Pulmonary surfactant protein D (SP-D), a lung host defense protein, is assembled as multimers of trimeric subunits. Trimerization of SP-D monomers is required for high affinity saccharide binding, and the oligomerization of trimers is required for many of its functions. A peptide containing the α-helical neck region can spontaneously trimerize in vitro. However, it is not known whether this sequence is necessary for the complete cellular assembly of disulfide-cross-linked, trimeric subunits and dodecamers. For the present studies, we synthesized mutant cDNAs with deletions or site-directed substitutions in the neck domain of rat SP-D, and examined the assembly of the newly synthesized proteins after transfection of CHO-K1 cells. The neck domain contains three “classical” heptad repeat motifs with leucine residues at the “d position,” and a distinctive C-terminal repeat previously suggested to drive trimeric chain association. Deletion of the highly conserved core of the latter repeat (FSRYLKK) did not interfere with the secretion of dodecamers with lectin activity. By contrast, deletion of the entire neck domain or deletion of one or two amino-terminal repeats resulted in defective molecular assembly. The secreted proteins eluted in the position of monomers by gel filtration under non-denaturing conditions. In addition, the neck + carbohydrate recognition domain of SP-D was necessary and sufficient for the trimerization of a heterologous collagen sequence located amino-terminal to the trimeric coiled-coil. These studies provide strong evidence that the amino-terminal heptad repeats of the neck domain are necessary for the intracellular, trimeric association of SP-D monomers and for the assembly and secretion of functional dodecamers.

Pulmonary surfactant protein D,1 like other members of the collectin subfamily of C-type lectins, is believed to play important roles in the innate defense against a variety of respiratory pathogens (1–3). In addition, recent in vivo studies strongly suggest that SP-D plays important roles in immune and inflammatory regulation within the lung and in the regulation of surfactant homeostasis (4–7).

Most collectins, including SP-D, are assembled as multimers of trimeric subunits (8, 9). SP-D is predominantly assembled as a dodecamer consisting of four subunits cross-linked by disulfide bonds. Each SP-D subunit consists of four major domains: an amino-terminal cross-linking domain, an uninterrupted triple helical collagen domain, a trimeric coiled-coil or neck domain (N), and a mannose subtype, C-type lectin carbohydrate recognition domain (CRD).

Trimerization of SP-D monomers (43 kDa, reduced) is a prerequisite for high affinity saccharide binding, and the oligomerization of trimeric subunits is required for many of its known biological activities (10). A trimeric CRD constitutes a high affinity saccharide-binding site, and multimers of trimeric subunits are required to mediate bridging interactions between multivalent particulate ligands, such as bacteria and viral particles. A mutant form of SP-D that cannot form amino-terminal disulfide cross-links (RrSP-Dser15,20) is secreted as trimeric subunits and binds to known saccharide ligands but lacks the ability to aggregate viral particles or other particulate ligands (11, 12).

SP-D is synthesized and secreted by type II pneumocytes and nonciliated bronchiolar cells in vivo. However, the ability of isolated epithelial cells to synthesize and secrete SP-D is rapidly lost during cell culture. We have, therefore, utilized CHO-K1 cells transfected with SP-D cDNAs as a model system for studying the assembly and constitutive secretion of SP-D (11, 13). Biosynthetic studies have shown that the rate of secretion of wild-type SP-D by CHO-K1 cells is comparable with that observed for freshly isolated rat type II cells (13). Furthermore, recombinant wild-type SP-Ds are not distinguishable from the natural protein by a wide variety of biochemical and biological criteria (11, 12, 14, 15). In particular, recombinant rat and human SP-Ds synthesized in the presence of ascorbic acid are secreted as disulfide-cross-linked dodecamers with normally hydroxylated and glycosylated, pepsin-resistant, triple helical collagen domains. These molecules are able to participate in CRD-dependent interactions with microorganisms and phagocytic cells.

Subcellular fractionation and pulse-chase experiments using this model demonstrated that the newly synthesized chains of wild-type recombinant rat SP-D rapidly associate within the cell to form disulfide-cross-linked trimers with lectin activity (13). These trimeric subunits subsequently undergo a much slower process of subunit oligomerization with disulfide rear-

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1 The abbreviations used are: SP-D, surfactant protein D; CRD, carbohydrate recognition domain; MBL, mannose-binding lectin; rSP-D, recombinant SP-D; PCR, polymerase chain reaction; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis.
angement leading to the formation of disulfide-cross-linked dodecamers. Assembly of dodecamers occurs within the rough endoplasmic reticulum, with rapid transit through the Golgi immediately prior to secretion. Thus, trimerization is a rapid event that appears necessary for the subsequent formation of disulfide-cross-linked dodecamers.

Other studies have shown that a 35-aa polypeptide corresponding to the sequence immediately carboxy-terminal to the collagen domain of human SP-D is sufficient for the stable but reversible association of the peptides to form trimeric complexes in vitro (16). This sequence, which lacks cysteine, predicts an α-helical coiled-coil containing four uninterrupted heptad repeats with hydrophobic residues in a 3–4–3–4 spacing. When cDNAs encoding the contiguous neck + CRD domains are expressed in bacteria as glutathione S-transferase fusion proteins, the recombinant proteins are isolated as trimeric CRDs (17, 18). Such fusion proteins show lectin activity and demonstrate some of the CRD-dependent activities of the intact protein, including chemotactic activity (19) and certain binding properties. Such fusion proteins show lectin activity.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of rSP-D—Site-directed mutagenesis was performed by the PCR overlap extension method using a full-length rat SP-D cDNA (11, 13). Briefly, forward and reverse primers containing the desired substitutions or splice junctions were synthesized (Washington University School of Medicine, Oligonucleotide Synthesis Center) (see Table 1 and Table II). PCRs were performed using ~200 ng of ScaI-linearized rSP-D/pGEM-3Z template in reaction buffer containing 2 mM MgCl₂, 2 mM DTT, and 5 units of Pfu DNA polymerase (Roche Molecular Biochemicals). Separate reactions containing the required primer pairs (Table I, PCR1 and PCR2) were performed for ~25 cycles at an annealing temperature of 52 °C. The products were then gel-purified, and the final reaction with the outside primers (Table I, PCR3) was performed for 30 cycles at an annealing temperature of 55 °C. The resulting DNA was purified using QiAquick Gel Extraction Kit (Qia- gen), digested with EcoRI, and subcloned into pGEM-3Z. Clones were sequenced to verify the presence of the desired mutations and the absence of any additional mutations.

A chimeric cDNA encoding the entirety of the human type IIA pro-collagen amino-terminal peptide and the neck and CRD domains of rat SP-D was constructed by overlap extension (Table I). cDNA encoding full-length human amnion peptide (exons 1–8) of human type IIA pro-collagen was amplified by reverse transcription-PCR from RNA isolated from normal adult articular chondrocytes grown in culture. Specific upstream and downstream primers (5’-TAGGATCCGCGGTGAGCCG-3’ and 5’-CCGCTTACAGTACTTGGTCTTCCATCCACT-3’) were designed using the human pro-α1 type II collagen gene sequence (L10847).

Expression of Rat SP-D cDNA Mutants in CHO-K1—Mutated rSP-D cDNAs and chimeric sequences were excised from pGEM-3Z with EcoRI and subcloned into the corresponding site within the multiple cloning site of pEE14 (14). Restriction mapping and/or DNA sequencing were used to determine the orientation of the subclones. Transfection of pEE14 constructs into CHO-K1 cells and selection of stably expressing cell lines was performed as previously described (14). To collect secreted recombinant proteins for isolation and structural analysis, 50 confluent 100-mm plates were incubated for 24 h in serum-free Dulbecco’s modified Eagle’s medium supplemented with 50 μg/ml fresh ascorbic acid. Conditioned medium containing secreted recombinant proteins was collected, and any insoluble material was removed by centrifugation for 20 min at 10,000 × g at 4 °C. Phenylmethanesulfonyl fluoride was added to a final concentration of 0.1 mM. Proteins were identified by direct enzyme-linked immunosorbent assay and characterized by immuno blotting using a polyclonal antibody to rat SP-D (26).

Transient Transfection Assays—CHO-K1 cells were transfected to six-well plates at 0.5 × 10⁶ cells/well in Glasgow’s minimum essential medium and allowed to grow to ~50% confluence. The cells were then transfected with 3 μg of purified DNA in Glasgow’s minimum essential medium containing 10 μl of LipofectAMINE (Life Technologies, Inc.) and in the absence of serum, antibiotics, or phenol. Iodination reactions were performed for 24 h. The cells were washed twice with Dulbecco’s modified Eagle’s medium and then incubated for 16–24 h in the presence of fresh ascorbic acid (50 μg/ml). The medium was collected and clarified by brief centrifugation, and phenylmethylsulfonyl fluoride was added to a final concentration of 0.1 μM. For some experiments characterizing the mutants, 10 mM iodoacetamide was also added to prevent disulfide bond formation between unpaired sulfhydryls present in the incompletely oligomerized proteins. For immunoblotting, 35 μl

| Plasmid         | Rsn   | Primes                               | Template |
|-----------------|-------|--------------------------------------|----------|
| RsSP-Dd203–230  | 1     | Sp6 + Oligo 46 (5'-aggagaagectgtaggcttaaattattcgtct-3') | rSP-D    |
| RsSP-Dd205–230  | 2     | T7 + Oligo 47 (5'-aggagaagectgtaggcttaaattattcgtct-3') | rSP-D    |
| RsSP-Dd220–214  | 1     | Sp6 + Oligo 33283 (5'-cttccagctgactgtaggcttaaattattcgtct-3') | rSP-D    |
| RsSP-Dd225–230  | 2     | T7 + Oligo 33283 (5'-cttccagctgactgtaggcttaaattattcgtct-3') | rSP-D    |
| RsSP-Dd241–221  | 1     | Sp6 + Oligo 33214 (5'-aggagaagectgtaggcttaaattattcgtct-3') | rSP-D    |
| RsSP-Dd250–230  | 2     | T7 + Oligo 33215 (5'-aggagaagectgtaggcttaaattattcgtct-3') | rSP-D    |
| II/SP-D         | 1     | Oligo 57 (5'-aggagaagectgtaggcttaaattattcgtct-3') | IIA      |
|                 | 2     | T7 + Oligo 58 (5'-aggagaagectgtaggcttaaattattcgtct-3') | rSP-D    |
|                 | 3     | Oligo 57 + T7                         | rSP-D    |
out of a total volume of 1 ml was resolved on a 0.75-mm minigel. To examine intracellular proteins by immunoblotting, cells from each well were harvested directly into 500 μl of SDS-PAGE sample buffer and immediately boiled to inactivate proteases. Samples were briefly stored at −20 °C pending SDS-PAGE and immunoblotting.

Metabolic Labeling of Transfected Cells—Nearly confluent cell cultures were briefly washed in fresh serum-free Dulbecco’s modified Eagle’s medium and then incubated for 16 h in Dulbecco’s modified Eagle’s medium containing 1% (v/v) dialyzed fetal calf serum, 50 μg/ml ascorbic acid, and 5 μCi/ml 1,1′-14C)proline (283 mCi/mmol; PerkinElmer Life Sciences). The medium was harvested as described under “Transient Transfection Assays,” and secreted recombinant protein was isolated from the culture medium by gel filtration chromatography. Liquid scintillation counting was used to monitor the elution of radiolabeled proteins. Aliquots of the fractions were examined by SDS-PAGE and autoradiography.

Maltosyl-Agarose Chromatography—Secreted recombinant proteins containing the SP-D CRD were isolated from the culture medium using maltosyl-agarse affinity chromatography as previously described. The medium was dialyzed extensively against 0.15 M NaCl, 50 mM Tris-HCl, pH 7.5 (TBS), containing 10 mM EDTA, recalcified immediately before chromatography, and applied to a column of maltosyl-agarse equilibrated with TBS containing 20 mM CaCl2. Bound proteins were resolved by SDS-PAGE and visualized by silver staining, immunoblotting, or autoradiography as appropriate.

Gel Filtration under Nondenaturing Conditions—Proteins were concentrated by maltosyl-agarse chromatography and subsequently characterized by gel filtration chromatography under nondenaturing conditions on A15m (27). For some experiments characterizing incompletely oligomerized proteins, an A5m column was used. The gel filtration columns were calibrated with blue dextran, purified rat SP-D dodecamers, trypsinogen, rat SP-D trimeric subunits (RrSP-Dser15,20), bovine serum albumin, and lysozyme. The state of oligomerization of SP-D dodecamers and trimeric subunits was originally established by ultrstructural and biochemical analysis of natural and recombinant SP-D proteins resolved under identical conditions (27). Eluted proteins were resolved by SDS-PAGE and visualized by silver staining or autoradiography as required. Yields were calculated based on the recovery of purified proteins. Proteins were quantified using a dye-binding assay with bovine serum albumin as a standard.

Purification of IIA Aminopeptidase—Approximately 100 μg of IIA/SPD fusion protein was digested overnight at 37 °C with recombinant human matrix metalloproteinase 9 (MMP-9; a gift from Dr. R. Senior, Department of Internal Medicine, Washington University, St. Louis) at an enzyme/substrate ratio of 1:100. MMP-9 cleaves within the neck domain. In particular, we compared the structure of wild type and mutant SP-D by CHO-K1 cells transiently or stably transfected with the wild type and mutant cDNAs. A mammalian expression system is essential for such studies. Prokaryotic and insect cells do not support normal post-translational modification and folding of the collagen domain. In order to simplify the analysis, we utilized rat SP-D cDNAs because the secreted protein is almost exclusively assembled as dodecamers (14). By contrast, the human SP-D cDNA is secreted as a mixture of dodecamers, higher order oligomers, and trimeric species (15).

As shown in Fig. 1, the primary sequence of the neck domain is highly conserved among all known SP-D proteins. There is nearly complete conservation of residues predicted to contribute to the formation of a stable α-helical coiled-coil, including the unique aromatic residues (Phe1225 and Tyr1226) of the C-terminal heptad repeat. Deletion analysis was first used to examine the functional role of the neck as well as the contributions of specific sequences within the neck domain.

Deletion of the Neck Domain Results in the Secretion of Monomers That Do Not Bind to Maltosyl-agarse—We initially examined the importance of the neck domain for SP-D secretion and assembly by stably expressing a deletion mutant extending from the C-terminal end of the collagen domain through Lys219 (RrSP-Dd203–230). This site of termination corresponds to the last residue encoded by exon 7 of the human gene (28) and position f of the C-terminal heptad repeat of the neck domain.

Both transient and stable transfections gave recoveries of medium protein comparable with that obtained for parallel transfections using the wild-type rSP-D cDNA (data not shown; see also Fig. 6). Analysis of the secreted protein by SDS-PAGE and immunoblotting showed a major band migrating near the position of wild-type monomers in the region of sulfhydryl reduction (Fig. 2, lane 2). Disulfide-cross-linked trimers that characterize secreted wild-type dodecamers were not seen; however, less intense bands with the expected mobility of dimers were observed (Fig. 2, lane 2, arrow D). When examined by SDS-PAGE in the presence of dithiothreitol, a single component was observed; this species migrated slightly more

| Table 1 | Molecular Assembly of Surfactant Protein D |
|---------|---------------------------------|
| **SP-D consensus** | *LRR* | *L Q Q L Q A F Y K* |
| N | Collagen L | CRD |
| 203 | 231 |
| rat SP-D | DSAALRDOMEANGLGRLEAF4YSYKKA |
| human SP-D | DVASLGRELQADKQLDLSLQAS4YSYKKA |
| mouse SP-D | DSAALRDEMEANGLGRLEAF4YSYKKA |
| porcine SP-D | GIALGRELQADKQLDLSLQAS4YSYKKA |
| bovine SP-D | EYRALQREGGGLGRLEAF4YSYKKA |
| bovine CL-43 | EVRALQREGGGLGRLEAF4YSYKKA |

**Fig. 1.** The schematic diagram at the top of the figure illustrates the relative size and position of key domains of SP-D. These include the amino-terminal cross-linking domain (N), collagen domain, linking or neck domain (L), and CRD. Each molecule of rat SP-D consists of four, homotrimeric subunits. The positions of intrachain bonds within the CRD are illustrated. The neck regions of several collectins are aligned, and the positions of key residues in the four heptad repeats are identified. Note the complete conservation of hydrophobic residues in the d position. The bottom identifies regions deleted or mutated in the various rat SP-D constructs.
slowly than the unreduced protein (not shown, but see below). In subsequent experiments, we found that the proportion of the cross-linked components was reduced by the addition of iodoacetamide to the culture medium prior to SDS-PAGE (data not shown). Because untrimerized, mutant monomers must contain free sulfhydryls in their amino-terminal domains, cross-linking probably occurs when the protein is concentrated in the stacking gel.

In order to assess the lectin activity of the protein, conditioned medium was applied to malsosyl-agarose. Secreted wild-type SP-D or trimeric subunits of SP-D (e.g. RrSP-Dser15,20) bind tightly and are recovered following elution with EDTA or competing sugar. However, in preliminary experiments, essentially no protein was recovered in the EDTA-eluate, and the assessment of the unbound fractions was complicated by the presence of serum proteins. We therefore monitored the elution of the protein by immunoassay. As shown in Fig. 3, essentially all of the immunoreactive RrSP-Dd203–230 was unbound and recovered in the wash.

Because monomers do not bind efficiently to malsosyl-agarose, our data suggested the absence of trimeric subunits, rather than the production of trimers lacking disulfide cross-links in their amino-terminal domains. To examine the extent of noncovalent oligomerization, we performed gel filtration chromatography under nondenaturing conditions on A15m- or A5m-agarose. For these studies, we directly applied metabolically labeled proteins in dialed conditioned medium to the column. This approach was used to circumvent the need to concentrate the medium and to preclude interference by serum proteins expected to elute near the recombinant protein. As predicted, radiolabeled RrSP-Dd203–230 eluted late and within the included volume, after the position of elution of authentic trimeric subunits and slightly earlier than bovine serum albumin (68 kDa) (Fig. 4A). Although the theoretical molecular mass of monomers is ~40 kDa, even non-triple helical collagenous sequences assume a more extended conformation in solution and have a higher hydrodynamic radius than globular proteins of comparable mass. However, given the absence of corresponding dimeric standards, the elution profile alone does not allow us to definitely distinguish between monomers or dimers. As indicated above, these preparations also contained a small fraction of disulfide-cross-linked dimers (Fig. 4A, fractions 62–66). These species were resolved from the monomer peak and eluted between authentic trimers and BSA. Comparable results were obtained using less porous A5m gel beads (data not shown). Thus, the data are most consistent with a predominance of monomers that lack the ability to bind to malsosyl-agarose.

As suggested by SDS-PAGE of the fractions that bound to malsosyl-agarose, purified monomers migrated more slowly in the presence of dithiothreitol, consistent with the presence of intrachain disulfide bonds within the CRD (Fig. 4B). Interestingly, the reduced protein did not migrate more rapidly than the wild-type protein. Given that the sequence of protein was reconfirmed by cDNA sequencing, we suspect that this in part reflects overhydroxylation and/or glycosylation of the unfolded collagen domains. It is well established that mutations in matrix collagens that interfere with helix formation are associated with overhydroxylation of hydroxyproline and hydroxylysine residues and a decreased mobility on SDS-PAGE (29). However, differences in SDS binding could also play a role.

Deletion of the C-terminal Heptad Repeat Does Not Alter the Assembly of Trimeric Subunits or Dodecamers—Given the importance of the neck domain for trimeric assembly, we initially focused our attention on the distinctive C-terminal heptad repeat. As indicated in the Introduction, Hakansson et al. (22) previously suggested that this region, and the conserved aromatic residues in particular, are critical for trimerization. We therefore expressed a more limited deletion (RrSP-Dd225–230) containing the carboxyl-terminal heptad repeat. As for the full neck deletion, stably or transiently transfected cells efficiently secreted the mutant. However, in contrast with RrSP-Dd203–230, RrSP-Dd225–230 bound efficiently to malsosyl-agarose, migrated near the position of RrSP-D trimers in the absence of reduction (Fig. 5A), and eluted from A15m gel filtration columns in the position of wild-type dodecamers (Fig. 5B). Substitutions of serine for Phe225 and/or Tyr228 (RrSP-Dser225, RrSP-Dser228, RrSP-Dser225,228) gave identical results (data not shown). Thus, this heptad repeat is not critical for stable...
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Fig. 4. Characterization of the radiolabeled neck deletion mutant (RrSP-Dd203–230) by gel filtration chromatography. A, stable transfectants expressing RrSP-Dd203–230 were metabolically labeled with L-[14C]proline, which preferentially labels collagenous proteins, as described under "Experimental Procedures." After dialysis to remove unincorporated isotope and newly synthesized low molecular weight proteins, the medium was applied to a calibrated column of A15m-agarose and chromatographed under non-denaturing conditions. A, column fractions were examined by SDS-PAGE and fluorescence autoradiography. The fraction number is indicated at the bottom, and the positions of elution of RrSP-Dser15,20 trimeric standards and BSA are indicated at the top. Species corresponding to SP-D dimers (D) and monomers (M), and the approximate position of migration of unreduced trimers (T) are indicated at the right. Most of the radiolabeled recombinant protein elutes in a position consistent with SP-D monomers. B, radiolabeled monomers eluting in fractions 68, 70, 72, were resolved by SDS-PAGE in the absence or presence of DTT. The decreased mobility of the reduced protein is consistent with the presence of intrachain disulfide bonds within the SP-D CRD.

Oligomerization or trimeric assembly. In addition, the findings are consistent with the previously cited published data indicating that sequences amino-terminal to the CRD are not required for the acquisition of C-type lectin activity.

Transient transfection assays indicated that the protein was expressed at levels comparable with wild type (e.g., see Fig. 6, compare lanes 1 and 2, below). However, recoveries following saccharide affinity chromatography and gel filtration chromatography were sometimes lower than for the wild-type protein or other mutants. In addition, we often observed the appearance of bands and immunoreactive components of lower apparent molecular mass on SDS-PAGE following maltosyl-agarose chromatography (Figs. 5A and 5B). Thus, the mutation may render the protein more susceptible to proteolytic degradation under our usual conditions of protein isolation; however, additional studies are required.

The Amino-terminal Heptad Repeats of the Neck Domain Are Required for the Formation of Trimeric Subunits and Dodecamers—Given the unexpected findings for RrSP-Dd225–230, we next focused our attention on the first three heptad repeats and generated constructs with deletions involving the first and second (RrSP-Dd207–214) and second and third (RrSP-Dd214–221) repeats. The deletion of residues 214–221 almost totally eliminated predicted coil-coil structure using the Coils software on the ISREC server when analyzed with the minimum window of 14 residues, and the deletion of residues 207–214 showed a major, but less marked, decrease in predicted coiled-coil formation.

Both constructs were initially examined in transient transfection assays. The cells were transfected with identical amounts of cDNA, and identical amounts of conditioned medium were analyzed by immunoblotting. As shown in Fig. 6, the accumulation of RrSP-Dd207–214 and RrSP-Dd214–221 was comparable with wild-type RrSP-D, consistent with similar rates of secretion, and comparable amounts of immunoreactive protein were also observed in the cell lysates (data not shown). When the two mutants were examined by SDS-PAGE in the absence of sulfhydryl reduction followed by immunoblotting, both showed a predominance of monomers, with only minor...
bands migrating in the positions of disulfide-cross-linked dimers (Fig. 6, lanes 7 and 8). Comparable results were obtained for a construct with a deletion involving the first three repeats (RrSP-Ddel207–221) (data not shown). By contrast, parallel transient transfections with wild-type RrSP-D gave the expected disulfide-cross-linked trimers (Fig. 6, lane 6).

To further confirm the state of multimerization of these proteins, clones stably expressing one of these deletion mutants, RrSP-Ddel207–214, were isolated. Although the protein could be visualized by silver staining, immunoblotting was again used to limit the confounding effects of contaminating serum proteins. As for the full neck deletion, the secreted protein did not bind to maltosyl-agarose (Fig. 7A) and eluted from A15m after trimers (Fig. 7B), near the position of RrSP-Ddel203–230.

Production of a Chimeric Protein Containing a Heterologous Collagen Domain—The amino-terminal propeptide domains of the interstitial collagens contain a short, triple helical collagen domain (30). Folding of this helical domain is believed to require the prior folding of the major collagen helix, which in turn depends on the noncollagenous, carboxyl-terminal propeptide domains for the correct alignment of chains and subsequent helix formation (29, 31, 32). Once formed, the triple helix of the propeptide is likely to be stable, given that trimeric, amino-terminal type I and type II propeptides have been isolated from developing bone (34, 35). Consistent with this generalization, the amino-terminal propeptide of type IIA procollagen contains a short, interrupted, triple-helical collagen domain encoded by exons 3–7 (30), and recombinant IIA propeptides expressed in bacterial or eukaryotic systems in the absence of the major collagen helix and carboxyl-terminal propeptide are recovered as monomers or dimers.3

The deletion analysis of the neck domain of SP-D indicates that more than two complete heptad repeats are necessary for the formation of trimeric subunits and dodecamer assembly. However, as indicated under “Discussion,” the mechanism of trimeric association is quite varied among collagenous proteins. In order to determine whether the carboxyl-terminal domains of SP-D are sufficient for trimerization of an amino-terminal, collagen-containing sequence, we constructed a chimera consisting of the amino-terminal propeptide of type IIA procollagen and the neck + CRD domain of SP-D utilizing the normal exon boundaries (Fig. 8).

The chimeric protein was efficiently secreted by transiently or stably transfected cells and was readily isolated from the culture medium of stably transfected cells by saccharide affinity chromatography (data not shown). When examined by SDS-PAGE, the chimera migrated at a position appropriate for its predicted molecular mass (~45 kDa, unreduced). In the presence of sulfhydryl reduction, the protein showed a decrease in mobility consistent with the presence of intrachain disulfide bonds in the CRD and exon 2 of the IIA propeptide. In addition, immunoblotting demonstrated reactivity with antibodies to exon 2 of the propeptide, and with antibody to rat SP-D (see below).

The SP-D Neck Domain Is Sufficient for Trimerization of a
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**Discussion**

These studies provide the first direct evidence that specific subsequences within the neck domain are required for the trimerization of SP-D monomers and their subsequent intracellular oligomerization to form disulfide-cross-linked dodecamers. Trimeric assembly was blocked by deletion of the neck domain sequences and almost totally abrogated by more limited deletions confined to the amino-terminal heptad repeat. A 7-amino acid deletion involving two consecutive repeats interfered with the formation of disulfide-cross-linked trimers, and the secreted species consisted of monomers that lack the ability to bind to maltosyl-agarose.

As indicated in the Introduction, Hakansson et al. (22) suggested that the aromatic residues in the carboxy-terminal repeat, in particular the aromatic ring of Tyr228, are required to drive oligomerization to a trimeric assembly. This suggestion was in part based on the reported effects of exogenous benzene on the oligomerization of an engineered GCN4 leucine zipper protein. Nevertheless, deletion of the core of this repeat or more limited substitutions of serine for Phe225 and/or Tyr228 did not interfere with assembly of trimers, oligomerization to form dodecamers, or the ability to bind to maltosyl-agarose. Experiments designed to further assess the functional activity of the mutant and the role of this highly conserved sequence are in progress.

Given the predicted marked perturbation in protein structure, we anticipated that proteins with neck deletions might...

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**Fig. 8. Schematic diagram of RIIA/SP-D chimera.** The chimera consists of an amino-terminal human type IIA procollagen amino-propeptide sequence (IIA) joined to a carboxy-terminal sequence encoding the neck and CRD (L + CRD) of rat SP-D. The positions of the collagen sequence encoded by exons 3–7 of the IIA propeptide domain and the SP-D trimerization domain are indicated in black. The IIA peptide sequences, which are amino-terminal to the major collagen helix of type II procollagen, do not spontaneously trimerize. However, we hypothesized that trimerization mediated by the SP-D neck domain would permit trimerization of the IIA propeptide domain. The positions of the major predicted MMP-9 cleavage site (thick arrow) and a secondary cleavage site identified in these studies (thin arrow) are indicated. Cleavage with MMP-9 is predicted to liberate two major species: the amino-terminal propeptide and the neck + CRD of SP-D, which is known to have lectin activity.

**Fig. 9. Purification of MMP-9-cleaved IIA/SP-D.** The secreted chimera was isolated by sequential maltosyl-agarose chromatography and gel filtration chromatography under non-denaturing conditions as for SP-D. The purified protein was incubated with MMP-9 as indicated under “Experimental Procedures,” and the liberated IIA peptide was resolved from the trimeric lectin domain of SP-D by maltosyl-agarose chromatography. Peptides in the wash (Wash) and EDTA-eluate (Bound) were visualized by silver staining (lanes 1 and 2) or by immunoblotting with antibody to IIA or SP-D as indicated. The unbound IIA peptide was recovered in the wash (lane 3), whereas the trimeric neck + CRD of SP-D was bound to the column and eluted with EDTA (lane 5).

**Fig. 10. Characterization of IIA/SP-D and IIA by chemical cross-linking.** The MMP-9-generated IIA propeptide was purified by maltosyl-agarose chromatography as described in the legend to Fig. 9 and subjected to chemical cross-linking as described under “Experimental Procedures.” The reaction products were then denatured in SDS sample buffer and analyzed by SDS-PAGE. The concentration of bis(sulfosuccinimidyl)suberate cross-linker (BSS) is indicated. The positions of monomers (M), dimers (D), and trimers (T) are indicated at the right; the position of migration of globular standards is shown at the left. The faint band (>) is believed to represent a degradation product of MMP-9. In any case, it did not react with antibodies to IIA or SP-D.

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4. N. Fukui, A. McAlinden, and L. Sandell, unpublished data.
5. J. G. Bann, unpublished data.
not be secreted. Nevertheless, the mutants were efficiently secreted into the medium (Fig. 6), and immunoblots showed comparable signals for SP-D in lysates of wild-type cells and those transfected with the neck deletion constructs (data not shown). It is unlikely that the cells are generally defective in their capacity to retain or degrade abnormal proteins. Our previous studies have shown that the substitution of serine for Cys15 or for Cys20 results in cellular retention and the absence of detectable protein in the culture medium (13). In addition, the prevention of collagen hydroxylation with 2,2′-dipyridyl inhibits the assembly and secretion of RrSP-D by the CHO-K1 cells, as observed for the secretion of natural SP-D by freshly isolated type II cells.

The failure of the full neck (RrSP-Ddel203–230) and amino-terminal neck deletion (RrSP-Ddel207–214) mutants to efficiently bind to maltosylagarose could theoretically reflect a loss of lectin activity secondary to subtle perturbations in CRD structure but more likely reflects differences in CRD valency. Both preparations contained a small subpopulation of dimeric molecules that bound to maltosyl-agarose. Furthermore, protease digestion studies of cell-free translated C-type lectins have shown that the CRD domains are self-folding modules and that the exon boundary coincides with the amino-terminal extent of the functional CRD (38). Last, monomeric forms of wild-type C-type lectins show a markedly reduced ability to interact with their ligands. For example, the Kd for the interaction of a single C-type CRD with a monosaccharide ligand is on the order of $10^{-8}$ M, whereas the binding of collectin trimers and dodecamers to polylvalent ligands is on the order of $10^{-11}$ M, respectively (8).

Most but not all collagenous sequences, including the interstitial fibrillar collagens and type IV collagen, require additional, globular sequences C-terminal to the triple helical domain to mediate chain association (29, 31, 32, 37). By contrast, the trimeric assembly of type XII collagen is dependent on the collagen domain (38), and chain association for XIII collagen, a membrane-associated collagen, occurs amino-terminal to the collagen domain (39). It is likely that members of the macrophage scavenger receptor family also undergo chain association in their amino-terminal coiled-coil domains (40). Previous studies have shown that deletion of the entire collagen domain of rat SP-D results in the secretion of trimers cross-linked at their amino termini (41); however, the contributions of the amino- and carboxyl-terminal domains were not directly examined. Furthermore, the neck + CRD domains of RrSP-D isolated following digestion with bacterial collagenase (i.e. lacking both the collagen and amino-terminal domains) consisted of monomers rather than stable trimers (41).

In order to determine whether the SP-D carboxyl-terminal domain is sufficient for trimerization of a collagen sequence, we utilized a chimera consisting of the amino-terminal propeptide domain of type II procollagen and the neck + CRD domains of SP-D. This unconventional approach was chosen for several reasons. First, the major SP-D epitopes are located within the CRD, and we have no antibodies reactive with the relatively nonimmunogenic, amino-terminal peptide or collagen domains of rat SP-D. Second, a cDNA encoding the human type IIA propeptide sequence and antibodies reactive with the exon 2 sequence of the IIA propeptide are currently available (42, 43). Third, preservation of the SP-D CRD provides a means for rapidly purifying the chimera by saccharide affinity chromatography. Fourth, preliminary studies showed that the isolated IIA propeptide does not spontaneously trimerize in HEK 292 cells, although these cells can support the secretion of triple helical collagens (44). Last, the propeptide contains at least one site for MMP-9 cleavage carboxyl-terminal to the IIA collagen domain (Fig. 6). Notably, fusion of the IIA propeptide to the SP-D “trimerization domain” allowed the formation of propeptide trimers.

The mechanisms by which the carboxyl-terminal domains of other collagenous proteins promote the trimerization of collagen domains are poorly understood. Although coiled-coils contribute to the trimerization of several different classes of proteins (45), they may also contribute to the trimerization of several different classes of collagenous proteins including the collagenous C-type lectins and macrophage scavenger receptors (40). The neck domain of bovine conglutinin, which is evolutionarily derived from SP-D, can trimerize in vitro (46), and a similar trimeric coiled-coil is present in the mannos-binding lectin (24). In addition, deletions that include the neck region of SP-A prevent the normal oligomerization and secretion of recombinant SP-A by COS-7 cells (47).

The current studies show that more than two heptad repeats are required for the formation of a stable trimeric SP-D subunit, consistent with the observation that most stable coiled-coils contain four or more repeats. However, given that RrSP-Ddel225–230 forms cross-linked multimers of trimeric subunits, the mechanism that determines trimeric versus dimeric assembly remains uncertain. Frank et al. (40) recently suggested that proteins containing dimeric or trimeric coiled-coils contain specific “trigger sequences” that facilitate chain association. For example, a consensus (IV/V/L)(N/D/E)IN(R/K)N or (IV/L)(D/E)XIX(R/K)N was identified in the macrophage scavenger receptor and certain other proteins with trimeric coiled-coils. However, such a sequence is not present in the neck region of SP-D. Earlier studies by Harbury emphasized the potential importance of β-branched side chains at the α position in the formation or stabilization of trimeric coiled-coils (48, 49). Given the frequent occurrence of valine in the α and δ positions of the human neck domain, Hoppe et al. (16) suggested that this might drive trimerization. However, as shown in Fig. 1, rat SP-D, unlike human SP-D, contains no branched amino acids at these positions.

Together, the available data suggest that the primary role of the neck domain in molecular assembly is to align the collagen chains and thereby facilitate nucleation events suitable for the subsequent “zipper-like” folding of the collagen helix (50). In this regard, it was recently shown that triple helix of type III procollagen can fold when the carboxyl-terminal propeptide is replaced with a transmembrane domain (51). At least in the case of SP-D, the trimerization and collagen domains need not be contiguous, given that 30 amino acids separate these domains in the IIA/SP-D chimera. Because deletion of the neck domain of SP-D prevents the formation of trimeric subunits as well as the association of trimeric subunits to form functional dodecamers, we infer that defective folding of the collagen triple helix prevents the formation of intra- and intersubunit disulfide cross-links by limiting the interaction of the amino-terminal peptides. By contrast, in the complete absence of the collagen domain, trimerization of the neck domain is sufficient for the trimerization of the contiguous amino-terminal peptide domain but insufficient for the association of trimeric subunits to form stable dodecamers (41).

In summary, our studies provide strong evidence that the amino-terminal heptad repeats of the coiled-coil domain of the neck region of SP-D are necessary, and possibly sufficient, for the assembly of trimeric subunits with triple helical collagen domains. Given the ability to express IIA/SP-D chimeras, we will be able to efficiently isolate and characterize mutant proteins lacking all or part of the lectin domain. Such constructs

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6 N. Fukui, A. McAlinden, and L. Sandell, unpublished data.
should allow us to further define the minimal sequences required for trimeric assembly. Knowledge of the structural requirements for collectin oligomerization will assist with the development of recombinant collectins with novel or enhanced functional properties (33, 52).

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