to form spider-like molecules with four collagenous arms and peripheral, globular sugar-binding domains (9, 10). However, single arm forms and higher order multimers of dodecamers have also been identified (10). Each M₉, 43,000 monomer consists of a noncollagenous NH₂ terminus containing two conserved cysteine residues (Cys²⁵ and Cys²⁸), a collagenous domain that contains the single consensus sequence for N-linked oligosaccharide, a short linking or neck sequence, and a COOH-terminal, C-type carbohydrate recognition domain (CRD) (11–13). The CRD contains four additional cysteine residues; however, these residues participate in intra-chain bonds that stabilize the conformation of the CRD (1).

In vitro studies of SP-D interactions with influenza virus suggest that the degree of subunit oligomerization is an important determinant of SP-D function. Trimeric subunits are comparable in potency to dodecamers in lectin-mediated hemagglutination inhibition activity but are defective as viral agglutinins and are unable to enhance the respiratory response of leukocytes to bound influenza virus (14). By contrast, natural and recombinant human SP-D multimers, which consist of up to eight dodecamers associated at the NH₂-terminal hub, are even more potent than human dodecamers in viral agglutination and in modulating the leukocyte response to bound virus (15).

Previous biochemical and molecular studies have identified at least three structural features that could play critical roles in the assembly and secretion of SP-D. These include the conserved asparagine-linked oligosaccharide at Asn⁷⁰ (16), the triple helical conformation of the collagenous domain, and the conserved NH₂-terminal cysteine residues.

The N-linked sugar is conserved and utilized in all known SP-Ds and is located near the NH₂-terminal end of the collagen domain near the point of divergence of the trimeric arms at the hub of the dodecamer (9). Asn-linked sugars within the collagen helix have been implicated in the registration of trimeric collagenous domains during the assembly of type IV collagen tetramers (16). In addition, some studies suggest that Asn-linked carbohydrates are necessary for the intracellular targeting and secretion of a related protein, SP-A (17, 18).

Prolyl hydroxylation is required for the formation of a stable collagen helix and for the secretion of many collagenous proteins. For example, 2,2'-dipyridyl (DP), an iron chelator that inhibits prolyl and lysyl hydroxylase, inhibits the secretion of most matrix collagens. DP-induced retention in the RER may involve the association of specific binding proteins, such as protein disulfide isomerase and HSP47, to the nonfolded chains (19, 20). DP also inhibits the secretion or normal assembly of several other collagenous proteins, including the macrophage scavenger receptor (21) and the collectins myelin basic protein,

*‡To whom correspondence should be addressed: Dept. of Pathology, Jewish Hospital at Washington University Medical Center, 216 S. Kingshighway, St. Louis, MO 63110. Tel.: 314-454-8462; Fax: 314-454-5505; E-mail: crouche@medicine.wustl.edu.

1 The abbreviations used are: SP-D, surfactant protein D; rSP-D, natural rat SP-D; RrSP-D, recombinant wild-type rat SP-D; RrSP-Dser15/20, simultaneous substitution of serine for Cys⁵⁰ and Cys⁸⁰ in rat SP-D; CRD, carbohydrate recognition domain; DP, 2,2'-dipyridyl; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle's medium; TM, tunicamycin; endoH and endoF, endoglycosidase H and F, respectively; ER, endoplasmic reticulum; RER, rough endoplasmic reticulum; DTT, dithiothreitol.

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SP-A, and SP-D (18, 22, 23).

Finally, recent biochemical studies have shown that the two NH₂-terminal cysteine residues of SP-D participate in the formation of interchain disulfide cross-links, which are critical for stabilizing interactions between trimeric subunits (9). These studies also suggest that the disulfide-bonded trimers observed when SP-D dodecamers are resolved by SDS-PAGE in the absence of reduction contain chains derived from two trimeric subunits. Consistent with these findings, the simultaneous substitution of serine for Cys₁⁵ and Cys²⁰ in rat SP-D (RrSP-Dser₁⁵/²⁰) results in the secretion of trimeric subunits corresponding to a single arm of the SP-D dodecamer (14). These single-arm mutants are structurally similar to trimeric forms of natural human SP-D.

Because oligomerization influences the functional properties of secreted SP-D and different sizes of SP-D oligomers accumulate in vivo, processes that regulate the covalent association of trimeric SP-D subunits could constitute important regulatory steps in SP-D biosynthesis. Given the above considerations, we formulated the following working hypotheses relating to SP-D trimeric SP-D subunits could constitute important regulatory pathways in type II and Clara cells, respectively (25, 26). We therefore, hypothesized that c) the formation of specific inter-subunit disulfide cross-links is required for dodecamer secretion.

Unfortunately, studies of the assembly and secretion of natural SP-D are limited by the marked phenotypic instability of isolated type II pneumocytes (24) and further complicated by the probable existence of constitutive and regulated secretory pathways in type II and Clara cells, respectively (25, 26). We have attempted to at least partially circumvent these difficulties by using CHO-K1 cells stably transfected with a rat SP-D (rSP-D) as a model system for studying assembly and constitutive secretion. Our previous studies demonstrated that recombinant wild-type rat SP-D (RrSP-D) synthesized by these cells is chemically and morphologically indistinguishable from natural rSP-D (27). This expression system, used in combination with site-directed mutagenesis of the SP-D cDNA and selected metabolic inhibitors, has allowed us to begin examining these hypotheses.

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 (12), using polymerase chain reaction overlap extension (28). Primers for mutating Ser⁻¹⁵ of the NGS consensus sequence to Ala72 were: CAAAGGAGAAAACGGCGCTGCTGGAGAACC (forward) and GTTCTCCAGCADGCGGCTTTCTCCTTG (reverse). Primers for mutating Cys⁻¹⁵ to Ser⁻¹⁵ (RrSP-Dser⁻¹⁵) were: CAATAAACCAAACGACAGCCACTGCT (forward) and GCTCAGGTTGCTGGTGGTTATGG (reverse); and primers for mutating Cys⁻²⁰ to Ser⁻²⁰ (RrSP-Dser⁻²⁰) were: CCTAGTCTCCTGAGATCCTGCA (forward) and CTTGTTGACTACGAGACTAGG (reverse). Sp6 and T7 sequences were used as outside primers. The double cysteine mutant (RrSP-Dser₁⁵/²⁰) was generated as described previously (14). The SacI fragment from wild-type rSP-D spanning most of the collagenous domain and the linking and CRD domains was subcloned into the corresponding site in the mutant RrSP-D/pGEM-3Z. Subcloning of the wild-type sequence of all mutant constructs was verified by DNA sequencing.

Expression of Rat SP-D cDNA Mutants in CHO-K1 Cells—Mutated rSP-D cDNAs were excised from pGEM-3Z with EcoRI and subcloned into the corresponding site within the multiple cloning site of pEE14 (27, 29, 30). Transfection of pEE14 constructs into CHO-K1 cells and selection of stably expressing cell lines were performed as described previously. Cell lysates and conditioned medium of transfected cell lines were assayed for SP-D production by immunoblotting and immunoprecipitation with polyclonal anti-rat SP-D (31).

Metabolic Labeling and Purification of Recombinant SP-D—Confluent cultures were washed in serum-free DMEM then incubated for 16 h in DMEM containing 1% dialyzed fetal calf serum, 50 μg/ml ascorbic acid, and 5 μCi/ml ³⁵S-proline (Dupont NEN; 283 mCi/mmol). Conditioned medium was collected in the presence of protease inhibitors added to sequential agarose affinity and size selection chromatography (9). The elution of radio labeled protein was monitored by liquid scintillation counting and/or SDS-PAGE.

Immunoprecipitation—Cells were plated in 6-well tissue culture plates and grown to confluence. Metabolic labeling was performed with 1-³⁵S-proline as described above or with Tran³⁵S-label (ICN, 1255 Ci/mmol). For labeling, cells were washed and preincubated for 30 min with DMEM deficient in cysteine and methionine supplemented with 50 μg/ml ascorbate. Cells were then incubated in 1 ml DMEM—(Cys/Met) plus 10 μCi/ml Tran³⁵S-label for up to 8 h. In experiments where cells were treated with tunicamycin (TM) or 0.1 mM DTT, these reagents were added to both the starvation medium and the labeling medium to ensure complete inhibition. TM was prepared as a 1000 × stock in methanol and was diluted to a 0.5–6 μM/ml working concentration in medium. A 100 mM stock of DTT was prepared in ethanol and used at a 0.1 mM working concentration. In pulse/chase experiments, cells were labeled as described above. After labeling, medium was removed, the cell layer was washed three times with DMEM, and label was chased for up to 8 h with DMEM containing excess cold proline or methionine/lysine.

Following labeling, conditioned medium was harvested and concentrated by ultrafiltration at 1000 × g to pellet any cells or debris. For analysis of intracellular protein, cell layers were washed 2 × with cold DMEM and lysed with 1 ml immunoprecipitation buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 0.5% (v/v) sodium deoxycholate, 1% (v/v) Triton X-100, 0.1% (v/v) SDS, 2.5 mM EDTA, and 0.1 mM phenylmethylsulfonfyl fluoride) supplemented with 50 μg/ml aprotinin to trap free sulfhydrils. Cell lysates were incubated on ice in the dark for 30 min and centrifuged at 12,000 × g to pellet cell debris. Immunoprecipitations were performed by mixing 200–800 μl cell lysate or conditioned medium with 500 μl immunoprecipitation buffer and 5 μl polyclonal rat SP-D antibody for 2 h at room temperature or overnight at 4 °C. Samples were then mixed with 300 μl of a 10% (v/v) suspension of IgGsorb (The Enzyme Center, Malden, MA) for 1 h at room temperature. After centrifugation at 12,000 × g for 30 s, the pellets were washed three times with immunoprecipitation buffer, and the precipitated proteins were resolved by SDS-PAGE and visualized by fluorography.

Size Fractionation of Secretory Intermediate—For each experiment, confluent monolayers of CHO-K1 cells were grown to confluence, pulse-labeled for 30 min with 1-³⁵S-proline, and chased for various periods or continuously labeled for up to 6 h. Following labeling, cells in each flask were scraped in 2 ml lysis buffer (150 mM NaCl, 50 mM Tris, pH 7.5, containing 1% (v/v) Triton X-100, 2.5 mM EDTA, 0.1 mM phenylmethylsulfonfyl fluoride, and 50 mM iodoacetamide), and the lysate was incubated in a microcentrifuge tube in an ice bath for 30 min in the dark. Lysates were clarified by centrifugation at 10,000 × g for 20 min, and the supernatant was adjusted to 5 mM CaCl₂ immediately prior to chromatography on maltosyl-agarose. After thorough washing of the column, bound SP-D was eluted with EDTA. The SP-D-containing fractions were identified by liquid scintillation spectrometry and by immunoblotting (dot blots). Pooled fractions were applied to a column of 4% agarose (Bio-Rad A15 M) equilibrated in the usual TBS/EDTA buffer containing 0.1% (v/v) Triton X-100.

Degradative Analysis—Immunocomplexes were washed with distilled water and then resuspended in 50 μl glycosidase buffer (100 mM sodium phosphate, pH 6.1, 50 mM EDTA, 1% (v/v) β-mercaptoethanol, 0.1% (w/v) SDS, 1% (w/v) Nonidet P-40, and 2 μg phenylmethylsulfonfyl fluoride). Samples were boiled and then incubated overnight at 37 °C with 0.05 unit endo-N-acetylglucosaminidase H or endoglycosidase F.

Subcellular Fractionation—Subcellular fractionation of rSP-D expressing CHO-K1 cells was performed using the method of Morand and Kent (32) with 16% Percoll gradients. Cells were labeled with Tran³⁵S-label as described above. Percoll was obtained from Sigma. Microsomal membranes (2,700 units/mg) were isolated from the homogenate and centrifuged for 20 min at 100,000 × g at 4 °C. The pellets were dissolved in 1% SDS and subjected to SDS-PAGE. After degradation, the fragments were analyzed by immunoblotting with antibodies to SP-D.

RESULTS

Rat SP-D Is Secreted at Comparable Rates by Stably Transfected Cells and Freshly Isolated Pneumocytes—To further ver-
ify the validity of the model system, we initially compared the synthesis/secretion time (i.e., the time interval between the addition of label and the appearance of radiolabeled SP-D in the culture medium) for stably transfected CHO-K1 cells (Fig. 1A, top) and freshly isolated rat type II cells (Fig. 1A, bottom).

The estimated synthesis/secretion time, as determined by extrapolation to the x axis, was between 1 and 1.5 h for both cell populations.

In pulse-chase experiments, RrSP-D demonstrated a t<sub>1/2</sub> of secretion of approximately 3 h. Interestingly, a small fraction of the total radioactive SP-D was retained in the cell for up to 8 h. Comparable results were obtained when the chase was performed in the presence of 30 μg/ml cycloheximide or many fold higher concentrations of unlabeled amino acid (data not shown).

RrSP-D Shows a Prolonged Transit Through the RER—To monitor the extent of oligosaccharide processing and to determine the probable subcellular localization of RrSP-D accumulating in the cell, cultures were continuously labeled for up to 4 h. The cells were then lysed and subjected to immunoprecipitation with anti-SP-D prior to incubation of parallel aliquots with endoH or endoF (Fig. 2). At all time points, the vast majority of the intracellular RrSP-D was endoH-resistant but sensitive to endoF and neuraminidase. Comparable results were obtained when cells were pulse labeled for 10 min and chased for up to 8 h (data not shown). At all time points, the vast majority of the intracellular immunoreactive protein was endoH-sensitive and neuraminidase-resistant.

Because some endoH-sensitive glycoproteins can accumulate in the cis-Golgi (35), we further confirmed the cellular localization by subcellular fractionation. Cells were labeled for 30 min with Trans<sup>35</sup>S-label and chased for 1 h or harvested immediately for subcellular fractionation. Fig. 3B shows the localization of Golgi galactosyltransferase and endoplasmic reticulum NADPH-cytochrome c reductase activities, and Fig. 3A shows SP-D immunoprecipitated from the corresponding fractions. With or without a 1-h chase, most of the SP-D codistributed...
with ER resident enzyme activity at both zero and 1 h of chase, confirming that the rate-limiting step in SP-D biosynthesis occurs in the RER.

Intracellular SP-D Consists of Covalent Complexes of Two to Four Trimeric Subunits—To examine the state of multimerization of the major intracellular species, cells were lysed in the presence of iodoacetamide (to block free sulfhydryls and prevent disulfide interchange) after different periods of continuous labeling or following a 30-min pulse and various periods of chase. The extracted proteins were subjected to sequential maltosyl-agarose affinity chromatography and gel filtration chromatography under nondenaturing conditions on 4 or 6% agarose. Preliminary experiments demonstrated that essentially all of the intracellular immunoreactive material bound to maltosyl-agarose, consistent with rapid folding of the CRD. After short periods of chase, the majority of the extracted immunoreactive protein eluted near the expected position of SP-D trimers (Fig. 4A, upper panel). Following several hours of continuous labeling (Fig. 4A, lower panel), or after 1 h or more of chase (data not shown), components eluting in the position of trimers were still identified. However, the majority of the radiolabeled and immunoreactive protein eluted as a broad peak between the position of authentic dodecamers and trimers (fractions 55–75), and only a small fraction eluted in the position of dodecamers.

Indirect immunoprecipitation assays demonstrated that the vast majority of the radioactive protein in each of the two peaks (i.e. the trimer peak and the broad peak intermediate in size between dodecamers and trimers) migrated in the expected position of disulfide-bonded SP-D trimers when examined by SDS-PAGE in the absence of DTT (Fig. 4B). Although monomers and disulfide-bonded dimers were also present in most of the fractions, they were most conspicuous in the trimer peak (fractions 72–82). Assuming interchain disulfide bonds are required for the stable association of subunits, (9) these observations are consistent with our previous conclusion that there are two classes of interchain disulfide bonds (intra- and inter-trimeric) in mature SP-D. Disulfide-bonded trimers eluting in the late fractions (trimer peak) are derived from a single subunit (3 × M₀, 43,000) and contain intra-trimeric interchain cross-links. By contrast, disulfide-bonded trimers eluting in the earlier fractions are derived from cross-linked subunits (i.e. contain chains derived from two separate trimers).

RrSP-D Is Assembled and Secreted Normally in the Absence of Asn-linked Glycosylation—To examine our hypothesis that
the conserved N-linked sugar at Asn\textsuperscript{70} is important for assembly and secretion, we examined the effect of blocking N-linked glycosylation by treatment of the transfected cells with varying concentrations of TM. At 4 \textmu g/ml TM, RrSP-D glycosylation was completely blocked, as evidenced by increased mobility on SDS-PAGE and comparison to endoF-treated RrSP-D (data not shown). However, contrary to our hypothesis, TM showed no detectable effect on the rate or level of SP-D secretion as compared to controls at TM concentrations as high as 6 \textmu g/ml.

To further confirm these results, we examined the biosynthesis of a mutant RrSP-D containing a Ser to Ala mutation at residue 72 of the mature protein (RrSP-D\textsubscript{ala72}), altering the Asn-X-Ser glycosylation recognition site. Asn-linked glycosylation was indeed prevented by this mutation, as shown by increased mobility of secreted RrSP-D\textsubscript{ala72} on SDS-PAGE and the absence of a mobility shift with endoF digestion (Fig. 5). Also, treatment of wild-type RrSP-D immune complexes with endoF resulted in a shift to the same mobility as RrSP-D\textsubscript{ala72}. Consistent with the TM results, mutation of the N-glycosylation site did not prevent SP-D secretion (Fig. 5), and the extrapolated synthesis/secretion time was similar to wild-type (Fig. 1B, top).

Although radiolabeled RrSP-D\textsubscript{ala72} eluted at the expected position of nonglycosylated dodecamers when examined by gel filtration chromatography, the assembly of functional molecules was further confirmed by saccharide affinity chromatography. RrSP-D\textsubscript{ala72} bound efficiently to maltosyl-agarose affinity support and was eluted with EDTA treatment. It also showed hemagglutination inhibition and viral aggregation titers comparable to wild-type RrSP-D for several strains of influenza A (data not shown).

DP Interferes with Interchain Disulfide Bond Formation—We next sought to determine whether the collagen helix is required for the assembly of cross-linked trimeric subunits by treating cells expressing wild-type RrSP-D with DP, an inhibitor of prolyl and lysyl hydroxylation (and collagen helix

**Fig. 4.** Size fractionation of intracellular SP-D. Four T75 flasks of RrSP-D-transfected CHO-K1 cells were grown to confluence and pulse-labeled for 30 min with \textsuperscript{14}C-proline as described in Fig. 3 or continuously labeled for up to 6 h. Following labeling, cells in each flask were lysed and subjected to sequential maltosyl-agarose affinity chromatography and gel filtration chromatography on 4% agarose as described under "Materials and Methods." A, representative chromatograms are shown. Upper panel, 30 min pulse/10 min chase. Lower panel, 6 h continuous labeling. The positions of elution of unglycosylated SP-D dodecamers and trimers are indicated. The position of elution of trimers was initially determined by reduction and alkylation of dodecamers under nonnaturating conditions combined with correlative ultrastructural studies (9). The upper panel also indicates the position of elution of thyroglobulin (TG), which elutes after SP-D trimers. The profile obtained after 1 h of chase was similar to that shown in the lower panel. B, fluorescence autoradiogram of intracellular proteins fractionated by gel filtration chromatography (A, bottom panel). Proteins in every second fraction (56–88) were resolved by SDS-PAGE in the absence of sulphydryl reduction. Note that disulfide-bonded trimers account for most of the protein in each peak. Minor components with the mobility of monomers and disulfide-bonded dimers are also identified in the region of the trimer peak.

**Fig. 5.** Ser to Ala mutation at residue 72 abolishes RrSP-D N-linked glycosylation. Cells transfected with either wild-type SP-D (RrSP-D) or SP-D containing a Ser to Ala mutation at residue 72 (RrSP-D\textsubscript{ala72}) were plated in 6-well plates and grown to confluence. Prior to labeling, cells were washed three times in DMEM (deficient in proline) and preincubated for 30 min at 37°C in DMEM plus 5 \textmu g/ml ascorbic acid. The cells were incubated in 1 ml DMEM plus 5 \textmu Ci/ml \textsuperscript{14}C-proline for 4 h at 37°C. Following labeling, SP-D was immunoprecipitated from the conditioned medium with a polyclonal anti-SP-D. The immunoprecipitated SP-D was incubated with endoF as described under "Materials and Methods." The digested proteins were resolved by SDS-PAGE in the presence of reduction and visualized by fluorography.
formation). As reported previously, DP almost completely inhibited the secretion of RrSP-D (27). However, pulse-chase studies of DP-treated cultures also demonstrated an associated abnormality in disulfide bond formation for the retained protein, as assessed by SDS-PAGE in the absence of sulfhydryl reduction (Fig. 6). Under control conditions, the vast majority of intracellular RrSP-D accumulated as disulfide-bonded trimers. By contrast, the intracellular RrSP-D in DP-treated cultures migrated predominantly as monomers and disulfide-bonded dimers on SDS-PAGE.

**DTT Reversibly Inhibits Secretion and Disulfide Bond Formation**—Previous experiments by other investigators have shown that disulfide bond formation can be reversibly blocked by treatment of cells with DTT (36–38). To further examine the role of disulfide bond formation in SP-D biosynthesis, RrSP-D producing CHO-K1 cells were pulse-labeled and chased in the presence or absence of 2 mM DTT (Fig. 7). With a 10-min pulse in the absence of DTT SDS-PAGE of immunoprecipitated protein in the absence of reduction showed species with the expected mobility of monomers (Fig. 7, lane 1). Some dimeric and trimeric disulfide-bonded intermediates were also identified. The addition of DTT during the pulse prevented all disulfide bond formation (Fig. 7, lane 2). The lower mobility of the monomer form in the presence of DTT is consistent with the absence of intrachain disulfide bonds in the CRD domain. Cells pulsed and chased in the presence of DTT did not secrete detectable SP-D (Fig. 7, lane 8), and the retained SP-D was recovered predominantly as a monomer, a small amount of which was present as the more rapidly migrating form that contains intrachain disulfide bonds within the CRD (Fig. 7, lane 4). Some of the immunoreactive protein migrated as high molecular weight aggregates or low molecular weight degradation products. This DTT-mediated inhibition of interchain disulfide bonding and secretion was completely reversible when cells were pulsed with DTT then chased in the absence of DTT. After 4-h chase, the accumulation of SP-D in the medium was comparable to control levels (Fig. 7, lane 7). In cells that were pulsed in the absence of DTT then chased plus DTT, we also observed inhibition of oligomerization and secretion (Fig. 7, lanes 6 and 10).

**Site-directed Mutagenesis of Cys**

Site-directed mutagenesis of Cys20 (or Cys25) blocks SP-D secretion. CHO-K1 cells were stably transfected with RrSP-Dgly20-pEE14, a construct encoding a mutant RrSP-D with glycine substituted for cysteine at position 20. Confluent cultures of a representative mutant clone were metabolically labeled with Trans35S-label for 4 h as described previously. Aliquots of cell lysate and culture medium were immunoprecipitated with anti-rSP-D, and aliquots of cell lysate and culture medium proteins were resolved by SDS-PAGE in the absence of reduction and visualized by fluorography.

**FIG. 6.** DP interferes with interchain disulfide bond formation and blocks RrSP-D secretion. RrSP-D-producing CHO-K1 cells were grown to confluence in 6-well plates and preincubated for 30 min in DMEM (−Cys/Met) supplemented with 0.1 mM DP. The cells were then labeled in 1 ml of the same medium containing 10 μCi/ml Trans35S-label and chased for up to 4 h in the presence of DP. Cell lysates (C) and medium (M) were then harvested. Immunoreactive proteins were collected by immunoprecipitation, resolved by SDS-PAGE in the presence of reduction, and visualized by fluorography.

**FIG. 7.** DTT reversibly blocks RrSP-D secretion. Cells were pulse labeled in the absence (−) or presence (+) of DTT, and aliquots of cell lysate and culture medium were immunoprecipitated as described in the legend for Fig. 6. Immunoprecipitates of cell and culture medium proteins were resolved by SDS-PAGE in the absence of reduction and visualized by fluorography.

**FIG. 8.** Site-directed mutagenesis of Cys20 (or Cys25) blocks SP-D secretion. CHO-K1 cells were stably transfected with RrSP-Dgly20-pEE14, a construct encoding a mutant RrSP-D with glycine substituted for cysteine at position 20. Confluent cultures of a representative mutant clone were metabolically labeled with Trans35S-label for 4 h as described previously. Aliquots of cell lysate and culture medium were immunoprecipitated with anti-rSP-D, and the immunoreactive proteins were resolved by SDS-PAGE in the absence (−) or presence (+) of DTT and visualized by fluorography. The mutant protein was not secreted and accumulated in the cell as monomers and disulfide-bonded dimers. Comparable results were obtained for cell lines expressing a mutant with an amino acid substitution of serine for cysteine at position 15 (data not shown). Right, expected positions of migration of reduced, mature, SP-D monomers, dimers, and trimers. Some of the immunoreactive protein migrated as high molecular weight aggregates or low molecular weight degradation products. This DTT-mediated inhibition of interchain disulfide bonding and secretion was completely reversible when cells were pulsed with DTT then chased in the absence of DTT. After 4-h chase, the accumulation of SP-D in the medium was comparable to control levels (Fig. 7, lane 7). In cells that were pulsed in the absence of DTT then chased plus DTT, we also observed inhibition of oligomerization and secretion (Fig. 7, lanes 6 and 10).

**Site-directed Mutagenesis of Cys**

Site-directed mutagenesis of Cys15 or Cys20 Prevents SP-D Secretion—Previous studies with RrSP-Dser15/20 indicated that the NH2-terminal cysteine residues are not required for
secretion or the formation of trimeric subunits (Ref. 14; Fig. 1B, bottom). For this reason, the inhibitory effects of DP and DTT on RsSP-D secretion suggested that cellular retention resulted as a consequence of incomplete or incorrect pairing of cysteine residues, rather than from the absence of cross-links. To further explore the possible effects of unpaired cysteines on SP-D secretion, we generated mutants with the substitution of serine for Cys15 (RsSP-Dser15) or glycine for Cys20 (RsSP-Dgly20) in the encoded protein. (The presence of a glycine at residue 20 rather than the desired serine in RsSP-Dgly20 was likely due to a Taq polymerase-generated mutation.) Neither of the mutants showed detectable secretion into the medium by enzyme-linked immunosorbent assay or immunoprecipitation assays (Fig. 8). As predicted, the largest intracellular covalent oligomers migrated in the position of disulfide-bonded dimers. The abnormal secretory phenotype of the two mutants was further confirmed in transient transfection assays. Immunoprecipitation assays of the culture medium and cell lysates 48 h following transfection showed no detectable secretion of either RsSP-Dgly15 or RsSP-Dser15, despite efficient secretion of RsSP-Dser15/20 and wild-type RsSP-D (data not shown).

RsSP-Dser15/20 Secretion Is Not Inhibited by DP—To assess the contribution of the collagen helix to the secretion of trimeric, independent of NH₂-terminal disulfide cross-links, we incubated RsSP-Dser15/20-expressing cells in the presence of DP. However, to our surprise, the mutant was efficiently secreted under conditions that inhibited the secretion of the wild-type protein (Fig. 9). The secreted underhydroxylated mutant eluted from gel filtration columns near the expected position of trimeric subunits but was readily degraded by pepsin under conditions in which the triple helical domain of hydroxylated RsSP-Dser15/20 remained intact (data not shown). By contrast with the wild-type protein, secretion of RsSP-Dser15/20 was inhibited by 6 μg/ml TM.

**DISCUSSION**

We have used stably transfected CHO-K1 cells expressing wild-type and selected mutant RsSP-Ds as a model system for examining the biosynthesis of SP-D. Previous work has shown that wild-type rSP-D synthesized using this system is structurally and functionally indistinguishable from the native protein (27). Furthermore, the synthesis/secretion time for SP-D by CHO-K1 cells is comparable to that observed for freshly isolated rat type II cells. Studies performed using this model support our hypothesis that the secretion of wild-type SP-D is dependent on the formation of dodecamers stabilized by specific inter- and intra-subunit disulfide bonds and that the formation of cross-linked dodecamers requires prior folding of the collagen helix. However, contrary to our initial hypothesis, the N-linked sugar at Asn70 is not required for the alignment of subunits or subsequent dodecamer secretion.

Three independent observations suggest that detection of abnormal disulfide bonds or unpaired sulfhydryls is the primary means by which CHO cells monitor the molecular assembly of wild-type SP-D dodecamers. DTT resulted in reversible retention of RsSP-D; and mutants with single NH₂-terminal cys substitutions were not secreted. We also noted that expression of RsSP-D in the presence of DP, which was accompanied by impaired disulfide bonding, resulted in retention (Fig. 6).

To our surprise, RsSP-Dser15/20 was secreted in the presence of DP. The latter observation strongly suggests that the DP-induced inhibition of secretion does not involve the direct detection of the improperly folded collagenous domains by specific binding proteins, as suggested previously for several other collagenous proteins (19, 20). Rather, the failure of the collagenous domain to fold leads to inhibition of secretion by interfering with the correct formation of interchain disulfide bonds. We speculate that this results in prolonged binding to protein disulfide isomerase or other ER-resident binding proteins to conformation-dependent determinants within the NH₂-terminal domain. Pulse/chase and continuous labeling studies
showed that the vast majority of the intracellular pool of SP-D is endoH-sensitive and copurified with RER markers in subcellular fractionation studies. Thus, our data in aggregate strongly suggest that subunit association and cross-linking in the RER are rate-limiting in SP-D biosynthesis (Fig. 10).

Interestingly, TM treatment inhibited the secretion of RsSP-Dser15/20. These preliminary data suggest that although N-linked sugars are not necessary for dodecamer formation or secretion, they may still contribute to the regulation of SP-D secretion under certain circumstances. One possibility is that the removal of NH2-terminal cysteine residues in RrSP-Dser15/20 results in the selective loss of the binding determinants for chaperones involved in NH2-terminal disulfide interchange and unmask normally transient and/or redundant interactions with other classes of binding proteins. In this regard, calnexin and calreticulin are known to transiently associate with many TM-sensitive proteins in the ER and appear to promote oligomerization and disulfide cross-linking of some glycoproteins (4, 39–43). Such sugar-dependent mechanisms might be required for the secretion of SP-D trimers, which have been identified in preparations of natural and recombinant human SP-D, or influence targeting in specific cell types (e.g. to secretory granules in Clara cells). Additional studies using inhibitors of Golgi transport, inhibitors of sugar-processing enzymes, and antibodies to specific ER-resident binding proteins will help to define the molecular mechanisms regulating assembly and secretion.

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