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IN VITRO TRANSLATION OF RABBIT LUNG CLARA CELL SECRETORY PROTEIN mRNA

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SUMMARY The major secretory product of Clara cells is a low molecular weight protein (CCSP) whose extracellular function, at this time, is not known. The primary translation product of its mRNA is a protein with molecular weight approximately 1 kD greater than that of the native secreted protein (6.0 kD). The primary translation product is not detected in incubated lung tissue, only the secretory protein is found. The primary translation product is trypsin sensitive whereas the secretory protein is not. Cell free translation of the mRNA in the presence of microsomes results in cleavage of the signal peptide and the appearance of the lower molecular weight trypsin-resistant secretory protein. These data indicate that the low molecular weight Clara cell secretory protein is synthesized as a larger, trypsin sensitive, protein. Passage of the protein into the cisternae of the endoplasmic reticulum results in loss of the signal peptide and alterations to the tertiary structure of the protein rendering it trypsin insensitive.

Clara cells are nonciliated epithelial cells that are distributed throughout the pulmonary airways and trachea of many mammalian species although the cells are concentrated within the bronchioles (1). Clara cells are characterized by an absence of cilia, abundance of smooth endoplasmic reticulum, and the presence of cytoplasmic osmiophilic granules (2). Clara cells have, for many years, been considered secretory although the nature of their secretions has, until recently, been obscure. The osmiophilic granules of Clara cells are considered to be the storage sites of secretory materials (3) and decreases in the volume density of those structures has been considered strong putative evidence for Clara cell secretion.

Recently, the major secretory product of Clara cells from the lungs of rabbits was identified as a low molecular weight protein (CCSP, Mr 6 kD on SDS gels under reducing conditions) (4,5). Secretory proteins, after synthesis on polysomes attached to endoplasmic reticulum, pass through the lumina of the endoplasmic reticulum to the Golgi complex and then to secretory granules or

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vesicles (6). Discharge of proteins into extracellular space, however, depends on the nature of the cells (7). For instance, in endocrine and exocrine cells, proteins are stored in secretory granules and wait for proper stimulation for exocytosis (8-10) while in other cells such as, hepatocytes, fibroblasts, muscle cells, macrophages, plasma cells (7,11,12) vesicles release their proteins continuously. In order to gain further insight into the metabolic and secretory nature of the Clara cell secretory protein we performed its cell free synthesis and identified the primary translation product of its mRNA.

MATERIALS AND METHODS

Reagents: The following materials were used: guanidinium thiocyanate (Fluka purum grade, Switzerland); sodium N-lauroylsarcosine, sodium citrate, 2-mercaptoethanol, 30% antifoam A, aprotinin, protein A attached to non-crosslinked agarose, goat serum, rabbit IgG, rabbit anti-goat IgG attached to agarose, phenylmethylsulfonyl fluoride (PMSF), N-alpha-p-tosyl-1-lysine chloromethyl ketone (TLCK), pepstatin A, TPCK (L-1-tosylamide-2-phenylethyl chloromethyl ketone) treated trypsin, peroxidase antiperoxidase (developed in goat), antigoat IgG (developed in rabbit) (Sigma Chemical Co., St. Louis, MO); sodium dodecyl sulfate (SDS), acrylamide, methylene bis-acrylamide, ammonium persulfate, glycerol, N,N,N',N'-tetramethylenediamine (TEMED), protein standards for electrophoresis (myosin, B-galactosidase, phosphorylase B, bovine serum albumin, ovalbumin, trypsin inhibitor and lysozyme), Coomassie brilliant blue R-250 (Bio-Rad Laboratories, Richmond, CA); oligo(dt)cellulose, rabbit reticulocyte lysate (Bethesda Res. Laboratories, Gaithersburg, MD); disodium ethylenediaminetetraacetate (EDTA) (Fisher Scientific Company, Fair Lawn, NJ); ultra pure guanidine hydrochloride, cesium chloride optical grade (Schwarz/Mann Biotech, Cleveland, Ohio) canine pancreatic microsomal membranes (Amersham Corporation, Arlington Heights, IL); En3hance (NEN Research Products, Boston, MA).

Animals: Adult male rabbits of New Zealand strain weighing between 2-2.5 kg were obtained from Dutchland Laboratories Animal, Inc., Denver, PA. They were maintained on standard diet and had free access to food and water.

Isolation of RNA: Ribonucleic acid was prepared essentially by the cesium chloride method of Chirgwin et al.(13). A rabbit was sacrificed by intravenous injection of 3 ml of Nembutal (50 mg/ml) and 6 g of lung was homogenized with polytron homogenizer (Brinkmann Instruments, Westbury, NY) in 10 volumes of guanidium thiocyanate reagent. The lysate was passed 12 times through a 19 gauze needle to sheer DNA and reduce the viscosity of the solution (14). The solution was centrifuged at 15,000 x g for 20 min. The supernatant was layered over one third volume of cesium chloride-EDTA solution, pH 7.0, in a centrifuge tube and spun at 100,000 x g_{max} for 18 hr. The upper layer was removed and cesium chloride layer was rinsed two times with 2 ml of guanidinium thiocyanate reagent and once with 2 ml of water. The tubes were inverted and 2 ml of guanidine hydrochloride solution was added. The solution was transferred to another tube with 23 ml of guanidine hydrochloride solution and its pH was adjusted to 5.0 with 1 M acetic acid. 12.5 ml of ethanol was then added and the mixture kept at -20 °C until the next day. The pellet was washed two times with cold ethanol: 2 M NaCl solution. The ethanol was drained and the pellet dissolved in 6 ml of elution buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.2% SDS). An aliquot (10 ul) was diluted to 1 ml and readings were taken at 280 and 260 nm.

Isolation of mRNA: Oligo(dt)-cellulose (0.32 g) was allowed to swell in binding buffer (elution buffer containing 0.5 M NaCl) for 1 hr, transferred to a column and washed with 30 ml of elution buffer. The absorbance of the effluent buffer was about 0.03 at 260 nm. The column was equilibrated with

binding buffer (30 ml). The solution of RNA in elution buffer was heated at 65 °C for 5 min, chilled in ice, mixed with an equal volume of elution buffer containing 1.0 M NaCl and loaded on the column. The effluent was again heated for 5 min at 65 °C, chilled and passed through the column. The effluent was once more passed through the column, but without heating at 65 °C. The column was washed with binding buffer (30 ml) till the effluent gave a minimum reading at 260 nm. The column was washed with 4 ml of elution buffer containing 0.1 M NaCl to remove any bound tRNA and then eluted with 8 ml of elution buffer. One milliliter fractions were read at 280 and 260 nm, and fractions containing mRNA were pooled (6 ml). The pooled fractions (6 ml) were mixed with 0.1 volume (0.6 ml) of 20% potassium acetate, pH 6.5, and 2.5 volumes (16.5 ml) of ethanol and kept overnight at -20 °C. The tubes were centrifuged at 100,000 x g_{max} for 15 min and the pellet was washed two times in the same way with cold 70 % ethanol. The alcohol was drained and the pellet was dried by lyophilization. The pellet was dissolved in a suitable aliquot of water and kept at -70 °C. The prepared mRNA had A260/A280 ratio of greater than 2.0.

In vitro translation of mRNA: Cell free synthesis was performed using the rabbit reticulocyte in vitro translation system according to the instructions supplied by the manufacturers (Bethesda Research Laboratories, Gaithersburg, MD). The total volume of the reaction mixture was 30 μl and 30 μCi of [^{35}S]-methionine (1200 Ci/mmole, Amersham Corporation, Arlington Heights, IL) was used for labelling the synthesized proteins. The samples were incubated for 60 to 90 minutes. Ten microliters of membrane mix (buffer containing canine pancreatic microsomal membranes) or blank mix (buffer used for suspending microsomal membranes) was also added to the above reaction mixture when translation was performed in the presence of canine pancreatic microsomal membranes. The reaction was stopped by adding 3 μl of RNAse per 30 μl of reaction volume. In certain experiments, the translation product was incubated with $CaCl_2$ (2 mM) and trypsin (0.5 mg/ml) for 1 hr at 0 °C in the presence or absence of 1% Triton X-100. An aliquot (2 μl) of the sample was added to 0.5 ml of 1 M sodium hydroxide solution containing 5 % v/v of 30 % hydrogen peroxide and incubated at 37 °C for 10 min. They were then placed in ice and 2 ml of ice cold 25 % TCA containing 2 % casein hydrolysate was added and left in ice bath for 30 min. The precipitates were filtered through millipore filters (0.45 μm, Millipore Corporation, Bedford, MA). The filters were washed with two 3 ml aliquots, dried and counted in hydrofluor (National Diagnostics, Manville, NJ).

Labeling of lung tissue: The rabbits were killed by intravenous injection of 3 ml of pentobarbital solution and lungs were kept in cold HEPES-BSS (150 mM NaCl, 5 mM KCl, 4 mM KH_2PO4, 5.5 mM glucose, 25 HEPES, pH 7.4). One millimeter thick slices (600 mg) of lungs were incubated for 1 hr in 2 ml of methionine-free Ham's F12 medium at 37 °C under 95 % O_2 and 5 % CO_2 in a Dubnoff metabolic shaker (Fisher Scientific, Springfield, NJ). The medium was removed and 1.5 ml of fresh methionine free medium and 2 mCi of [^{35}S]-methionine (1200 Ci/mmol, Amersham Corporation, Arlington Heights, IL) were added to the slices. The incubation was continued for 2 hr as above. The medium was separated and slices were homogenized in 0.25 M sucrose with Potter Elvehjem homogenizer (12 passes of pestle). The homogenate was centrifuged at 100,000 xg for 1 hr to get the soluble fraction and the tissue residue. The tissue was again suspended in 3 ml of 0.25 M sucrose solution. The medium was centrifuged at 580xg for 10 min. Two microliter each of PMSF (0.5 M in DMF), TLCK (0.5 M in DMF) and pepstatin A (1 mg/ml in ethanol) were added per milliliter of all the fractions, and kept at -70 °C.

Immunoprecipitation of CCSP and SDS-PAGE: An aliquot of the translation products was made to 0.5 ml with water and then 0.5 ml of double strength lysis buffer and 1 μl of anti-CCSP serum were added. The lysis buffer contained 30 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1 % Triton X-100, 0.1 % SDS, 0.5 % sodium deoxycholate, 0.2% NaN_3 and 0.5% Nonidet P-40. The solution was incubated at 4 °C overnight followed by the addition of 0.2 ml (packed volume) of rabbit anti-goat IgG bound to agarose. The mixture was again mixed overnight at 4 °C on a vertically rotating platform and

centrifuged to sediment the immunoprecipitate bound to agarose. A slightly different protocol was used for immunoprecipitating CCSP from labelled lung slices (the residual tissue was first dissolved in 1 % SDS and then used for immunoprecipitation). However, the final concentration of SDS in the immunoprecipitation mixture was the same as that in normal strength lysis buffer. The sample from labeled lung slices was first cleaned by immunoprecipitating with 5 μl of non-immunized goat serum and 80 μl of protein A-agarose (50% suspension) overnight. The solution was centrifuged and the supernatant again immunoprecipitated with 5 μl of anti-CCSP serum and 50 μl of protein A-agarose (50 % suspension). The immunoprecipitate was washed six times with lysis buffer with 5 min of mixing each time. The sample was heated with SDS sample buffer for 3 min and used for SDS-PAGE (15). The gels were treated with En3hance solution (1 hr), 1 % glycerol (1 hr), and dried for fluorography.

Preparation of antiserum: Low molecular weight Clara cell secretory protein was purified from lavage effluents as described elsewhere (5). The purified protein showed a single major band on Coomassie staining and was more than 95 % pure on scanning by densitometer. The protein solution in water (500 μg) was sonicated with an equal volume of complete Freund's adjuvant (Difco Labs., Detroit, Mich.) and intramuscularly injected into the haunches and shoulders of a goat. Three injections were repeated at one month intervals with 250 μg of protein in incomplete Freund's adjuvant. The goat was bled 10 days after the last injection and serum was separated.

RESULTS

Specificity of Anti-CCSP Serum: Antiserum developed in goats against purified CCSP was twice absorbed with liver acetone powders. The reaction of this antiserum with Clara cell proteins, pulmonary lavage effluents and purified CCSP is shown in Figure 1. The antiserum reacted only with the low molecular weight protein present in Clara cells and pulmonary lavage effluents. This antiserum was used in all of the immunoprecipitations.

Translation of mRNA: Immunoprecipitation of in vitro translation product of mRNA isolated from rabbit lungs resulted in the identification of nascent synthesized CCSP (Figure 2). Lane 1 shows total proteins synthesized, and lane 2 shows the nascent CCSP synthesized and immunoprecipitated by anti-CCSP serum. The specificity of immunoprecipitation is documented in lanes 3 and 4. Lane 3 shows the results of immunoprecipitation in the presence of 1 μg of purified CCSP. It is evident that pure protein is able to compete with the nascent synthesized CCSP and thus markedly reduce the intensity of the labeled band. Immunoprecipitation using non-immunized goat serum did not yield CCSP (lane 4). The newly synthesized protein was digested by trypsin and did not appear on immunoprecipitation (lane 5). Lane 6 shows the molecular weight of CCSP purified from lavage effluents. Molecular weight standards indicated that the nascent synthesized protein was about 1 kd larger than the secreted protein.

Translation of mRNA in the Presence of Microsomal Membranes: When translation of mRNA by the rabbit reticulocyte lysate was performed in the in the presence of canine pancreatic microsomal membranes, two proteins were immunoprecipi-

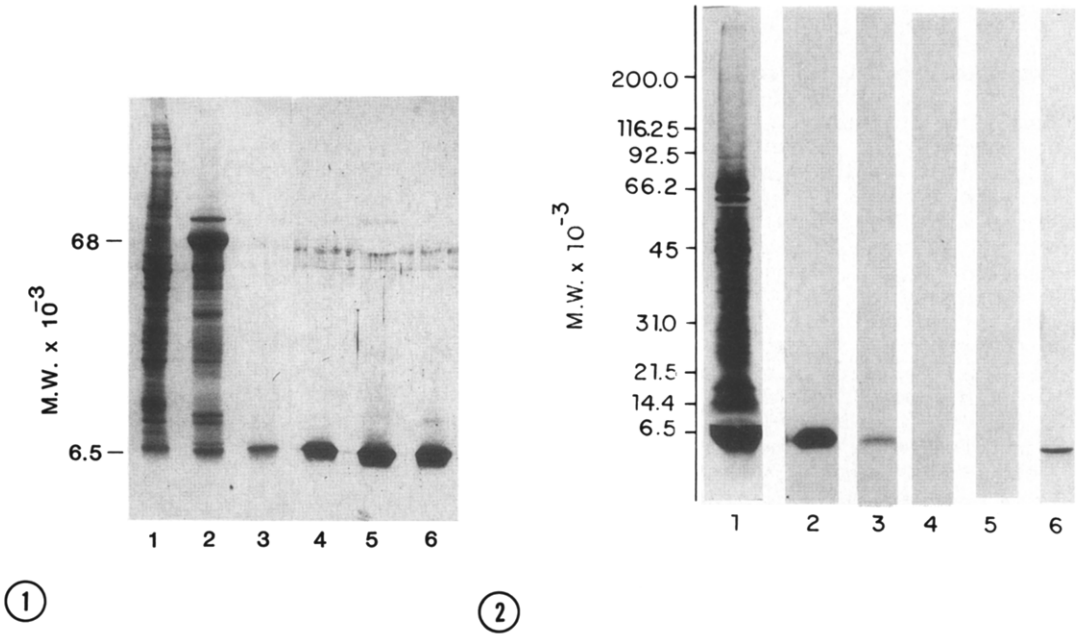

Figure 1. Immunoblots using antiserum developed in a goat against purified CCSP. SDS-gels prepared as described under Materials and Methods. Lane 1, proteins from Clara cells (87.4% pure) isolated from rabbit lungs (134 μg protein). Lane 2, lavage effluent proteins from the lungs of rabbits (35 μg protein). Lane 3, CCSP purified from pulmonary lavage effluents (3 ug protein). Lane 4, immunoblot with Clara cell proteins (same as lane 1). Lane 5, immunoblot using pulmonary lavage effluent proteins (same as lane 2). Lane 6, immunoblot with purified CCSP (3 μg). Antiserum absorbed twice with rabbit liver acetone powders. Primary antiserum diluted 1:100, followed by rabbit antigoat IgG (whole molecule) (diluted 1:50), followed by peroxidase antiperoxidase (developed in goat)(diluted 1:100). Lanes 1-3 stained with Coomassie stain.

Figure 2. Cell free translation products in the absence of microsomal vesicles. Protein was synthesized using about 2 μg of mRNA in a rabbit reticulocyte lysate system. [^{35}S]-Methionine (30 μCi) was used for labeling the protein and incubation was performed for 1 hr. An aliquot of the reaction mixture was subjected to SDS-PAGE under reducing conditions. Lane 1; total proteins synthesized. Lane 2; CCSP immunoprecipitated by anti-CCSP serum. Lane 3; immunoprecipitation of CCSP in the presence of 1 μg of pure protein. Lane 4; immunoprecipitation with non-immunized goat serum. Lane 5.; digestion of the protein by trypsin (10 μg) for 1 hr at 0 °C before immunoprecipitation by anti-CCSP serum. Lane 6; pure CCSP from lavage effluents. Lanes 1 through 5 are fluorograms and 6 is Coomassie stained.

tated (Figure 3, lane 2). Immunoprecipitation did not occur when nonimmune serum was used in the place of antiserum (lane 5) and immunoprecipitation of both proteins was inhibited by adding 1 and 5 ug of pure protein during immunoprecipitation (lanes 3 and 4). If microsomes were added to the translation system after 90 min when most of the cell free synthesis was over, and the reaction continued for another 90 min after adding microsomes, most of the nascent synthesized protein did not convert into secreted protein (lane 7). The molecular weight of the larger protein was the same as that obtained when translation was performed in the absence of microsomes (lane 6). The

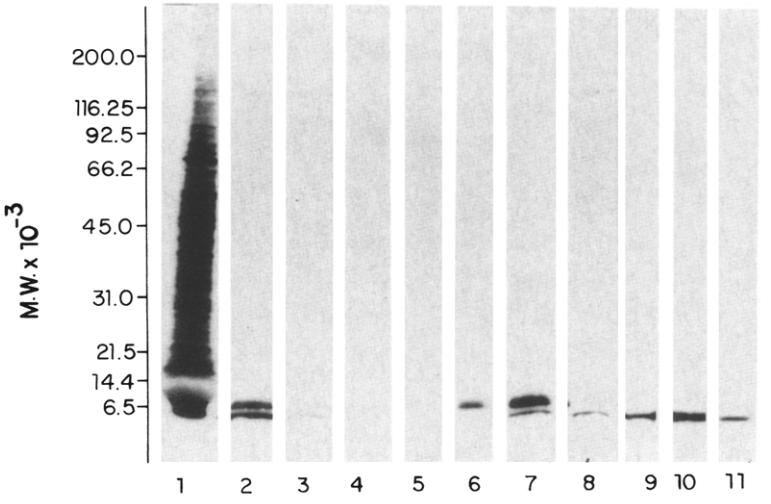

Figure 3. Cell free translation in the presence of microsomal vesicles. Synthesis of protein was performed for 1.5 hr in the presence of 10 μl of microsomal vesicles as described in legend to Figure 1. Lane 1, total proteins synthesized. Lane 2, CCSP immunoprecipitated from total proteins using anti-CCSP serum. Lane 3, immunoprecipitation in the presence of 1 ug of pure protein. Lane 4, immunoprecipitation in the presence of 5 ug of pure protein. Lane 5, immunoprecipitation using non-immunized goat serum. Lane 6, synthesis of protein in absence of microsomal vesicles (blank contained only buffer in place of microsomal vesicles) followed by immunoprecipitation by anti-CCSP. Lane 7, microsomal vesicles were added to the reaction mixture 1.5 hr after initiation of reaction by mRNA and the reaction further continued for 1.5 hr. Then CCSP was immunoprecipitated as in lane 2. Lane 8, digestion of the reaction mixture used in lane 7 by trypsin (10 μg) before immunoprecipitation. Lane 9, digestion of the reaction mixture used in lane 2 by trypsin before immunoprecipitation. Lane 10, digestion of the reaction mixture used in lane 2 by trypsin in the presence of 1% Triton X-100 followed by immunoprecipitation. Lane 11, pure CCSP isolated from lavage effluents. Lanes 1 through 10 are the fluorograms and 11 is the Coomassie stained CCSP.

molecular weight of the smaller protein was the same as that of the secreted protein (lane 11).

Digestion of the two proteins obtained following translation in the presence of microsomes indicated that only the higher molecular weight product was susceptible to trypsin. The lower molecular weight protein was not susceptible to trypsin (Figure 3, lanes 8, 9, and 10). Similarly, the secreted protein was highly resistant to the action of trypsin as shown in Figure 4. Under similar conditions albumin was completely digested.

CCSP Synthesis by Lung Slices: Synthesis of CCSP was also performed using lung slices and compared with the purified protein after immunoprecipitation. Proteins released from the tissue during the incubation period were separated from the tissue (Figure 5, lane 3). The tissue was homogenized and the insoluble residue (lane 1) separated from the soluble phase (lane 2). Proteins obtained on immunoprecipitation from the tissue residue, soluble phase, and secretions are shown in lanes 4, 5 and 6, respectively. The CCSP bands (5.8 kd) seen in lanes 4, 5 and 6 are nearly invisible in lanes 1, 2 and 3 because they are present in only small amounts. Immunoprecipitation performed in the presence of 20 μg of purified CCSP resulted in loss of radiolabelling in the

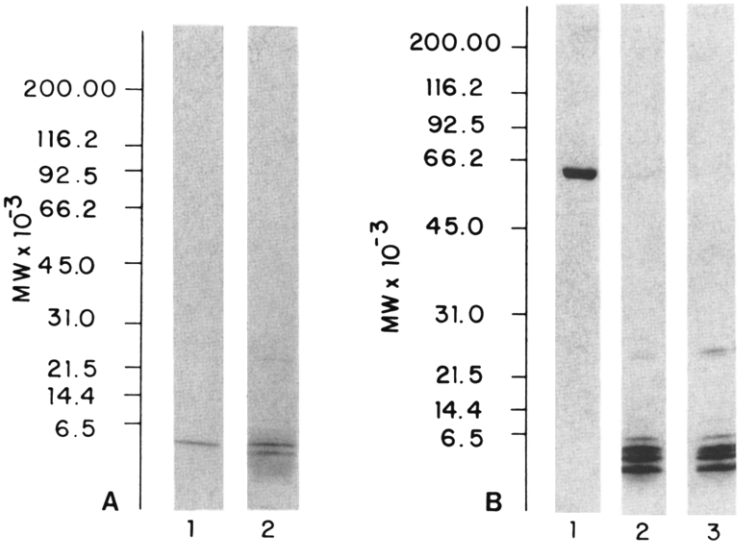

Figure 4. Digestion of CCSP with trypsin. A. CCSP isolated from lavage effluents (1 μg) was incubated with trypsin (0.5 mg/ml) and $CaCl_2$ (2 mM) in a total volume of 20 μl for 1 hr at 0 °C. The reaction products were subjected to SDS-PAGE and Coomassie staining. Lane 1, pure CCSP. Lane 2, CCSP after digestion with trypsin (10 μg). B. The bovine serum albumin (5 μg) was incubated with trypsin and $CaCl_2$ in a total volume of 40 μl as above. Lane 1, bovine serum albumin. Lane 2, bovine serum albumin after treatment with trypsin (20 μg). Lane 3, trypsin only (20 μg).

immune precipitate (lanes 7,8, and 9). The other bands at about 55 kd were non-specific bands as they did not disappear on adding pure CCSP during immunoprecipitation. The CCSP obtained on immunoprecipitation from lung slices, secretions, cell free synthesis in the presence of microsomes (the lower molecular weight protein), and purified from lavage effluents showed the same molecular weight and specificity of immunoprecipitation.

The nonspecific reaction of antiserum with the 55 kD protein was further examined. A similar 55 kD band was obtained when the immune serum was replaced with nonimmune serum. This was not due to simply absorption because washing the immune precipitate with detergent did not reduce the intensity of the radioactive band. We think that this protein may be IgG synthesized in the lung slices and consistent with this idea is that rabbit IgG under the same conditions resulted in a similar band with molecular weight of 55 kD (data not shown).

DISCUSSION

Most secretory proteins have an NH2-terminal extension of 15 to 30 amino acids that are rich in hydrophobic residues (16) and this NH2-terminal extension has been called the "signal sequence" (17). The difference between the molecular weights of nascent synthesized protein and secreted protein

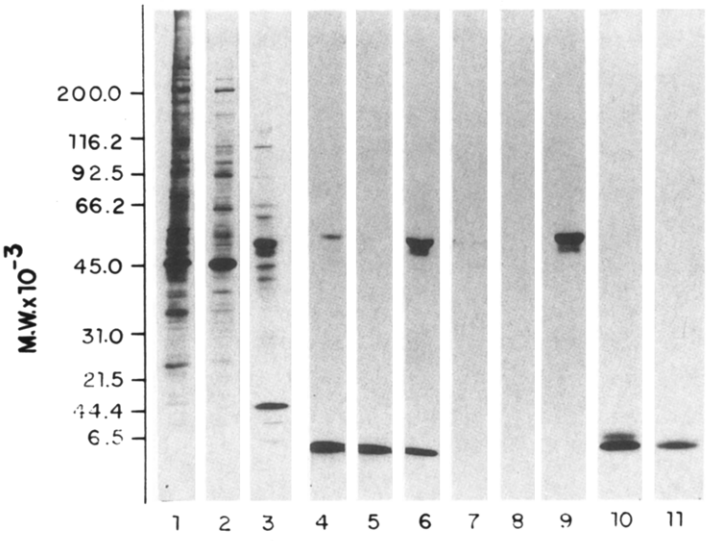

Figure 5. Synthesis of CCSP by lung slices and comparison with translated and isolated protein. Lung slices (600 mg) were incubated with 2 mCi of [^{35}S]-methionine in methionine-free Ham's F12 medium for 2 hr. The residual tissue, soluble fraction and medium were used for immunoprecipitation using anti-CCSP. See Materials and Methods for details. Lane 1, total radiolabeled proteins present in the lung tissue. Lane 2, total proteins present in the soluble fraction. Lane 3, total proteins secreted into the medium. Lanes 4, 5 and 6, CCSP from tissue, soluble fraction and medium, respectively, immunoprecipitated by anti-CCSP serum. Lanes 7, 8 and 9, immunoprecipitation as in lanes 4, 5 and 6 in the presence of 20 μg of pure LMW protein. Lane 10, translation in presence of microsomal vesicles followed by immunoprecipitation by anti-CCSP serum. Lane 11, pure isolated CCSP. Lanes 1 through 10 are fluorograms and 11 has been stained with Coomassie Brilliant Blue R 250.

reflects post translational modifications of the protein prior to or during secretion (18-20).

We have shown that the major secretory protein of pulmonary Clara cells is synthesized with a molecular weight approximately 1 kd higher than that of the secreted protein. This primary translation product appears to contain a signal peptide that is cleaved when translation is performed in the presence of microsomes. These findings are consistent with the signal peptide hypothesis of Blobel and Dobberstein (17). Secretory proteins are synthesized with signal sequences that direct proteins into the cisternae of the endoplasmic reticulum. The finding of a signal peptide is consistent with our contention regarding the secretory nature of CCSP.

The antiserum used in this study was developed against a low molecular weight protein isolated from the pulmonary extracellular lining of the lungs of rabbits (5). We showed that this protein is immunochemically similar to that synthesized by Clara cells (5) and it appears to be the major secretory product of those cells (4). In addition, we have shown that this low molecular weight protein found in the pulmonary extracellular lining is immunochemically similar to that secreted by lung slices.

We have demonstrated previously that the Clara cell secretory protein is resistant to digestion with trypsin and that complex formation of CCSP with trypsin did not occur (5). In contrast, the primary translation product appears to be digestable by trypsin. Quite possibly a transformation in tertiary structure has occurred during the passage of the protein through the endoplasmic reticulum and that this transformation has rendered the protein resistant to trypsin. This lability of nascent synthesized CCSP is also consistent with the signal peptide hypothesis (17) where it has been proposed that under conditions where availability of ribosome binding sites in the membrane may be limiting and in the case that synthesis might continue on free ribosomes, the completed peptide may be liberated into the cytosol and then degraded. The degradation occurs probably because nascent protein is not able to acquire native structure through enzymatic modification and cleavage of signal peptide.

In summary, cell free synthesis of CCSP indicates that this protein is not a proteolytic product of some high molecular weight membrane protein. The presence of a signal sequence attached to the CCSP supports the secretory nature of this protein, as well as that of Clara cells. The trypsin resistance of this protein is probably acquired during its passage into the lumen of the endoplasmic reticulum.

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