1983

A Transmembrane Precursor of Secretory Component

Keith Elliot Mostov

A Transmembrane Precursor of Secretory Component

A thesis submitted to the faculty of

The Rockefeller University

in partial fulfillment of the requirements

of the degree of Doctor of Philosophy

by

Keith E. Mostov

March, 1983

The Rockefeller University

New York, New York

Table of Contents

		page
List of Figures		v
List of Abbreviations		vii
Acknowledgments		viii
Abstract		1
Chapter 1	Introduction	3
Chapter 2	Experimental Procedures	9
	2.1 Immunological Reagents	9
	2.2 Cell-free Synthesis of Rabbit SC	10
	2.3 Synthesis of SC by HT29.E10 Cells	13
	2.4 Recombinant DNA Methods	16
	2.5 Materials	19
Chapter 3	Cell-free synthesis of Rabbit SC	20
	3.1 Introduction	20
	3.2 Results	20
	3.3 Discussion	25
Chapter 4	Synthesis of Human SC by HT29.E10 Cells	38
	4.1 Introduction	38
	4.2 Results	39
	4.3 Discussion	45
Chapter 5	Cloning and Sequencing of cDNA of Rabbit SC mRNA	56
	5.1 Introduction	56
	5.2 Results	56
	5.3 Discussion	59
Chapter 6	Conclusions	73

page

References 81

List of Figures

		page
1	Pathway of SC and pIg in Epithelial Cell	8
2	Cell-free Synthesis and Transmembrane Integration of Rabbit Mammary SC	29
3	Cell-free Synthesis and Transmembrane Integration of Rabbit Liver SC	31
4	Partial Fractionation of mRNA Coding for Rabbit SC	33
5	Core glycosylation of Cell-free Synthesized SC	35
6	Binding of Cell-free Synthesized Rabbit SC to Human IgA	37
7	Comparison of Human SC synthesized *in vitro* and *in vivo* by HT29.E10 Cells	49
8	Pulse-chase Analysis of SC Synthesized by HT29.E10 Cells	51
9	Transmembrane Orientation of SC Precursor Synthesized by HT29.E10 Cells	53
10	Partial NH_2-terminal Sequence Analysis of Various Forms of SC synthesized by HT29.E10 Cells	55
11	Translation of mRNAs Selected by Hybridization to p32.23 DNA	62
12	Analysis of Rabbit SC mRNAs	64
13	Restriction Map and Sequencing Strategy of cDNA inserted in p32.23.	66
14	DNA Sequence of p32.23 cDNA Insert and Amino Acid Sequence of COOH-terminus of SC Precursor	68
15	Partial Amino Acid Sequence Analysis of Cell-free Synthesized Rabbit SC	70

16 Relationship of SC mRNA, cDNA Inserted in p32.23, and SC Precursor 72

17 Comparison of Cytoplasmic Tails of Selected Proteins Presumed to Span the Plasma Membrane Once 80

List of Abbreviations

cDNA	complementary DNA
Con A-Sepharose	Concanavalin A-Sepharose
EDTA	ethylenediamine-tetra-acetic acid
IgA-Sepharose	human IgA-Sepharose
MEM	minimal essential medium
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pIg	polymeric IgA and IgM
RER	rough endoplasmic reticulum
SC	secretory component
SDS	sodium dodecyl sulfate

Acknowledgments

The work described in this thesis has been carried out in the laboratory of Günter Blobel. I thank him for this priceless opportunity and incomparable education.

Many other individuals contributed to my experience at Rockefeller. Vishwanath Lingappa provided my initial training in this laboratory and also sparked my interest in the secretory component problem. Mike McCune provided me with purified IgA as well as the benefit of his vast knowledge of immunology. Charlotte Cunningham-Rundles generously gave me secretory IgA. Jean-Pierre Kraehenbuhl provided purified rabbit SC, antiserum to this SC, and rabbit mammary tissue. Bruce Cunningham, John Hemperly, and Beth Weiner helped with the protein sequencing. Marion Boudreau made many of the polyacrylamide gels. Jim Kaput, Jeff Boeke, Sherry Goltz, Paul Fisher, Ken Krauter, and Andy Hiatt gave useful advice on recombinant DNA technology. I thank Marty Friedlander for his recent help in the work on the cDNA cloning and sequencing and for his patience as I wrote this dissertation. I also thank all of the members of the Blobel lab, past and present, for providing a stimulating environment.

Finally, I thank my family and my friends for their support.

Abstract

Secretory component (SC), a glycoprotein associated with polymeric IgA and IgM (pIg) in external secretions, is produced by certain epithelial cells and is thought to be the receptor mediating the transcellular transport of pIg. We studied the biosynthesis and processing of rabbit and human SC. Using translation of mRNA from rabbit mammary gland and liver in a cell-free system supplemented with dog pancreas microsomal vesicles, we discovered that the translation products of rabbit SC include at least four polypeptides. Moreover, we found that all four polypeptides are synthesized not as soluble secretory forms, but as larger transmembrane forms that are core glycosylated and asymmetrically integrated into the dog pancreas microsomal vesicles with an 11-15 kilodalton domain remaining in the cytoplasm. We studied the biosynthesis and processing of human SC in a cloned cell line (HT29.E10) derived from a colon adenocarcinoma. In both cell-free translations and pulse labelling of cells, SC is made as a single larger precursor with an approximately 15 kilodalton cytoplasmic domain. In pulse-chase experiments, the carbohydrate moieties of the precursor are first converted to the complex type and the precursor is then proteolytically cleaved to a form slightly larger than SC isolated from colostrum. This cleaved form is slowly released from the cell. Partial NH_2-terminal sequencing indicates that the cleaved form of SC is derived from the NH_2-terminal, ectoplasmic (non-cytoplasmic) domain of the precursor.

To determine the structure of the cytoplasmic and membrane spanning portions of the SC precursor, we cloned and sequenced DNA complementary to 1563 nucleotides at the 3' end of rabbit SC mRNA, and deduced the corresponding sequence of the COOH-terminal 163 amino acid residues of the SC precursor. The SC precursor has a putative membrane spanning segment of

23 non-charged amino acid residues, followed by a cytoplasmic tail of 103 amino acid residues with a preponderance of charged and hydrophilic residues.

1. Introduction

Polymeric IgA and IgM (pIg) are specifically taken up by a variety of glandular epithelial cells and transported across the cell into external secretions, such as milk, bile, and intestinal fluid, where they form the first immunological defense against infection (1). During this transport, the epithelial cell adds an extra polypeptide, secretory component (SC) to the pIg (2, 3). It has long been supposed that SC somehow mediates the transcellular transport of pIg and, in fact, one of the earliest reports on SC called it "transport piece" (4).

Brandtzaeg first proposed that SC was the receptor for pIg at the basolateral surface of the epithelial cell (5). He found SC at this surface in intestinal epithelial cells (6) and this observation has been extended to epithelial cells in other tissues (7-9). pIg can bind to this basolateral (or in hepatocytes, sinusoidal) cell surface-associated SC (10-18). The SC-pIg complex formed there is then transported to the apical (or in hepatocytes, bile canalicular) surface where the complex is released into the secretion. The binding and transport of pIg can be blocked by adding either excess soluble SC or antibodies to SC (14-18). Antibodies to SC can even replace pIg and be transported into secretions (16).

On the basis of cytochemical, cell fractionation, and pIg uptake studies, the pathways of SC and pIg in the cell have been shown to be unusually complex (9, 15-23). SC is synthesized in the rough endoplasmic reticulum (RER), transported through the Golgi apparatus, and to the basolateral cell surface. Here it binds pIg, and the SC-pIg complex is endocytosed via clathrin coated pits. However, unlike most instances of receptor-mediated endocytosis (24, 25), the ligand is not degraded in lysosomes. Rather the SC-pIg complex is rapidly carried in vesicles across the cell, exocytosed

at the apical surface, and discharged into the secretion. This process of receptor-mediated transcytosis is an attractive model system for studying general aspects of protein traffic and sorting in the cell.

A second remarkable aspect of SC is its apparent dual character. SC functions first as a plasma membrane receptor for pIg. Normally such receptors are integral membrane proteins which are anchored in the lipid bilayer by a hydrophobic portion(s) and are generally not soluble in water in the absence of detergents. Yet SC isolated from secretions is hydrophilic and water-soluble.

One explanation, proposed by Kuhn and Kraehenbuhl, is that SC is secreted by cells and is bound to an unidentified membrane receptor on the cell surface (22, 26). SC would then act as a linker between this unidentified receptor and the pIg. In support of this model, they found high-affinity binding sites for SC on the surface of mammary cells. However, such sites were not found on liver cells and this was attributed to endogenous receptor occupancy.

As a result of our cell-free translation experiments (Chapter 3) we proposed (27) that SC was not made as a soluble, secreted protein, but rather as a transmembrane precursor (Fig. 1). When this precursor reaches the basolateral surface, a portion of the precursor, the ectoplasmic domain, is exposed on the outside of the cell and constitutes the ligand (pIg) binding domain. This domain is subsequently cleaved off from the remainder of the precursor and thus becomes the "mature" SC found in secretions. Cleavage probably occurs either in the endocytotic vesicle or after exocytosis. The cytoplasmic portion of the precursor may act as an effector domain and serve to guide the SC precursor through the cell. The ultimate fate of the membrane spanning and cytoplasmic portions of the precur-

sor after cleavage are unknown; presumably they are rapidly degraded.

Our model is similar to that first proposed by Brandtzaeg (5), except that it distinguishes between the transmembrane precursor of SC and the proteolytically generated, secreted form of SC.

Much of the work described in this dissertation has been directed towards testing predictions of this model. Three basic approaches were used. First, we studied the earliest steps in the biosynthesis of SC by cell-free translations of rabbit SC mRNA (27). Mature rabbit SC consists of a group of several polypeptides and we found four primary translation products. More importantly, we were able to show in a cell-free coupled translation and membrane insertion system that these SC precursors are transmembrane proteins, each of which have sizable (approximately 15 kilodaltons) cytoplasmic domains. As discussed in Chapter 6, this cytoplasmic domain may interact with other proteins in the cytoplasm and provide the information for guiding SC in its long pathway through the cell.

Second, we studied the complete process of biosynthesis, cleavage, and release of SC in a human colon cancer-derived cell line (28). These cells have been shown to transport pIg and SC in the "normal" manner (23). Humans have only one form of mature SC and we found one primary translation product. In pulse chase experiments, a core-glycosylated form in the RER is first observed. As in the rabbit, this form has a cytoplasmic domain of about 15 kilodaltons. After conversion of its carbohydrate moieties to the complex type, the precursor is proteolytically cleaved to a form which is slightly larger than authentic SC isolated from colostrum. This cleaved form is then slowly released from the cell. Direct protein sequencing indicated that mature SC is derived from the NH_2-terminal, ectoplasmic domain of the precursor.

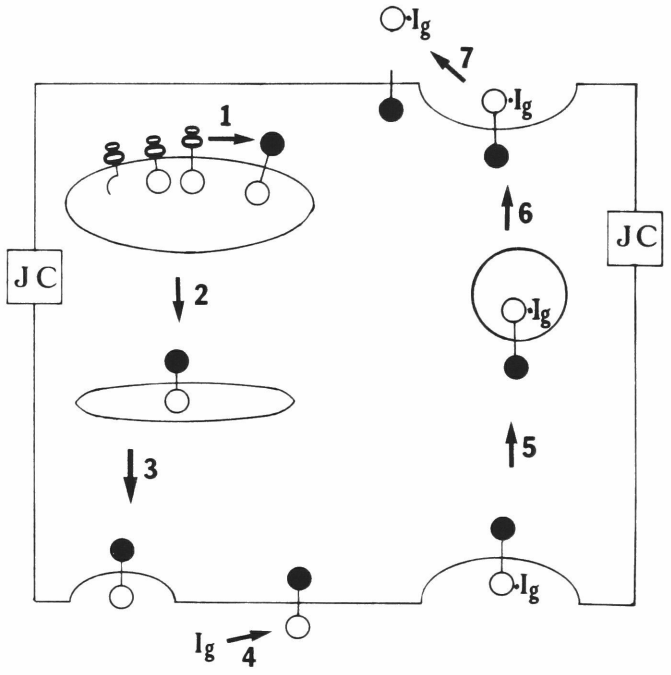
Finally, we investigated the primary structure of the cytoplasmic and membrane spanning portions of the SC precursor. As mentioned above, the cytoplasmic domain may contain the information for guiding the SC precursor through the cell. Determining its primary structure is therefore a first step towards elucidating the mechanisms involved in protein sorting in the cell. We cloned complementary DNA (cDNA) to SC mRNA and isolated a plasmid containing sequences corresponding to 1563 bases at the 3' end of SC mRNA. We sequenced the cDNA and found a 489 base open reading frame at the 5' end of this sequence which codes for 163 amino acids at the COOH-terminus of the SC precursor. The protein sequence contains a stretch of 23 non-polar amino acid residues which is likely to be a membrane spanning segment. The proposed cytoplasmic tail comprises 103 amino acid residues, with a preponderance of charged and hydrophilic residues.

Figure 1

Hypothetical model for the intracellular pathway of SC in a glandular cell.

The luminal or apical portion of the plasma membrane (upper half) is separated from the basolateral region of the plasma membrane (lower half) by the junctional complex (JC). In parenchymal liver cells, the bile canalicular region of the plasma membrane corresponds to the apical surface, and the sinusoidal region of the plasma membrane is equivalent to the basolateral region. SC is synthesized by membrane-bound polysomes as a transmembrane form(s) and is cotranslationally and asymmetrically integrated into the rough endoplasmic reticulum (step 1). A distinct ectoplasmic domain (open circle) is translocated into the rough endoplasmic reticulum lumen, another distinct cytoplasmic domain (solid circle) remains untranslocated, and a membrane-spanning segment(s) (bar) links the two domains. After transport to the Golgi complex (step 2), and subsequently to the basolateral region of the plasma membrane (step 3), the ectoplasmic domain of the SC binds to pIg (step 4). The transmembrane SC-pIg complex is then removed from the basolateral region of the plasma membrane via endocytosis (step 5). The endocytotic vesicle traverses the cell and fuses with the apical region of the plasma membrane (step 6). The ectoplasmic domain of transmembrane SC is endoproteolytically cleaved (step 7), most likely either in the endocytotic vesicle or at the apical surface, generating a soluble SC-pIg complex and a membrane bound remainder of transmembrane SC that includes the cytoplasmic domain and transmembrane segments(s). Various different intermediate transmembrane forms of SC resulting from sequential proteolytic or other modifications during its intracellular pathway may exist. Our model is similar to that proposed by

Brandtzaeg (5) except that it distinguishes between the larger, intracellular transmembrane form(s) of SC and the smaller, proteolytically generated, secreted form(s) of SC.



2 Experimental Procedures

2.1 Immunological Reagents

SC purified from rabbit milk, as well as goat antiserum to this SC, were the gift of J.-P. Kraehenbuhl (22). Goat antiserum to rabbit secretory IgA was purchased from Cappel Laboratories, Dowington, PA. and immunoprecipitated the same SC primary translation products as the antiserum provided by Kraehenbuhl.

Human colostral secretory IgA was obtained initially as a generous gift from Dr. C. Cunningham-Rundles, Sloan Kettering Institute for Cancer Research, and was later purchased from Sigma, St. Louis, Mo., and Cappel Laboratories,

To purify human SC for use as an immunogen, 50 mg of human colostral secretory IgA (Sigma) was dissolved in 10 ml of 70% formic acid. 250 mg CNBr was added, and the sample digested for two hr at room temperature. Since human SC contains no methionine (29), it was not attacked by the CNBr, while contaminating methionine containing proteins were digested to smaller fragments. The reaction was diluted with 10 volumes of H_2O, lyophilized, and the products subjected to preparative sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 5-10% polyacrylamide gradient slab gels (30). The gels were stained with Coomassie blue for five min and the SC band was cut out. One-fifth of the total gel slice was homogenized with 4 ml H_2O and 6 ml complete Freund's adjuvant. The homogenate was injected subcutaneously into 15 sites on the back of a female New Zealand white rabbit. Booster injections using the same amount of antigen in incomplete Freund's adjuvant were given 4 and 6 weeks later. The rabbit was bled 2 weeks after the second boost.

Affinity purified antibodies to human SC were used to immunoprecipitate SC precursor synthesized in the cell-free translation system (see below). SC was purified by gel electrophoresis as described above and electroeluted from the gel. Approximately 2 mg SC was coupled to CNBr-Sepharose according to the manufacturer's instructions. The serum was passed over the column and specific antibodies were eluted with 0.1 M glycine-HCl, pH 2.3, and immediately adjusted to pH 7.5 with Tris base. Commercial rabbit anti-human SC antisera were from Dako (Accurate Chemicals, Hicksville, N.Y.); Boehringer Mannheim, Indianapolis, Ind.; Atlantic Antibodies, Scarborough, Me.; and Behring Diagnostics, Sommerville, N.J. All of these commercial antisera recognized human SC, although their titers against SDS-denatured SC was lower than that of the antiserum prepared in this study.

2.2 Cell-Free Synthesis and Analysis of Rabbit SC.

Cell-free translation of SC. Total cellular RNA was extracted by the SDS/proteinase K/phenol/chloroform/isoamyl alcohol method (31) from rabbit liver and lactating mammary gland. Two New Zealand White rabbits were used. Rabbit A (mammary gland only) was from Cunicentre (Jongny, Switzerland); the mammary gland was shipped frozen to New York. Rabbit B (mammary gland and liver) was from Dutchland Laboratories, Denver, PA. In certain experiments mRNA was further purified by selection of poly (A)-containing strands on oligo(dT)-cellulose (32), and size fractionation on a sucrose gradient (33). The wheat germ extract used for cell-free translation experiments was prepared as described previously (34). RNA was included at a final concentration of 0.2 mg/ml. Translations were generally in a volume of 0.02 ml, unless otherwise specified. A radioactive amino acid, usually

[^{35}S]cysteine, was included at a final concentration of 1 mCi/ml.

Membrane Integration of SC. Dog pancreas microsomal vesicles were prepared as described (35). When specified, they were included in the cell-free translation reaction at a final concentration of 2 A_{260} units/ml. (1 A_{260} unit is that amount which has an absorbance of 1 at 260 nm when contained in a volume of 1 ml and measured with a pathlength of 1 cm.) In certain experiments, after the synthesis was completed, the mixture was adjusted to 2 mM in $CaCl_2$, using a 100 mM stock, and 0.1 volumes of 3 mg/ml trypsin was added. The reaction was incubated on ice for 1 hr.

Immunoprecipitation and Product Analysis. After these reaction were completed, SDS was added to 2% and the samples were heated to 100°C for 5 min, cooled, and diluted with 4 volumes of 2.5% Triton X-100/150 mM NaCl/20 mM triethanolamine-HCl, pH 8.1/5 mM EDTA/1% Trasylol. Antiserum to rabbit SC was added to a final concentration of 0.1 % and the samples were kept at 4°C for 16 hrs. Protein A-Sepharose was added to a concentration of 1% and the samples agitated for 1 hr. The beads were then washed with six 1.4 ml portions of 1% Triton X-100/0.2% SDS/150 mM NaCl/20 mM triethanolamine-HCl, pH 8.1/5 mM EDTA/0.1% Trasylol, and once with the same buffer lacking detergents. After removal of all of the final wash buffer, 0.02 ml of loading buffer (5% SDS/0.2M Tris base/1 M dithiothreitol/ 0.3 M sucrose/0.002% bromophenol blue) was added and the samples incubated at 100°C for 5 min. The samples were then analyzed by SDS-PAGE on gels containing a 5-10% linear gradient of polyacrylamide (30). Gels were generally impregnated with diphenyloxazole (36) and exposed to Kodak XAR-5 film at -70° for 5-60 days.

Binding of Cell-Free Synthesized SC to Con A-Sepharose. Cell-free translations (0.1 ml) were carried out in the presence of microsomal vesi-

cles. SDS was added to 2% and the sample incubated at 100°C for 5 min. The sample was then diluted with 4 volumes of 2.5% Triton X-100/150 mM NaCl/1 mM $MgCl_2$/20 mM triethanolamine-HCl, pH 7.5. Con A-Sepharose (0.2 ml of beads) was then added and the sample agitated at 22°C for 3 hrs. The beads were then washed with twelve 1.4 ml portions of 0.05% SDS/150 mM NaCl/20 mM triethanolamine-HCl, pH 7.5. Bound proteins were eluted by adding 0.1 ml of 1 % SDS and boiling for 5 min. The eluate was immunoprecipitated as described above. As a control for non-specific binding, the same experiment was performed, except that incubation with the beads was carried out in the presence of 0.4 M alpha-methyl mannoside.

Sequencing of Tryptic Peptides of Cell-Free Synthesized SC. A 1 ml cell-free synthesis was carried out, using [^{35}S]methionine as the labeled amino acid. The reaction was immunoprecipitated and products analyzed by SDS-PAGE. The gel was dried directly onto 3MM paper and exposed to Kodak XAR-5 film. The film was aligned with the gel by spotting radioactive ink on the gel before exposure. The M_r =90,000 band of precursor SC was excised with a razor blade. The band was incubated in 0.2 ml of 0.1% SDS for 2 days at 37°C. An equal volume of 0.2% Triton X-100 as well as 0.06 mg of trypsin were added and the incubation continued for 2 more days. The supernatant above the gel slice was then loaded directly into the reaction cup of a Beckman 890C sequenator and sequenced (37).

Binding of Cell-Free Synthesized SC Precursors to IgA-Sepharose. Purified polymeric, J chain-containing, human myeloma IgA was kindly provided by J. M. McCune, Rockefeller Univ. This IgA was coupled to CNBr-Sepharose according to the manufacturer's directions, using a final concentration of 1 mg protein/g Sepharose. Translations (0.1 ml) were performed in the presence of microsomal membranes. After translation was completed,

the membranes were recovered from the translation mixture by layering the mixture in a Beckman Airfuge tube over a two-step sucrose gradient consisting of 0.08 ml of 0.5 M sucrose and 0.025 ml of 2 M sucrose. Both sucrose solutions also contained 150 mM NaCl and 20 mM Na phosphate (pH 6.5). After centrifugation for 15 min at 160,000 x g, the membranes were recovered at the 0.5 M/2 M interface in a volume of 0.025 ml. They were diluted to 0.2 ml with 150 mM NaCl/20 mM Na phosphate, pH 6.5, and adjusted to 1 % in Triton X-100. One per cent of this material was analyzed directly by SDS-PAGE and fluorography. To the remaining 99% of the material, 0.02 ml of IgA-Sepharose was added and the samples agitated at 4°C for 16 hrs. The beads were washed five times wih 1.2 ml of 1% Triton X-100/150 mM NaCl/20mM Na phosphate, pH 6.5. Bound proteins were eluted by boiling with loading buffer, and the samples analyzed by SDS-PAGE and fluorography.

2.3 Synthesis of SC by the Cell Line, HT29.

Cell culture. The cell line HT29, originally derived from a human colon adenocarcinoma, was obtained from Dr. J. Fogh, Sloan Kettering Institute for Cancer Research. Cells were maintained as previously described (38) in McCoy's 5a medium, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 ug/ml streptomycin, and 2.5 ug/ml fungizone.

Cell-free translation. Cells from eight 100 mm dishes were scraped off with a rubber policeman in a minimal volume of phosphate-buffered saline (PBS, 150 mM NaCl/20 mM Na phosphate, pH 7.4). After a brief centrifugation, the pellet was frozen and kept at -80° until used. Total RNA was prepared as described above for rabbit tissues. It was translated in the wheat germ cell-free system in a volume of 0.1 ml. In indicated experiments, dog pancreas microsomes were included in the translation and the

reaction scaled up to 0.4 ml; in some cases the translation products were post-translationally digested with trypsin. Translation products were immunopreciptated with affinity purified antiserum to human SC, and analyzed by SDS-PAGE and fluorography.

Pulse-chase experiments. Cells in 35 mm dishes were rinsed twice with PBS. Cells were starved for 20 min with minimal essential media (MEM) made without cysteine and supplemented with 10% dialysed fetal bovine serum. This was replaced with 0.3 ml of the same media containing 0.05 mCi [^{35}S]cysteine and the cells were pulsed for various periods. In chase experiments the labelling media was then removed, the cells washed with PBS, and 0.8 ml McCoy's 5a medium supplemented with serum and antibiotics (which contains a >1000 fold excess of cold cysteine) was added. At various time points the cells and media were havested by adding 0.5 ml of 0.5% SDS/150 mM NaCl/20 mM triethanolamine-HCl, pH 8.1. The cells were scraped off of the plate using a rubber policeman, and the lysate transferred to a 1.5 ml centrifuge tube. The tube was placed in a boiling water bath for 5 min. Lysis of the cells with SDS, followed immediately by boiling, was used to prevent artifactual proteolysis (32). After cooling, the sample was sonicated for 20 sec with a Branson sonicator at a power setting of 4, using a microprobe. The sample was then diluted with an equal volume of Triton dilution buffer. Immunoprecipitation was then carried out as described as above, using 4 ul of anti-SC per 35 mm dish. In some experiments, immunoprecipitated products were treated with endoglycosidase H prior to electrophoresis (40,41).

Proteolytic digestion of cell homogenate. A 60 mm dish of cells was pulse-labeled for 30 min. The media was removed and the monolayer washed 3 times with ice-cold 0.25 M sucrose/5 mM N-2-hydroxyethylpiperazine-N'-2-

ethanesulfonic acid (pH adjusted to 7.4 with KOH). The monolayer was scraped off in 0.5 ml of this buffer with a rubber policeman and transferred to a 1 ml Dounce homogenizer (Wheaton Glass) with a tight fitting ("A") pestle. After 20 strokes on ice the homogenate was divided into 0.1 ml aliquots. Certain samples received pronase (final concentration 0.02 mg/ml) and/or Triton X-100 (final concentration 0.4%). Samples were incubated on ice for 1 hr. Reactions were terminated by boiling for 5 min, followed by solubilization with SDS (1% final concentration) and immunoprecipitation.

Partial amino-terminal sequence analysis of products. In each of the amino-terminal sequencing experiments, cells were grown in four to eight 60 mm dishes. Labelling for the isoleucine experiment was done with MEM made up without isoleucine; the proline experiments used MEM containing all essential amino acids. Each dish was labeled with 0.65 ml of media containing 0.25 mCi of tritiated amino acid for 30 min. In certain experiments, after the 30 min labelling, cells were chased for 24 hr. Where indicated, cells were homogenized after labelling and then digested with pronase. Products for sequencing were immunoprecipitated, separated by SDS-PAGE, eluted from the gel, and sequenced as previously described (37).

Cloning of HT29 cell line. In preliminary labelling experiments using the HT29 line, the amount of SC which could be immunoprecipitated from the cells was rather low. Since previous reports (11, 23, 38) indicated that not all of of the cells were positive for SC (as assayed by immunofluorescence) it seemed likely that the HT29 line was a mixed population of cells. We therefore isolated individual clones and screened them for production of SC. A single cell suspension was prepared by trypsinization, diluted to 1-2 cells/ml, and 0.1 ml aliquots were placed in the wells of three 98 well

microtiter plates. A mixture of fresh and conditioned McCoy's 5a medium was used. Wells were inspected to insure that none contained 2 or more cells. After 3 months, the colonies were transferred to 25 cm^2 flasks, and after a further 2-3 months a portion of the cells in each clone were transferred to 35 mm dishes. Twelve clones were isolated in this way. These were pulse-labeled with [^{35}S]cysteine and the SC precursor immunoprecipitated and analyzed as above. Interestingly, all 12 of the clones synthesized some SC precursor; however there was a considerable variation in the amount produced. Two of the clones produced equally large amounts of SC precursor, and one of these, HT29.E10, was selected for further study. It should be pointed out that in preliminary studies using the uncloned HT29 line, the same pulse-chase and translation results reported here were obtained, except that the amount of SC precursor synthesized was less.

2.4 Recombinant DNA Methods.

Construction of a cDNA library from partially purified SC mRNA. As described above, mRNA was prepared from rabbit lactating mammary gland and the mRNA was size selected on a sucrose gradient. Fractions enriched in mRNA coding for the M_r =90-93,000 SC polypeptides were identified by in vitro translation. The first and second strands of cDNA were synthesized by standard methods (42) with reverse transcriptase, using 5 ug of partially purified SC mRNA in an initial reaction volume of 100 ul. The hairpin loop was then cleaved with S1 nuclease (42). The double stranded cDNA was sized on a sucrose gradient and material sedimenting faster than approximately 500 base pairs was collected (42). Approximately 10-20 residues of dCTP were then added to the 3' ends using terminal deoxynucleotidyltransferase (42). The tailed molecules were annealed to PBR 322 which had been digest-

ed with Pst 1 and tailed with 10-20 dGMP residues. The molecules were then transformed into E. coli cells, strain MC1061 (42), and spread onto tetracycline containing plates. About 600 tetracycline resistant, ampicillin sensitive clones resulted.

Identification of clones coding for SC. Eighteen clones were grown in individual 250 ml cultures, amplified with chloramphenicol, and the plasmid DNAs extracted (43) and purified by banding on CsCl ethidium bromide density gradients (44). Hybrid selected translation was performed by the method of Ricciardi (45). Ten ug of plasmid DNA was cut with Eco R1 and bound to 1 cm diameter nitrocellulose filters. The filters were hybridized with 20 ug of total rabbit liver or mammary RNA. After extensive washing to remove unbound RNA, the specifically bound material was removed by boiling for 90 sec with 400 ul of H_2O. The eluted mRNA was ethanol precipitated with 5 ug of carrier wheat germ tRNA, and translated as previously described in the wheat germ cell-free translation system. The translation products were immunoprecipitated with antiserum to rabbit secretory IgA (which recognizes rabbit SC) and the products analyzed by SDS-PAGE and fluorography. One of these initial clones specifically selected mRNAs for all of the SC translation products. Its insert was cut out by digestion with Pst 1, purified by agarose gel electrophoresis (46), nick translated (47), and used as a probe in a colony hybridization screen of the remainder of the cDNA library (48). Twelve additional clones were thus identified, and the largest (p32.23) had an insert of approximately 1650 nucleotides, including poly(A) and poly(G)-poly(C) tails.

Agarose gel electrophoresis and nitrocellulose blot analysis of SC mRNAs. One ug of total poly(A) containing mRNA from rabbit liver and lactating mammary gland was fractionated on a 1% agarose gel containing 2.2 M

formaldehyde (49). Gels were stained with ethidium bromide to visualize the ribosomal RNA molecular weight markers. The separated mRNAs were then transferred to nitrocellulose, and the filters baked in a vacuum oven at 80°C for 2 hrs. The filters were prehybridized for 3 hrs at 42° C in 15 ml of 50 % formamide/1 M NaCl/50 mM Na phosphate (pH 7.0)/ 10 mM EDTA/ 0.5% SDS/ 0.2% bovine serum albumin/0.2 % ficoll/0.2 % polyvinyl pyrrolidone/ 1 mM Na_4 pyrrophosphate. One million cpm of nick-translated p32.23 was then added to the reaction, and the incubation continued for 16 hrs. The filters were then washed in three changes of 500 ml each of 50 mM NaCl/10 mM NaPi (pH 7.0) at 50°C over a period of 2 hrs, and then exposed for 2 weeks to Kodak XAR-5 film with a Dupont Lightning plus intensifying screen at -70°.

DNA sequencing. We used the method of randomly subcloning restriction fragments of the DNA into the phages M13 Mp8 and Mp9 and sequencing the resultant subclones by the dideoxy chain termination technique using a universal M13 sequencing primer (50-53). Sequencing gels were 40 cm long, 30 cm wide, 0.4 mm thick, and contained 5.5% acrylamide/0.275% bis-acrylamide/8M urea. Running buffer was 0.1 M boric acid/0.1 M Tris base/2 mM Na_2EDTA. Gels were run at 43 watts for 2-10 hrs, dried onto 3MM paper, and exposed to either Fuji RX or Kodak SB-5 film at room temperature. To sequence through the G-C tail at the 5' end of the molecule, we substituted reverse transcriptase for the Klenow fragment of DNA polymerase 1 (54). This method did not work on the G-C tail at the 3' end of the insert, and so 63 bases, or 4% of the the total 1563 bases, were sequenced in only one direction. DNA sequence analysis was by the computer programs of Staden (55), as implemented at the Rockefeller University computer facility.

Materials.

[^{35}S]methionine, [^{35}S]cysteine, [^{3}H]proline, and [^{3}H]isoleucine were the highest specific activities available from New England Nuclear. [^{3}H]dCTP (specific activity 25 Ci/mMole) and [^{32}P]alpha-dATP (specific activity 800 Ci/mMole) were also from New England Nuclear. McCoy's 5A and minimal essential media, fetal bovine serum, penicillin, streptomycin, fungizone and phosphate-buffered saline (PBS) were from Gibco, Grand Island, N.Y. 98-well microtitre plates were from Flow Laboratories, Mclean, Va. Streptomyces griseus endo-beta-N-acetylglucosaminidase H (endoglycosidase H, EC 3.2.1.96) was the kind gift of Dr. P. Robbins, MIT. Pronase was from Boehringer Mannheim. Trypsin, pretreated with L-(tosylamido-2-phenyl)ethyl chloromethyl ketone, was from Worthington. Trasylol (aprotinin) was from Mobay Chemical, New York. Restriction enzymes were either from New England Biolabs, Beverly, MA., or Bethesda Research Laboratories, Bethesda, MD., and were used according to the manufacturers' instructions. Avian myeloblastosis virus reverse transcriptase was provided by J. Beard, Life Sciences, Inc., St. Petersburg, FL. Aspergillus oryzae S1 nuclease and the 15 base oligonucleotide universal sequencing primer were from Bethesda Research Labs. Deoxynucleotides and dideoxynucleotides were from PL Biochemicals. E. coli DNA polymerase 1, as well as its Klenow fragment, were from Boehringer Mannheim, Indianapolis, IN. Terminal deoxynucleotidyltransferase, and Pst 1 cut and oligo-dG tailed plasmid PBR 322 were from New England Nuclear, Boston MA.. Nitrocellulose filters, type BA85, were from Schleicher and Schuell, Keene, NH. E. coli strain MC1061 was the kind gift of K. Knight, Univ. of Illinois Medical Center, Chicago, Il. Concanavalin A-Sepharose (Con A-Sepharose), protein A-Sepharose, and CNBr-Sepharose were from Pharmacia.

3. Cell Free Synthesis of Rabbit SC

3.1 Introduction.

As discussed in Chapter 1, SC appears to have a dual character. It serves first as a pIg receptor at the basolateral surface of the epithelial cell, and, like other receptors, could be expected to be an integral membrane protein. Later, when released into the lumen in association with pIg, it is a soluble, secretory protein.

Only these latter secreted forms of SC have been isolated and characterized from various secretions. The earlier biosynthetic forms of SC have not been identified. Because characterization of these forms of SC can be expected to hold the key for an understanding of its dual character and its complex intracellular pathway, we investigated the earliest steps in SC synthesis. Using translation of mRNA from rabbit mammary gland or liver in a cell-free system supplemented with dog pancreas microsomal membranes, we discovered that the translation products of SC include at least four distinct polypeptides. More importantly, these early biosynthetic forms of SC are not synthesized as secretory proteins but as considerably larger transmembrane proteins. The mature secretory forms of SC therefore are proteolytic fragments derived from the transmembrane proteins.

3.2 Results

Characterization of SC-Related Primary Translation Products. Mature SC isolated from rabbit milk is heterogeneous. Depending on the individual rabbit of the same strain, two or three polypeptides that differ in apparent M_r by a few thousand can be resolved by SDS-PAGE (22). In the gel system used in this study, the mature SC forms migrated with an apparent M_r

of about 70,000 (Fig. 2, lane 1).

To compare these mature forms to the primary translation products, we translated mRNA from rabbit mammary glands in the wheat germ cell-free system. We used [^{35}S]cysteine as the labeled amino acid because SC is particularly rich in cysteine. Surprisingly, immunoprecipitation yielded four polypeptide chains (Fig. 2, lane 2). Moreover, two of these four polypeptides (referred to as the upper doublet) had an apparent M_r of about 90,000 and therefore are about 20 kilodaltons larger than mature SC. The other two bands (referred to as the lower doublet) were similar in mobility to the mature SC bands. All four of these cell-free synthesized polypeptides were immunologically related to the antigen because their immunoprecipitation was effectively blocked when carried out in the presence of unlabeled mature SC (Fig. 2, lane 7).

In analogy to the heterogeneity of mature SC from different rabbits, we also observed heterogeneity in the size of the translation products of mRNAs derived from different rabbits. mRNA from rabbit B yielded a less-well resolved upper doublet than did mRNA from rabbit A (Fig. 2, lanes 3 and 2, respectively). Moreover, the lower doublet from rabbit B appeared to be unresolved. Evidence in favor of an unresolved doublet rather than a single band is presented below.

The question, then, is: Are all four immunoprecipitated bands primary translation products and are they related to the mature SC forms, or is the observed size heterogeneity an artifact of immunoprecipitation or cell-free synthesis?

To investigate whether this heterogeneity was due to a contaminant(s) in the immunoprecipitation, we translated mRNA from liver, an organ that also synthesizes SC. Because the total translation products of liver

differ from those of mammary gland, one might also expect differences in the pattern of the immunoprecipitated bands from liver if any of the four bands were contaminants. However, translation of mRNA (from rabbit B) of mammary gland (Fig. 3, lane 1) and of liver (Fig. 3, lane 2) yielded identical patterns of immunoprecipitated products.

To investigate whether the lower doublet might represent an artifact of cell-free synthesis (due to degraded mRNAs or proteolysis of the upper doublet) we size-fractionated poly(A)-containing mRNA from mammary gland of rabbit B on sucrose gradients (Fig. 4). Our finding that the mRNAs coding for the lower doublet migrated slower (Fig.4, fractions 10-14) than those coding for the upper doublet (Fig. 4, fractions 8-12) argues strongly that the lower doublet is not derived proteolytically from the upper doublet. Furthermore, because the mRNAs have poly(A) at their 3' ends and presumably have intact initiation sites at their 5' ends, the lower doublet is unlikely to be the result of degraded mRNAs coding for the upper doublet.

Characterization of SC forms Translocated in the Cell-Free System into Dog Pancreas Microsomal Vesicles. Cell-free translation of mRNAs for secretory and transmembrane glycoproteins in the presence of dog pancreas microsomal membranes has been shown to reproduce with fidelity the *in vivo* events that are coupled to the cotranslational translocation of the polypeptide across the membrane (56). These events include core glycosylation at the cisternal side of the RER membrane (57-62). The cell-free translocated forms of secretory and transmembrane proteins thus appear to be analogous to the forms found *in vivo* in the cisternae of the RER. Because the mature forms of SC are glycoproteins, translation of mRNA in the wheat germ cell-free system supplemented with dog pancreas microsomal vesicles yielded core-glycosylated bands that moved slower than their counter-

parts synthesized in the absence of membranes (Fig. 2, lane 5). That these slower moving bands were in fact core-glycosylated was shown by their specific binding to Con A-Sepharose (Fig. 5, lane 1). A small population of chains was not glycosylated by the microsomal vesicles and did not bind to Con A-Sepharose (Fig. 5, lane 2). This binding was inhibited in the presence of alpha-methyl mannoside [Fig. 5, compare lane 3 (the material bound in the presence of alpha-methyl mannoside) to lane 4 (the material not bound in the presence of alpha-methyl mannoside)].

Similar observations were made with the SC-related chains synthesized in the membrane-supplemented system using liver mRNA from rabbit B. Although there was a shift-up of bands for the upper doublet (Fig. 3, lane 3), the banding pattern was complex, probably due to the presence of a significant amount of unglycosylated chains. Interestingly, there was a clearly resolved lower doublet (Fig. 3, lane 3) in which both bands had a slower mobility than what appeared to be a single band for the corresponding primary translation products (Fig. 3 lane 2). Our tentative interpretation of these data was that in rabbit B the two primary translation products of the lower doublet were of similar size and thus comigrated, whereas the core-glycosylated counterparts differed in mobility, perhaps due to differences in the number of core-glycosylated asparagine residues.

As in the case of previously studied secretory glycoproteins, we expected that the various cell-free translocated forms of SC would be segregated within the lumen of the dog pancreas microsomal vesicles and thus completely protected from externally added proteases. Surprisingly, this was not the case. A distinct portion of all four of the cell-free translocated forms of SC was accessible to digestion by externally added trypsin, and a distinct portion remained protected (see Fig. 2, lane 6, for rabbit

A, and Fig. 3, lane 4, for rabbit B).

Protection was dependent on the integrity of the microsomal vesicles because it was abolished when trypsin digestion was performed in the presence of detergents (Fig. 2, lane 9 and Fig. 3, lane 7). Moreover, when synthesized in the absence of membranes, the primary translation products were completely degraded even after a posttranslational incubation with microsomal vesicles (Fig. 2, lane 8 and Fig. 3, lane 6), suggesting that translocation cannot occur after translation is completed.

We have not, however, conclusively determined the origin of each of the protected fragments (Fig. 2, lane 6 and Fig. 3, lane 4) from their respective intact counterparts (Fig. 2, lane 5 and Fig. 3, lane 3).

These proteolysis data indicate that each of the four forms of SC contains an approximately 15-kilodalton portion that remains untranslocated by the dog pancreas microsomal membranes and is therefore accessible to externally added trypsin. The most important conclusion that can be drawn from these data, therefore, is that the various forms of SC are synthesized not as secretory glycoproteins, but as transmembrane glycoproteins.

Cell-Free Translocated SC Forms are Competent to Bind IgA. It is known that rabbit SC will bind specifically to human polymeric IgA (63). We investigated whether cell-free synthesized and translocated rabbit SC was competent to bind human IgA coupled to Sepharose (IgA-Sepharose). Dog pancreas microsomal vesicles into which SC and other proteins had been translocated were reisolated from the translation mixture by centrifugation through a sucrose gradient. Among the total products that sedimented with the microsomal membranes, the various forms of SC amounted to minor species (Fig. 6, lane 1). The isolated membranes were solubilized with Triton X-100 and incubated with IgA-Sepharose. These IgA-Sepharose beads were then

washed and the bound proteins were eluted with SDS and analyzed by SDS-PAGE and fluorography (Fig. 6, lane 2). The four SC forms became clearly discernible because they are selectively enriched relative to the other bound polypeptides. Moreover, only the binding of the four SC bands was specifically blocked by the addition of excess IgA (Fig. 6, lane 3) or SC (Fig. 6, lane 4), indicating that all four of the SC bands were bound specifically and that the other bands were bound non-specifically.

We also found that a considerable amount of SC did not bind to the IgA-Sepharose beads and could be immunoprecipitated from the supernatant (Fig. 6, lane 5). We do not know why only a fraction of the SC forms could be bound, albeit specifically, to IgA. It is possible that the competence to bind was not acquired by all molecules (due to improper folding) or that it was lost after detergent solubilization.

3.3 Discussion

Prior to the cell-free synthesis studies presented here, SC from rabbit was believed to be genetically and biochemically heterogeneous. Two allotypes of rabbit SC have been detected (64). Two or three immunologically related glycoproteins, slightly different in size and charge, have been isolated from rabbit milk (22). Moreover, a number of smaller forms of SC were immunoprecipitated from rabbit milk and were presumed to be degradation products.

Our data indicate that the primary translation products of rabbit SC consist of four polypeptides, distinct in size but immunologically related to each other. Each of the four polypeptides was shown to be translocated into dog pancreas microsomal vesicles by the criterion of being core-glycosylated. However, translocation occurred only for a specific segment

of each of the four polypeptide chains, leaving another distinct segment untranslocated and accessible to externally added trypsin. The untranslocated portion amounted to 15 kilodaltons for each of the four chains. Thus, the four SC forms are synthesized not as secretory proteins but as transmembrane proteins.

How are the four cell-free synthesized transmembrane forms related to the secreted forms in milk? Our data tentatively suggest the following relationship. When integrated into dog pancreas microsomal vesicles, the upper transmembrane doublet is tryptically digested to membrane-protected fragments (Fig. 2, lane 6) that still migrate slower than the polypeptides comprising mature SC (Fig. 2, lane 1). The somewhat smaller size of mature secreted SC can be rationalized if one assumes (Fig. 1) that it is generated by proteolytic cleavage at the ectoplasmic side of the plasma membrane (corresponding to the cisternal side of the RER). In contrast, the cell-free generated, membrane-protected fragments are cleaved at the cytoplasmic side of the membrane and therefore contain, in addition, the membrane-spanning portions which are absent from the mature, secreted forms. Hence, we propose that the upper doublet bands are the biosynthetic, transmembrane precursors to the M_r~70,000 secreted forms of SC. That the secreted forms from some rabbits can be resolved into three bands (Fig. 2, lane 1) may represent heterogeneity generated by posttranslational events. On the other hand, we cannot rule out that the upper doublet is made up of more than two primary translation products that are not resolved.

What, then are the "mature" counterparts of the lower doublet transmembrane forms? Again, if proteolytic cleavage also occurs for these forms, then the secreted counterparts may be represented by the smaller forms of SC that have been detected in milk, and that hitherto have been

assumed to be non-specific proteolytic degradation products of the "authentic" SC forms.

Figure 2.

Cell-free translation and transmembrane integration of rabbit mammary SC.

Lane 1 is a Coomassie blue-stained gel of authentic rabbit milk SC. The remaining lanes are from a fluorograph of cell-free synthesized and immunoprecipitated products. Lanes: 2, mammary mRNA from rabbit A translated in the wheat germ system; 3, as in 2, except the mRNA was from rabbit B; 4, as in 2, except that, after translation was completed (90 min incubation at 25°C), dog pancreas microsomal vesicles were added and the incubation continued for an additional 90 min; 5, as in 2, except that the microsomal vesicles were present at the start of translation (all four bands are shifted upward, due to cotranslational glycosylation by the membranes); 6, as in 5, except that, after translation was completed, trypsin was added to the reaction [all four bands are apparently shifted downward by about 15 kilodaltons; each of the two bands of the upper doublet appears to give rise to two closely spaced bands, which could be due to the presence of two nearby tryptic sites of which only one is cleaved in any given molecule (other explanations are possible)]; 7, as in 2, except that immunoprecipitation was carried out in the presence of 1 ug of the SC shown in lane 1 (the added SC completely blocked immunoprecipitation of all four bands); 8, as in 4, except that trypsin was added after translation was completed (because membranes were added after translation, the in vitro synthesized proteins were not translocated and were digested by trypsin); 9, as in 6, except that, when trypsin was added, the sample was also adjusted to 1% in Triton X-100 (even though the proteins were translocated, they were degraded when the microsomal vesicles were solubilized by detergent). The positions of the M_r marker proteins are indicated at the left margin, shown as

$M_r \times 10^{-3}$ (rabbit muscle phosphorylase B, 92,000; bovine serum albumin, 68,000; hen ovalbumin, 45,000).

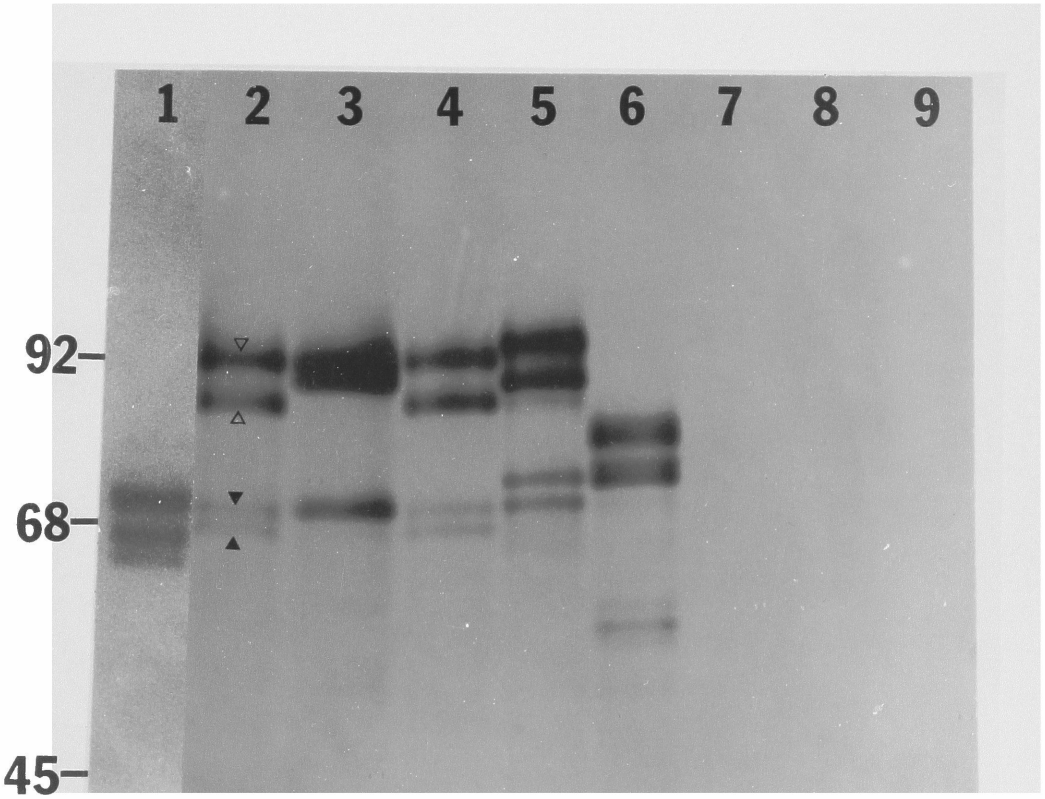

1
2
3
4
5
6
7
8
9
92
68
45

Figure 3.

Cell-free translation and transmembrane integration of liver SC.

Lanes: 1, as in lane 3 of Fig. 2 (to give the positions of the mammary SC bands of rabbit B); 2, liver mRNA from rabbit B translated in the wheat germ system; 3, as in 2, except that dog pancreas microsomal vesicles were present during translation; 4, as in 3, except that the sample was treated with trypsin after translation; 5, as in 2, except that immunoprecipitation was performed in the presence of 1 ug of SC; 6, as in 2, except that, after translation, microsomal vesicles were added and the incubation was continued for 90 min before the sample was treated with trypsin; 7, as in 4, except that, when trypsin was added, the sample was also adjusted to 1% in Triton X-100.

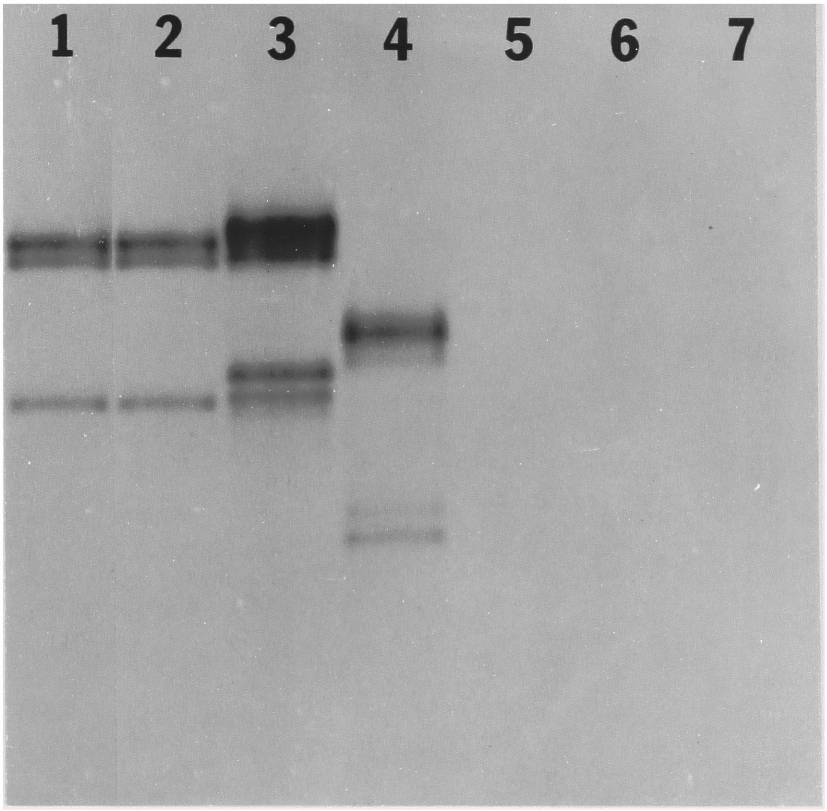

1
2
3
4
5
6
7

Figure 4.

Partial fractionation of mRNA's coding for SC.

Poly(A)-containing mRNA from rabbit B was fractionated on a sucrose gradient. Twenty-two fractions were collected and the mRNA from every second fraction was translated. In vitro synthesized products were immunoprecipitated and analyzed. The top of the gradient is toward the right. Only selected fractions are presented. The fraction number from which each lane is derived is indicated at the top of each lane. Lane T is an immunoprecipitate of a translation of total poly(A)-containing mRNA. The upper doublet is indicated by open arrowheads; the lower, unresolved, doublet, by closed arrowheads. Note that fraction 8 is enriched for the upper doublet whereas fraction 14 is enriched for the unresolved lower doublet. The faint band beneath the unresolved lower doublet in fractions 10 and 12 probably is a contaminant.

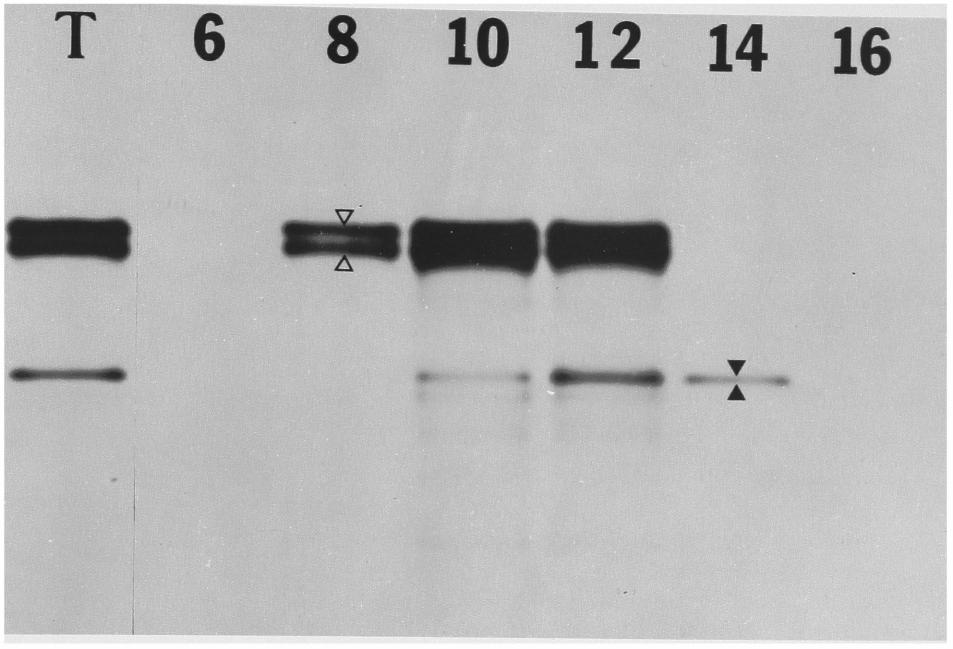

T
6
8
10
12
14
16

Figure 5.

Cell-free synthesized SC are core glycosylated by dog pancreas microsomal vesicles.

The newly synthesized chains (made in the presence of microsomal vesicles) were separated into (i) bound (lane 1) or not bound (lane 2) to Con A-Sepharose, and (ii) bound (lane 3) or not bound (lane 4) to Con A-Sepharose in the presence of 0.4 M alpha-methyl mannoside which blocks specific binding to the lectin. Binding of cell-free synthesized products to Con A-Sepharose, and analysis of products was performed as described in Chapter 2.

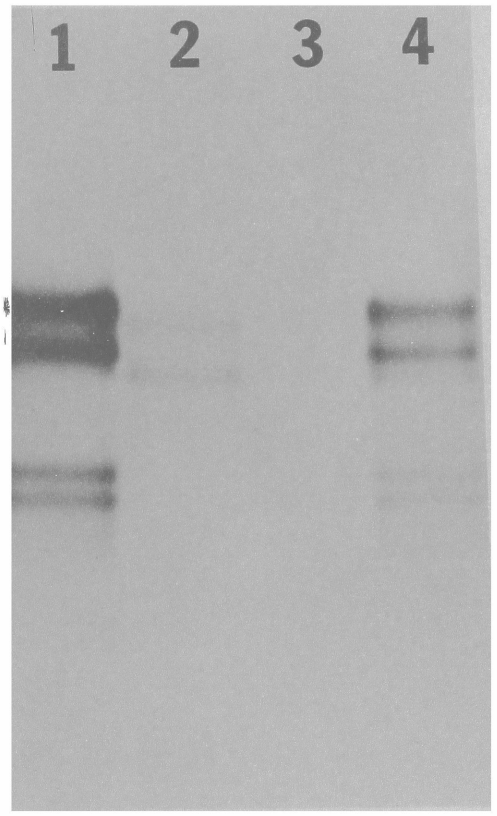
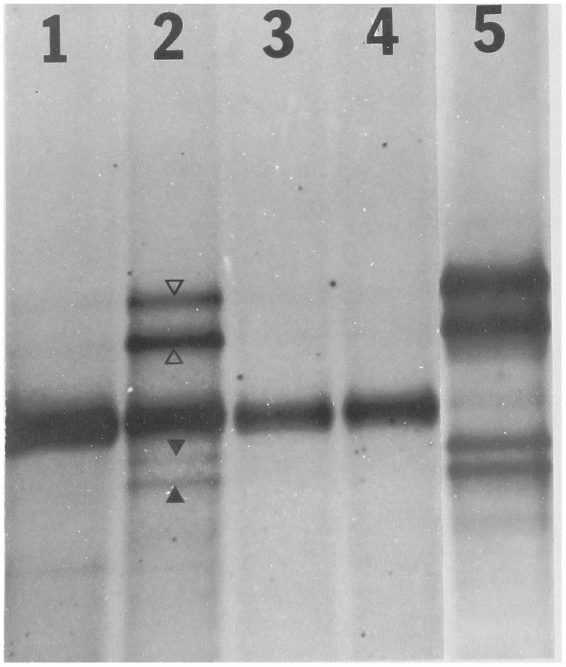

1
2
3
4

Figure 6.

In vitro translocated SC forms are competent to bind human polymeric IgA.

Translations (100 ul) were performed in the presence of microsomal vesicles. After translation was completed, membranes were recovered from the translation mixture by layering it in a Beckman Airfuge tube over a two step sucrose gradient consisting of 80 ul of 0.5 M sucrose and 25 ul of 2.0 M sucrose. Both sucrose solutions also contained 150 mM NaCl and 20 mM Na phosphate (pH 6.5). After centrifugation for 15 min at 160,000 x g, membranes were recovered at the 0.5/2.0 M interface in a volume of 25 ul. They were diluted to 200 ul with 150 mM NaCl/20mM Na phosphate, pH 6.5, and adjusted to 1% in Triton X-100. A 2 ul aliquot of this was analyzed in lane 1. This lane, therefore, represents 1% of the total *in vitro* synthesized products which cosedimented with the membranes. IgA-Sepharose (20 ul) was added to the 198 ul of solubilized membranes and the samples were agitated for 16 hr at $4^{o}C$. The beads were then washed five times with 1.2 ml of 1% Triton X-100/ 150 mM NaCl/20 mM Na phosphate, pH 6.5. Bound proteins were eluted by boiling in loading buffer, and the sample analyzed by electrophoresis and fluorography (lane 2; arrowheads as in Fig. 2, lane 2). Other lanes: 3, as in 2, except that 10 ug of IgA, not bound to Sepharose, was added at the same time as the IgA-Sepharose; 4, as in 2, except that 20 ug of SC was added at the same time as the IgA-Sepharose; 5, the material that did not bind to IgA-Sepharose (i.e. the supernatant from the IgA-Sepharose incubation) was adjusted to pH 8.1 and immunoprecipitated without prior denaturation with SDS.


1
2
3
4
5

4. Synthesis of SC by the Human Cell Line, HT29.E10

4.1 Introduction.

In the previous chapter, we found that in a cell-free translation system (supplemented with dog pancreas microsomal membranes) rabbit SC was synthesized not as a *secretory* protein, but as a much larger *transmembrane* protein. Larger, amphiphilic forms of SC were subsequently isolated from rabbit liver and mammary gland (65). We therefore proposed that the portion of the transmembrane protein exposed on the outside of the cell (the ectoplasmic domain) binds pIg, while the membrane spanning and cytoplasmic portions of the protein constitute the effector domain mediating transcytosis. The secreted form of SC found in association with pIg in glandular secretions is therefore the proteolytically severed ectoplasmic domain of this transmembrane receptor, cleaved sometime after the initiation of transcytosis.

We extended our cell-free translation experiments from rabbit to human SC, as the latter contains only one type of polypeptide chain, rather than the four types found in rabbit. Moreover, we carried out *in vivo* pulse and pulse-chase experiments, using a human adenocarcinoma-derived cell line (HT29) that is known to synthesize SC (11, 38) and transport SC and pIg in the "normal" manner (23). These *in vivo* experiments were facilitated by our isolation of a clone, HT29.E10, which produced SC in significantly larger quantities than the parent cell line. The various *in vitro* and *in vivo* synthesized forms of SC were characterized by using proteolytic enzymes as probes for their membrane orientation, by endoglycosidase H digestion, and by partial NH_2-terminal sequence analysis. Our data permitted us to relate the forms of SC synthesized *in vivo* to their *in vitro* synthesized

counterparts and permitted reconstruction of some of the events in the biosynthetic pathway of SC.

Our results demonstrate (i) that human SC (M_r= 77,000) is synthesized as a larger transmembrane precursor (M_r= 95,000) both *in vivo* and *in vitro* and (ii) that SC found in secretions represents a proteolytically severed fragment of this transmembrane precursor derived from its ectoplasmic domain and containing its NH_2-terminus.

4.2 Results

Cell-free translation and membrane integration of SC. Our first objective was to investigate whether human SC is synthesized as a larger transmembrane form. We followed the same experimental protocol that had been used in our previous study of rabbit SC. mRNA extracted from HT29.E10 cells was translated in the wheat germ cell-free system. The products were immunoprecipitated with affinity purified antibodies to human SC and analyzed by SDS-PAGE and fluorography. We found a single product of apparent M_r= 80,000 (Fig. 7, lane 3) which was about 3 kilodaltons larger than authentic SC from human colostrum (Fig. 7, lane 1, upper band). A control immunoprecipitate with nonimmune serum yielded a blank lane (Fig. 7, lane 2).

Integration of a transmembrane protein into the rough endoplasmic reticulum membrane, cleavage of its signal peptide, and core glycosylation can be faithfully reproduced in the cell-free system by including rough microsomal vesicles from dog pancreas in the translation reaction (56). When such microsomes were present during translation of HT29.E10 mRNA the M_r= 80,000 primary translation product was in part converted to several slower moving bands, the largest of which had an apparent M_r=95,000 (Fig. 7, lane

4, downward pointing arrow) and to a slightly faster moving band (upward pointing arrow in Fig 7, lane 4). By analogy to our data with rabbit SC (16) and other precedents (57, 66) we presumed that these additional bands represented membrane integrated polypeptides that were either fully core-glycosylated (Fig 7, lane 4, downward pointing arrow), partially glycosylated (Fig. 7, lane 4, dots), or unglycosylated (Fig. 7, lane 4, upward pointing arrow). Our conjecture that the M_r= 95,000 band represented a fully core-glycosylated form was based on its comigration with an in vivo synthesized counterpart detected after a 5 min (Fig. 8, lane 1) or 30 min (Fig. 7, lane 8 and Fig. 8, lane 2) labelling of cells (see below). Note that partially or non-glycosylated forms were not detected in vivo. We presumed that the fastest moving band (Fig 7, lane 4, upward pointing arrow) was a membrane integrated and signal peptide cleaved, but non-glycosylated form.

We demonstrated that the products were cotranslationally integrated into the dog pancreas microsomal membranes in a transmembrane configuration by post-translational incubation with trypsin (Fig. 7, lane 5). Each of the intact, membrane-integrated polypeptides (Fig. 7, lane 4) appeared to be degraded to a corresponding membrane-protected fragment (Fig. 7, lane 5) reduced in size by about 15 kilodaltons. (The bands of the proteolytic fragments of the partially and non-glycosylated forms were very faint and do not photograph well). Thus, each of the membrane-integrated polypeptides appeared to contain a trypsin-accessible domain of nearly identical mass. The resistance of these fragments to further trypsin digestion was not an intrinsic property, but depended on the integrity of the membrane: Post-translational incubation with trypsin in the presence of Triton X-100 resulted in their complete degradation (Fig. 7, lane 6). Moreover, in-

tegration was coupled to translation: When microsomal membranes were added to the translation mixture after translation was completed, (Fig. 7, lane 7), the products were completely digested. These results showed that human SC, like its rabbit counterpart, is synthesized in a cell-free system as a larger form that can be cotranslationally integrated into dog pancreas microsomal membranes in a transmembrane configuration.

In vivo pulse chase experiments. These experiments were carried out with the cloned human colon adenocarcinoma-derived cell line, HT29.E10 (see Chapter 2) grown to confluency and forming a polarized epithelial monolayer (23). Thus, the basolateral surface where transcytosis would be initiated faces the plastic of the culture dish, whereas the apical surface is exposed to the medium. Because of intracellular tight junctions pIg, which might be introduced into the medium by the fetal bovine serum, would probably not have direct access to the basolateral surface. However, it is likely that transcytosis can occur constitutively in the absence of the ligand (pIg), as suggested by in vivo studies with rat liver: during perfusion with pIg-free medium, SC continues to be released into the bile (67).

Pulse-labelling of these cells for 5 min (Fig. 8, lane 1) or for 30 min (Fig. 7, lane 8, and Fig. 8, lane 2) yielded a form of SC (M_r= 95,000) that is indistinguishable in apparent M_r from the fully glycosylated, membrane integrated form synthesized in vitro (Fig. 7, compare lane 4, downward pointing arrow, with lane 8).

To demonstrate that the pulse-labeled M_r= 95,000 form (like its in vitro synthesized counterpart) existed in a transmembrane orientation, we subjected a homogenate of pulse-labeled cells to digestion with pronase (Fig. 9). It can be seen that the pulse-labeled M_r= 95,000 form was reduced in mass by about 15 kilodaltons [Fig. 9, compare lanes 1 (not incu-

bated) and 2 (incubated without pronase) with lane 3 (pronase-incubated)]. This is similar to the reduction in mass of the *in vitro* synthesized counterpart by trypsin (see Fig. 7, lanes 4 and 5). The resistance of the remainder of the molecule to further degradation by pronase was dependent on the integrity of the membrane, since complete degradation occurred in the presence of pronase and Triton X-100 [Fig. 9, compare lane 4 (incubated with Triton X-100 alone) with lane 5 (incubated with Triton X-100 plus pronase)].

Labelling for 30 min, followed by chases for periods ranging from 0.5-48 hr (Fig. 7, lane 9, and Fig. 8, lanes 3-10) yielded a still larger form of SC (M_r= 100,000). Moreover, as early as after a one hour chase, (Fig. 8, lane 4, upward pointing arrow), though more clearly after a 2 hr chase (Fig. 8, lane 5), a M_r=80,000 form appeared. This M_r= 80,000 form increased in amount at the expense of the M_r= 100,000 form at chase periods of up to 24 hr (Fig. 8, lanes 4-9). This M_r= 80,000 form also appeared in the medium (Fig. 7, lane 10, and Fig. 8, lanes 13-18). It was soluble and not merely released by shedding of membrane fragments, since it resisted pelleting in a 30 min centrifugation in the Beckman airfuge at 160,000 x g, conditions which should pellet a 22 S particle (data not shown). The amount of M_r= 80,000 form associated with cells was higher than the amount in the medium, at least during the shorter chase periods [Fig. 8, compare 24 hr chased cells (lane 9) with the corresponding medium (lane 17)]. Only at the longest chase period of 48 hr was the M_r= 80,000 form predominantly in the medium rather than associated with the cells [Fig. 8, compare 48 hr chased cells (lane 12) with corresponding medium (lane 18)].

Taken together, the *in vivo* pulse and pulse-chase experiments showed

(i) that the earliest *in vivo* synthesized M_r= 95,000 form was indistinguishable from the *in vitro* synthesized, membrane integrated, and fully core-glycosylated form; (ii) that this M_r= 95,000 form is further converted within a period of 1-2 hr to a M_r= 100,000 form, presumably by conversion of the high-mannose core oligosaccharide moieties to the complex type (see below); (iii) that the M_r= 100,000 form is converted to a M_r= 80,000 form over a long period (2-48 hr); and (iv) that this M_r= 80,000 form is released into the medium from a cell-associated pool. These data suggest that constitutive transcytosis occurs in these cells and that the released M_r= 80,000 form of SC is proteolytically severed from the ectoplasmic domain of the M_r= 100,000 transmembrane form (see below).

The M_r= 80,000 form released into the medium is still somewhat larger than mature SC from human colostrum (Fig. 7, compare lanes 10 and 1, respectively). This may be due to cleavage at a different site on the ectoplasmic domain of the precursor in the mammary gland as opposed to the colon cancer cell line. Differences in the size of the cleaved form of SC have been observed in comparing liver and mammary SC from rabbits (65). Furthermore, the SC isolated from colostrum may have undergone a further trimming of its carbohydrate chains or a secondary proteolytic cleavage, since free colostral SC is rapidly degraded *in vivo* (68). Indeed, in the pulse-chase experiment the broad M_r= 80,000 band migrated somehwat faster after long chase times (Fig. 8, lanes 10 and 18).

Endoglycosidase H digestion. Endoglycosidase H digestion was carried out on the intracellular early and late forms as well as on the secreted form. Only the early intracellular form was affected by endoglycosidase H treatment, resulting in a reduction of apparent M_r from 95,000 to 80,000 [Fig. 7, compare control (lane 8) with endoglycosidase H-treated (lane

11)]. The later intracellular forms (24 hr chase) as well as the form secreted during this chase were all resistant to endoglycosidase H [Fig. 7, compare controls (lanes 9 and 10, respectively) and endoglycosidase H treated (lanes 12 and 13, respectively)]. This suggests that the conversion of the M_r= 95,000 form to the M_r= 100,000 form was due to the processing of high-mannose core oligosaccharides to the complex type (69).

Partial NH_2-terminal sequence analysis. If our conjecture is correct that secreted SC represents the ectoplasmic domain of the transmembrane form and, moreover, if the ectoplasmic domain contains the NH_2-terminus of the transmembrane form, then the intracellular forms of SC and the form released into the medium, as well as the membrane-protected fragment of SC generated by pronase digestion of the pulse-labeled cell homogenate, would be expected to have the same NH_2-terminal sequence as SC isolated from glandular secretions. That this is indeed the case is borne out by the data in Fig. 10. We carried out partial NH_2-terminal sequence analysis of (i) the pulse-labeled M_r= 90,000 form (panel A); (ii) the membrane-protected fragment generated by pronase digestion of the pulse-labeled form in homogenized cells (panel B); (iii) the biosynthetically later M_r= 100,000 form (panel C); (iv) the intracellular M_r= 80,000 cleavage product of the M_r= 100,000 form (panel D); and (v) the secreted M_r= 80,000 cleavage product of the M_r= 100,000 form (panel E). Of the NH_2-terminal residues analyzed, Pro occurs in all of the forms in positions 3 and 7. Furthermore, in the 30 min pulse form (M_r= 95,000) Ile occurs in position 4 (panel F). (We did not label the other forms with Ile). This partial sequence aligns with the known NH_2-terminal sequence of human SC isolated from colostrum, which has Pro in positions 3 and 7, and Ile in position 4 (70).

4.3 Discussion

The most generally significant result of our combined *in vitro* and *in vivo* studies on the biosynthesis of human SC is the demonstration of a precursor-product relationship between an integral membrane protein and a secreted protein. While our previous studies on cell-free synthesis and membrane integration of rabbit SC suggested such a relationship, our data on human SC provided definitive evidence. By partial NH_2-terminal sequence analysis we documented that human SC is a proteolytically derived fragment which represents most, if not all, of the ectoplasmic domain of a transmembrane protein precursor (M_r= 100,000), including the intact NH_2-terminus. Cleavage of the transmembrane precursor to generate secreted SC is a late event. The protease(s) responsible for generating SC from its transmembrane precursor is not known. The fate of the remainder (about 15 kilodaltons) of the transmembrane precursor (the transmembrane segment(s) and cytoplasmic domain) is unknown; most likely it is rapidly degraded.

Taken together with our earlier finding that the transmembrane precursor of rabbit SC can bind IgA, our data strongly support our previous proposal that this transmembrane precursor of SC is the receptor for transcytosis of pIg. This single polypeptide chain contains both functional domains expected of such a receptor: an ectoplasmic ligand binding domain and a cytoplasmic effector domain, which may be involved in mediating transcytosis. However, it is possible that the transmembrane precursor of SC constitutes only a part of the pIg receptor and that other, yet unidentified polypeptides are components of this receptor as well. The high affinity binding sites for SC found on mammary cell membranes (22) may represent such a protein(s).

Although we have no data on the subcellular localization of the vari-

ous forms of SC, we can make some speculative correlations of our biosynthetic data presented here with the pathway of SC and pIg observed cytochemically. The early M_r= 95,000 form probably resides in the endoplasmic reticulum (7), since it contains unprocessed oligosaccharides and comigrates with the form inserted into dog pancreas microsomal membranes *in vitro*. Processing to the terminally glycosylated M_r= 100,000 form presumably takes places in the Golgi apparatus (21), where the appropriate enzymes are located. Most likely, it is this M_r= 100,000 form that goes to the basolateral (or in hepatocytes, sinusoidal) surface of the cell, binds pIg, and is internalized. We do not know were cleavage to the M_r= 80,000 form takes place: The two most likely possibilities are in the transcytotic vesicle or at the apical (or in hepatocytes, bile canalicular) surface following exocytosis. It is possible that a second cleavage event occurs in certain tissues, since colostral SC is slightly smaller than the SC released by the HT29.E10 cells. Nevertheless, it is clear from our sequence data that the cleavage(s) remove material exclusively from the COOH-terminal portion of the precursor, as the precursor has the same NH_2-terminus as colostral SC.

We were surprised at both the slow rate of cleavage of the M_r= 100,000 form to the M_r= 80,000 form, as well as the slow release of the M_r= 80,000 form into the medium. *In vivo*, transport of pIg across liver cells occurs as rapidly as 10 min, although peak transport takes at least an hour (10, 13, 16, 19). Our experiments were done with transformed cells in culture which probably do not process and transport SC as rapidly as do cells *in vivo*. Although it appears that transport and processing of SC continues constitutively [i.e. in the absence of the pIg ligand (67)], binding of the ligand to the receptor at the basolateral surface may accelerate this pro-

cess. Furthermore, it is possible that after exocytosis and cleavage of the SC precursor, the SC-pIg complex normally remains bound to the apical surface of the cell for a period. The high affinity binding sites for SC found on mammary cells (22) may be responsible for this. A further, unknown event may be needed to release the complex from the membrane.

Figure 7

Comparison of SC synthesized in cell-free translations with SC made by HT29.E10 cells.

Lane 1 is a Coomasie blue stained gel of human colostral secretory IgA (Cappel). SC is the upper band. The lower band is the heavy chain of IgA. Lanes 2-7 are immunoprecipitated products from cell-free translations of HT29.E10 mRNA. Lanes 2 and 3 are from translations carried out in the absence of microsomal membranes. Lane 2: Immunoprecipitation with preimmune serum. Lanes 3-7: Immunoprecipitations with affinity-purified antibodies to SC. Lane 4: Translation performed in the presence of microsomal vesicles. Downward-pointing arrow indicates fully core-glycosylated SC precursor. Dots indicate partially glycosylated SC precursor. Upward-pointing arrow indicates minor amount of SC precursor which probably has had it signal sequence removed but has not been glycosylated. Lane 5: Translation performed in the presence of microsomal vesicles and post-translationally digested with trypsin (0.1 mg/ml, 1 hr, $0^{o}C$). Downward-pointing arrow indicates protected fragment generated from fully core-glycosylated form. Dots indicate very faint protected fragments probably generated from partially glycosylated forms. Upward-pointing arrow indicates very faint fragment probably generated from non-glycosylated, but membrane integrated and signal sequence-cleaved form. Lane 6: Microsomal vesicles were added only after completion of translation. The reaction was then digested with trypsin as in lane 5. Lane 7: Translation was performed in the presence of microsomal vesicles. The vesicles were post-translationaly solubilized by adding Triton X-100 to 1% and then the sample was trypsinized as in lane 5. Lanes 8-13 are anti-SC immunoprecipitates of products synthesized by

HT29.E10 cells during a pulse-chase experiment as described in Figure 2. (The samples run in lanes 8, 9, and 10 are duplicates of those Fig. 8, lanes 2, 9 and 17, respectively, and are provided here for comparison.) Lane 8: Immunoprecipitate from cells labeled for 30 min. Lane 9: Immunoprecipitate from cells labeled for 30 min and then chased for 24 hr. Lane 10: Immunoprecipitate from the chase medium of the cells in lane 9. Lanes 11-13 are duplicates of the samples in lanes 8-10, respectively, except that the immunoprecipitated products were treated with endoglycosidase H prior to SDS-PAGE. Lanes 2-13 are aligned from the same fluorographed gel. The positions of the molecular weight marker proteins used are indicated at the left margin as $M_r \times 10^{-3}$. Markers used were: E. coli beta-galactosidase, M_r=116,000; phosphorylase b, M_r=92,000; and bovine serum albumin, M_r=68,000.

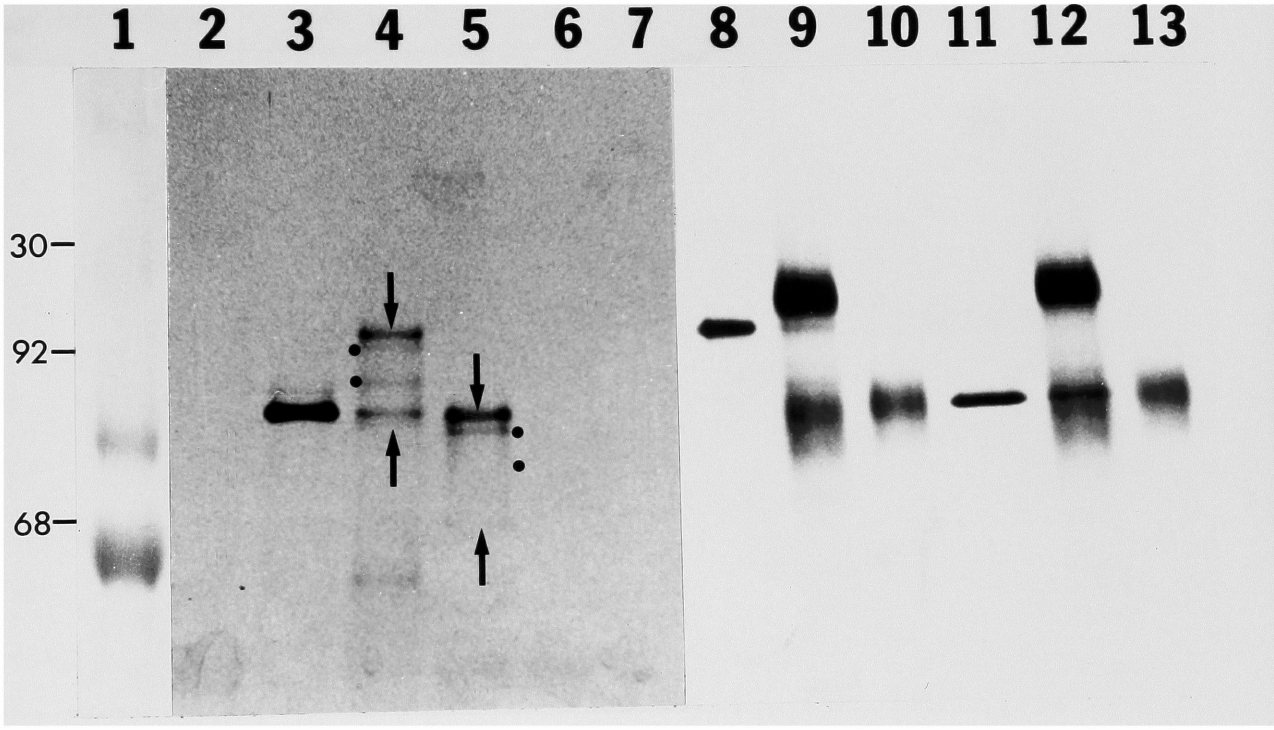

1 2 3 4 5 6 7 8 9 10 11 12 13
30
92
68

Figure 8.

Pulse-chase analysis of SC synthesis and processing.

Cells were labeled with [^{35}S]cysteine for either 5 min (lane 1) or 30 min (lanes 2-18). In lanes 3-18 after this labelling period the cells were washed with PBS, the medium replaced with supplemented McCoy's 5a medium, and the cells were chased for indicated times. Lanes 1-10 are anti-SC immunoprecipitates from the lysed cells. Lane 1: 5 min pulse. Lane 2: 30 min pulse. Lane 3: 30 min pulse, followed by 30 min chase. Lane 4: 1 hr chase. Lane 5: 2 hr chase. Lane 6: 4 hr chase. Lane 7: 6 hr chase. Lane 8: 10 hr chase. Lane 9: 24 hr chase. Lane 10: 48 hr chase. Lanes 11 and 12 are preimmune serum immunoprecipitates from cells pulsed for 30 min (lane 11) or pulsed for 30 min and chased for 48 hr (lane 12). Lanes 13-18 are anti-SC immunoprecipitates from the chase medium of cells which were labeled for 30 min and then chased for indicated times. Lane 13: 2 hr chase. Lane 14: 4 hr chase. Lane 15: 6 hr chase. Lane 16: 10 hr chase. Lane 17: 24 hr chase. Lane 18: 48 hr chase. The lanes in this figure were aligned from a 2 week exposure of one fluorographed gel.

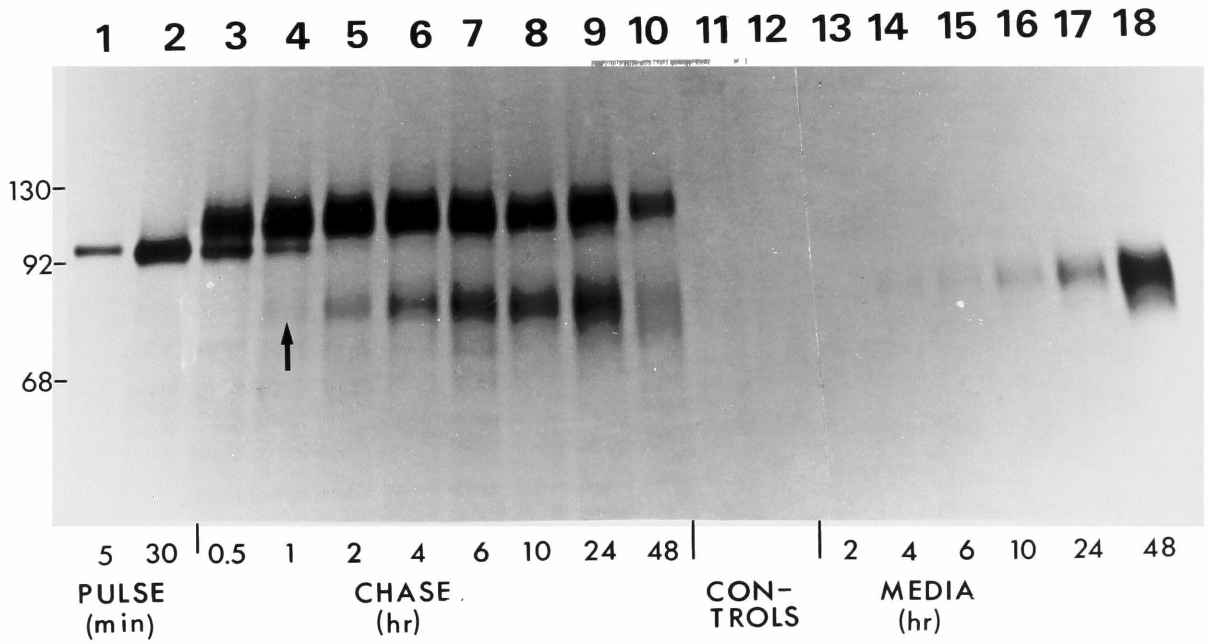

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
130-
92-
68-
5 30 0.5 1 2 4 6 10 24 48 2 4 6 10 24 48
PULSE (min)
CHASE (hr)
CON-TROLS
MEDIA (hr)

Figure 9.

Transmembrane orientation of SC synthesized by HT29.E10 cells. Cells were pulse-labeled for 30 min. Lane 1: Immunoprecipitate of cells which were not homogenized. Lane 2: Cells were homogenized after labelling and then incubated on ice for 1 hr without additions. Lane 3: Incubation was performed in the presence of 0.02 mg/ml pronase. Lane 4: Incubation was performed in the presence of 0.4% Triton X-100. Lane 5: Incubation was performed in the presence of both pronase and Triton X-100.

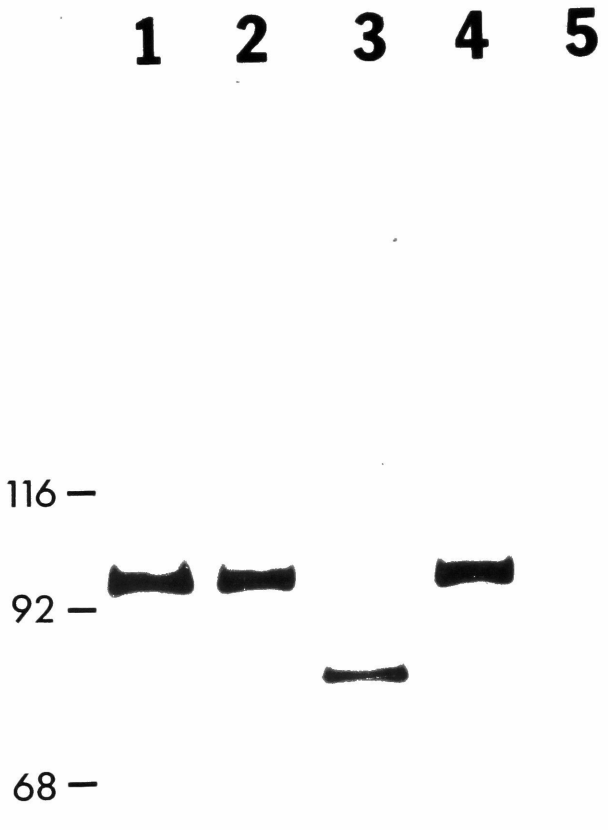
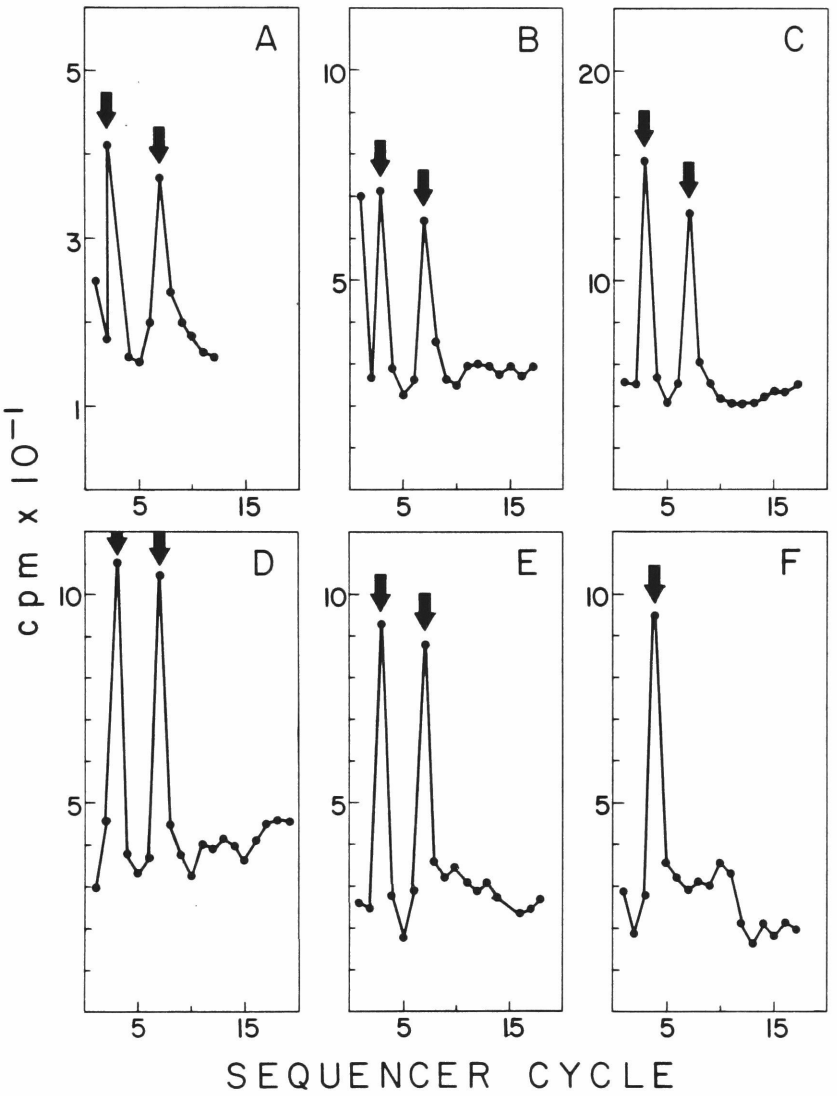

1 2 3 4 5
116
92
68

Figure 10.

Partial NH_2-terminal sequence analysis of the various forms of SC.

Cells were labeled with either [^{3}H]proline (panels A-E) or [^{3}H]isoleucine (panel F). Panels A and F are the M_r=95,000 pulse-labeled form. Panel B is the protected fragment generated by pronase digestion of pulse-labeled, homogenized cells. Panel C is the M_r=100,000 form associated with cells after 24 hr of chase. Panel D is the M_r=80,000 form associated with cells after 24 hr of chase. Panel E is the M_r=80,000 form released into the medium after 24 hr of chase. The data presented are radioactivity (uncorrected cpm) released at each sequencer cycle. Arrows indicate peaks corresponding to residues in SC from colostrum. The peak in panel B, sequencer cycle 1, is probably due to wash out of material from the sequencer reaction cup, which is commonly observed in radiosequencing experiments.


A
B
C
D
E
F
5
3
1
10
5
20
10
5
15
cpm x 10⁻¹
SEQUENCER CYCLE

5. Cloning and Sequencing of DNA Complementary to SC mRNA

5.1 Introduction.

In the two previous chapters, we described our finding that SC is made as a transmembrane precursor, which is proteolytically cleaved to yield mature SC. We proposed that this precursor is the cell surface pIg receptor, and that after transcellular transport, its ectoplasmic pIg binding domain is cleaved and is discharged bound to pIg. The precursor has a large cytoplasmic domain (about 11-15 kilodaltons). This domain may interact with cytoplasmic elements and provide the information for guiding the SC through the cell, first to the basolateral surface, and then via endocytosis, transcytosis, and finally exocytosis at the apical (or in liver, bile canalicular) surface.

As a first step towards understanding the mechanism of this protein transport process, we cloned and sequenced cDNA for SC mRNA and deduced the primary structure of the COOH terminal 163 amino acids of the transmembrane precursor, comprising its cytoplasmic and membrane spanning portions. The membrane spanning segment consists of 23 non-charged, predominantly hydrophobic amino acids, and is followed by a presumably cytoplasmically exposed hydrophilic segment of 103 amino acids.

5.2 Results

We have previously shown that rabbit SC comprises four primary translation products: an upper doublet of M_r =90-93,000, and a poorly resolved lower doublet of M_r =70-71,000.

We partially purified the mRNA coding for the upper doublet of SC by size separation of total poly(A) containing RNA from rabbit lactating mam-

mary gland on a sucrose gradient. This mRNA was converted to double-stranded cDNA by standard procedures, and the cDNA inserted into the plasmid PBR 322 by G-C tailing. The plasmids containing the cDNA inserts were transformed into the E. coli strain MC1061. Approximately 600 tetracycline resistant, ampicillin sensitive clones were obtained. Eighteen of these clones were initially screened by the ability of their plasmid DNA to specifically select mRNA coding for secretory component. The first plasmid so identified contained an insert of approximately 700 base pairs. It was used as a hybridization probe to screen the remaining clones. Twelve clones were found in this manner. The clone designated p32.23 contained the largest insert (approximately 1650 base pairs, including G-C tails) and was the only clone studied in further detail. The DNA of p32.23 specifically selected mRNA for each of the SC primary translation products from both liver (Fig. 11, lane 4) and mammary (Fig. 11, lane 7) mRNAs suggesting that these proteins are coded for by mRNAs which are very similar, at least in the region complementary to the sequence in p32.23. However, since the mRNA used for cloning was highly enriched in species coding for the upper doublet, it is likely that we have cloned the cDNA derived from mRNA coding for one of the polypeptides of this doublet.

To further characterize these mRNAs, we separated total liver and mammary mRNAs by formaldehyde-agarose gel electrophoresis, transferred the mRNAs to nitrocellulose paper, and hybridized with nick translated p32.23. Two broad bands, corresponding in size to an estimated 4200 and 3600 base pairs were found in both liver (Fig. 12, lane 1) and mammary (Fig. 12, lane 2) mRNAs. Each band may represent the two unresolved mRNAs coding for the corresponding protein doublet.

Our principal objective in this study was to deduce the primary struc-

ture of the cytoplasmic and membrane spanning portions of the SC precursor. We have previously shown by direct protein sequencing that these portions are located at the carboxy-terminal region of the molecule, and therefore should be coded for by the 3' part of the coding region of the mRNA. Since the cDNA clones were constructed by priming from the 3' poly(A) tail, it seemed likely that that p32.23 would represent the 3' portion of the mRNA. We therefore sequenced the insert in p32.23 by randomly subcloning fragments produced with several restriction enzymes into the phages M13 Mp8 and M13 Mp9 and then using the dideoxy chain termination method. The strategy used for sequencing is shown in Fig. 13 and the complete sequence in Fig. 14. The insert in p32.23 contains 1563 base pairs, plus a poly(A) tail at the 3' end and G-C tails at both ends. Note that the sequence contains the unusual poly(A) addition sequence AUUAAA located 20-25 residues from the poly(A) tail. This poly(A) addition sequence has so far been found only in lysozyme (71) and mouse pancreatic amylase (72) mRNAs.

Computer-assisted sequence analysis indicated that there was only one significant open reading frame, which extends from the 5' end of the sequence to nucleotide 489. The corresponding protein sequence is indicated in Fig. 4. This reading frame codes for 163 amino acids which constitute the carboxy-terminal end of the SC precursor. The remaining portion of the SC precursor would then be coded for by sequences which are not contained in this clone. The relationship between the mRNA, the cDNA insert in p32.23, and the deduced protein sequence is indicated in Fig. 15. Our data indicates that the SC mRNA contains an unusually large 3' untranslated region of 1074 bases. Since the primary translation product of the SC precursor has a M_r =90-93,000, we would expect that the coding region of the mRNA would contain about 3,000 bases. Since the mRNA is about 4,200 bases long

and 1074 bases are in the 3' untranslated region, it can be estimated the 5' untranslated region is about 125 bases long.

To test whether we made the correct reading frame assignment, we translated SC mRNA in the cell free system in the presence of [^{35}S]methionine, isolated the M_r=90,000 product by immunoprecipitation and SDS-polyacrylamide gel electrophoresis, digested it with trypsin, and subjected the total mixture of tryptic peptides to automated Edman degradation. The cleaved form of rabbit SC contains little or no methionine (73), so most or all methionine labeled tryptic peptides will be derived from either the carboxy-terminal extension of the precursor or a putative signal peptide. Three peaks of radioactivity were released at cycles 5, 12, and 18 (Fig. 16). The peaks at cycles 5 and 18 would result from peptides produced by cleavage after arginine at position 77, and lysine at position 37, respectively, and thus support our reading frame assignment. There are three possible explanations for the peak at cycle 12. First, there could be a methionine in a putative signal sequence. Second, there could be a methionine in the secreted form of SC. Third, because the carboxy-terminal sequence of the cleaved SC form is not yet known, we do not know at what position the cleavage of the SC precursor occurs; it is possible that this cleavage occurs in a region amino-terminal to that coded for by the sequences in p32.23. Hence, the methionine released in position 12 may be located between the COOH-terminus of mature SC and the sequence represented in p32.23.

5.3 Discussion.

The most striking feature of the sequence is a stretch of 23 uncharged, predominantly non-polar amino acids (amino acids 38-60), preceded

by a lysine (residue 37), and followed immediately by the basic sequence Arg-Ala-Arg-His-Arg (amino acids 61-66). These features are typical of membrane spanning segments from a variety of transmembrane proteins (74-79). It has been proposed that most membrane spanning segments are in an alpha-helical configuration (74, 78). Residues 46 to 60 certainly could be in such a structure. However, residue 45 is a proline, which is a strong helix breaker (81). [Proline has in fact been found in the membrane spanning segments of bacteriorhodopsin (92)]. These results suggest, but do not prove, that amino acids 38 to 60 constitute the membrane-spanning segment of the SC precursor. However, we cannot rule out that the precursor contains other membrane spanning segments that are further upstream and coded for by sequences not contained in p32.23. in clone 32.23.

The proposed cytoplasmic domain consists of 103 predominantly hydrophilic amino acids with a total molecular weight of 11,120. This is reasonably consistent with our previous estimate that about 15 kilodaltons at the carboxy-terminus of the SC precursor could be digested by proteases acting at the cytoplasmic surface of the membrane. The proteolysis data did not indicate whether this portion of the molecule might loop back into the lipid bilayer, or if it were a soluble, globular domain. The abundance of charged residues (16 basic residues and 20 acidic residues) as well as uncharged but hydrophilic residues (6 asparagines and glutamines, and 14 threonines and serines) suggests that this COOH-terminal portion of the molecule is indeed soluble. However, residues 140-154 contain no charged amino acids (although five hydrophilic residues) and might be inserted into the lipid bilayer.

Figure 11.

Translation of mRNAs selected by hybridization to p32.23 DNA.

Hybridization-selection, translation, immunoprecipitation, and product analysis were performed as described in Chapter 2. In lane 1, translation products of unfractionated rabbit liver mRNA (from rabbit B) were immunoprecipitated with goat antiserum to rabbit secretory IgA. This antiserum immunoprecipitates the same SC translation products as the antiserum used in Chapter 3. The bands around M_r =90-93,000 and M_r =70,000 are the primary translation products of SC. Lanes 2-4 were from translation of mRNAs selected by hybridization of total rabbit liver RNA to plasmid DNAs immobilized on nitrocellulose filters. Lane 2 was a control for specificity of hybridization in which PBR 322 DNA was immobilized on the filter and the translation products of the selected mRNAs were immunoprecipitated with antiserum to rabbit secretory IgA. Lane 3 was a control for specificity of immunoprecipitation in which the translation products of mRNAs selected by p32.23 were immunoprecipitated with non-immune goat serum. In lane 4 the products of mRNAs selected by hybridization to p32.23 DNA were immunoprecipitated by antiserum to rabbit secretory IgA. In lane 5-7 the same experiments were performed as in lanes 2-4, respectively, except that total lactating mammary RNA was used instead of total liver RNA.

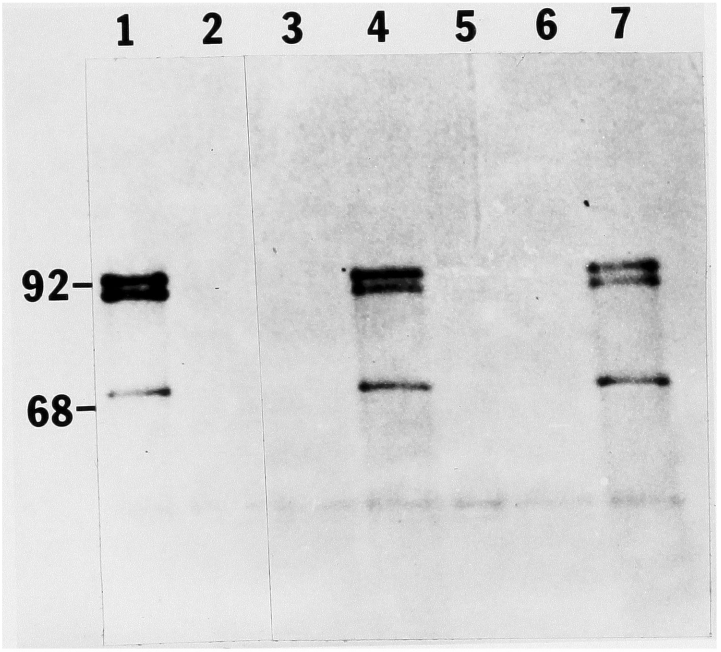

1
2
3
4
5
6
7
92
68

Figure 12

Analysis of SC mRNAs.

As described in Chapter 2, 1 ug of total poly(A)-containing mRNA from either liver (lane 1) or lactating mammary (lane 2) was fractionated on an agarose-formaldehyde gel, transferred to nitrocellulose, and probed with nick translated p32.23. The positions of the 18S and 28S ribosomal RNA markers, run in a parallel lane, are indicated at the left margin.

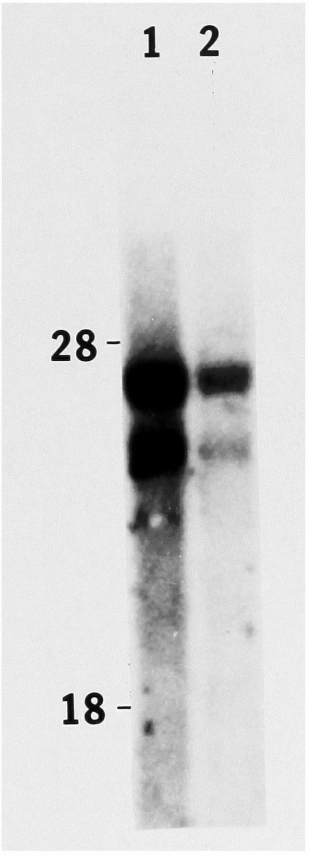

1
2
28
18

Figure 13

Restriction map and sequencing strategy of p32.23 insert DNA.

The scale at the top is in nucleotides. Only restriction sites used in the sequence analysis are shown. The G-C tails at both ends of the insert are indicated by \/\. Restriction fragments produced by the indicated enzymes were shotgun subcloned into M13 Mp8 or Mp9. Subclones were picked at random and sequenced. Arrows indicate direction and extent of sequencing of each subclone. The subclone indicated at the bottom line contained the entire insert, including G-C tails. We sequenced through the G-C tail at the 5' end of this clone by substituting reverse transcriptase for the Klenow fragment of DNA polymerase 1.

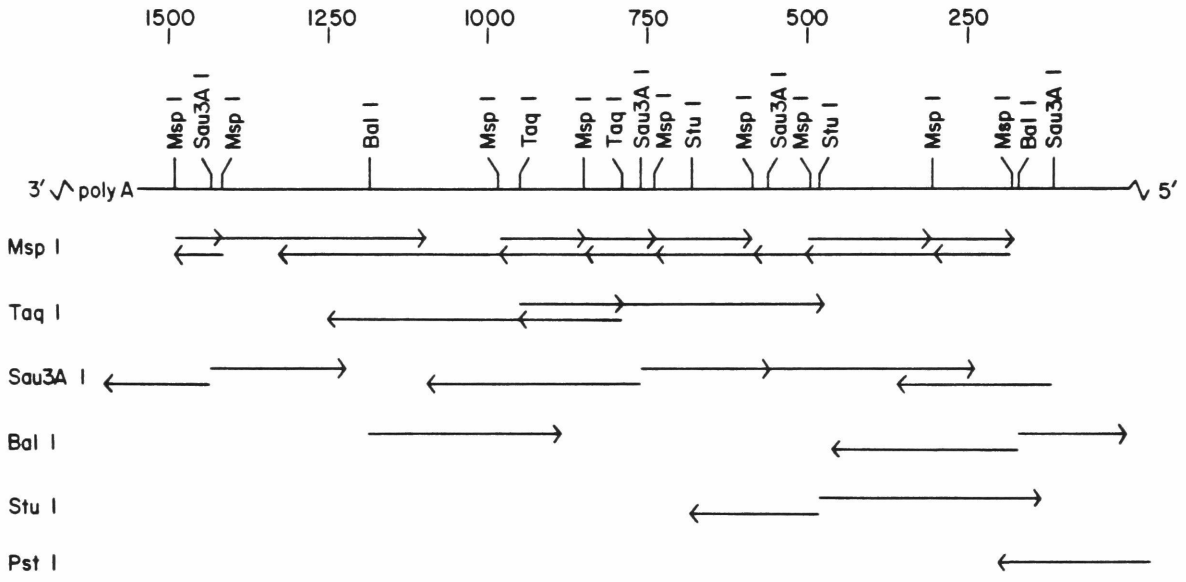

1500
1250
1000
750
500
250
Msp I
Sau3A I
Msp I
Bal I
Msp I
Taq I
Msp I
Taq I
Sau3A I
Msp I
Stu I
Msp I
Sau3A I
Msp I
Stu I
Msp I
Msp I
Bal I
Sau3A I
3'
poly A
5'
Msp I
Taq I
Sau3A I
Bal I
Stu I
Pst I

Figure 14.

DNA sequence of insert in p32.23 and translation into amino acid sequence of the carboxy-terminus of SC precursor.

Numbering of the nucleotide sequence is indicated on the left margin and numbering of the amino acid sequence is indicated on the right margin. The polyadenylation signal sequence at nucleotides 1538-1543 is indicated by overlining. The amino acid sequence of the putative membrane spanning segment (residues 38-60) is printed in lower case, while the remaining amino acids are in upper case. The proposed cytoplasmic domain was analyzed by the secondary structure prediction scheme of Chou and Fasman (81). Predictions are depicted as follows: alpha helix is underlined by a straight line, beta sheet by a wavy line, and beta turns by a dotted line. We wish to emphasize that these predictions are only probabilistic and that certain regions (e.g. amino acids 142-147) gave particularly ambiguous results.

```
    5'
1   CCT CGG CTC CTT GCG GAG GAG GTA GCA GTG CAG AGT GCG GAA GAC CCA GCC AGT GGG AGC
    PRO ARG LEU LEU ALA GLU GLU VAL ALA VAL GLN SER ALA GLU ASP PRO ALA SER GLY SER   20

61  AGA GCG TCT GTG GAT GCC AGC AGT GCT TCG GGA CAA AGC GGG AGT GCC AAA GTA CTG ATC
    ARG ALA SER VAL ASP ALA SER SER ALA SER GLY GLN SER GLY SER ALA LYS val leu ile   40

121 TCC ACC CTG GTG CCC TTG GGG CTG GTG CTG GCA GCG GGG GCC ATG GCC GTG GCC ATA GCC
    ser thr leu val pro leu gly leu val leu ala ala gly ala met ala val ala ile ala   60

181 AGA GCC CGG CAC AGG AGG AAC GTG GAC CGA GTT TCC ATC GGA AGC TAC AGG ACA GAC ATT
    ARG ALA ARG HIS ARG ARG ASN VAL ASP ARG VAL SER ILE GLY SER TYR ARG THR ASP ILE   80
    ————————~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

241 AGC ATG TCA GAC TTG GAG AAC TCC AGG GAG TTC GGA GCC ATT GAC AAC CCA AGC GCC TGC
    SER MET SER ASP LEU GLU ASN SER ARG GLU PHE GLY ALA ILE ASP ASN PRO SER ALA CYS   100
    ———————————————————————————————————————————————————————•••••••••••••••

301 CCC GAT GCC CGG GAG ACG GCC CTC GGA GGA AAG GAT GAG TTA GCG ACG GCC ACC GAG AGC
    PRO ASP ALA ARG GLU THR ALA LEU GLY GLY LYS ASP GLU LEU ALA THR ALA THR GLU SER   120
       ————————————————————————     •••••••••••••••     ————————————————————————~~

361 ACC GTG GAG ATT GAG GAG CCC AAG AAG GCA AAA CGG TCA TCC AAG GAA GAA GCC GAC CTG
    THR VAL GLU ILE GLU GLU PRO LYS LYS ALA LYS ARG SER SER LYS GLU GLU ALA ASP LEU   140
       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

421 GCC TAC TCA GCT TTC CTG CTC CAA TCC AAC ACC ATA GCT GCT GAG CAC CAA GAT GGC CCC
    ALA TYR SER ALA PHE LEU LEU GLN SER ASN THR ILE ALA ALA GLU HIS GLN ASP GLY PRO   160
    ——— ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

481 AAG GAG GCC TAG GCA CAG CCG GCC ACC GCC GCC GCC GCC ACC GCC GCC GCC GCC GCC ACC
    LYS GLU ALA stop
    •••••••

541 TGT GAA AAT CAC CTT CCA GAA TCA CGT TGA TCC TCG GGG TCC CCA GAG CCG GGG GCT CAA

601 CCG CCC TGC ACC CCC CAT GTC CCC ACC ACC TAA ACT TCC CTA CCT GTG CCC AGA GGT GTG

661 CTG GTC CCC TCC TCC ACG GCA TCC AGG CCT GGC TCA ATG TTC CCG TTG GGG TGG GGG TGT

721 GAG GGG TTC CTA CTT GCA GCC CGG TTC TCC CGA GAG AAG CTA AGG ATC CAG GTC CTG AGG

781 GAG GGG CCT CTC GAA GGC AGA CAG ACC AGA GAG GGG GGA GGA GCC CTT GGA TGG GAG GCC

841 AGA GGC GCT TTC CGG CCA CCC CCT CCC TCC CTG CCC CCA CCC TCC TTC CTT CAT TCA AAA

901 GTC CCA GTG GCT GCT GCC TAG GGT CCA GGC GCT GGC CGC ACG CCT CCT CGA AGC CGT TGT

961 GCA AAC ATC ACT GGA GGA AGC CAG GGC TCC TCC CGG GCT GTG TAT CCT CAC TCA GGC CTC

1021 CTG TCC TCC CCA GTA TCA GGA GAT GTC AAG CGT CTG AAG GCT GTG TGC CCT GGG CGT GTC

1081 TGC AAG TCA CCC CAG ACA CAT GTT CTC GCC ATT TTA CAG ATG AGA ACA CTG AGG TTG TAC

1141 TCA AGG GCA CCC TGC GAG ATG GAG CAA CAG CAA AGT AGA TGG GCT TCT GCT GTC CTC TTG

1201 GCC AGA GGT CTC TCC ACA GGA GCC CCT GCC CCT GTA GGA AGC AGA GTT TTA GAA CAT GGA

1261 AGA AGA AGA GGG GGA TGG CCC TGG ACG CTG ACC TCT CCC AAG CCC CCA CGG GGG AAA AGG

1321 CCC CCT CCT TTT CTG TCA CTC TCG GGG ACC TGC GGA GTT GAG CAT TCG TGC CCC GTG TGT

1381 CTG AAG AGT TCC CAG TGG AAA GAA GAA AAG AGG GTG TTT GTC AGT GCC GGG GAG GGC CTG

1481 ATC CCC AGA CAG CTG AAG TTT AAG GTC CTT GTC CCT GTG AGC TTT AAC CAG CAC CTC CGG

1501 GCT GAC CCT TGC TAA CAC ATC AGA AAT GTG ATT TAA TCA TTA AAC ATT GTG ATT GCC ACT

1561 GGG  poly(A)
          3'
```

Figure 15.

Partial amino acid sequence analysis of cell-free synthesized SC.

SC was translated in vitro using [^{35}S]methionine as the labeled amino acid. The M_r =90,000 band was purified by immunoprecipitation and SDS-polyacrylamide gel electrophoresis. This protein was digested with trypsin, and the total mixture of tryptic peptides was subjected to automated Edman degradation. The data presented are radioactivity released at each sequencer cycle. The low apparent repetitive yield is probably due to washout of the short peptides from the sequencer cup. In particular, the peptide producing the peak of radioactivity in cycle 18 is derived from amino acids 38-61; this peptide is unusually hydrophobic and is probably very poorly retained in the sequencer cup.

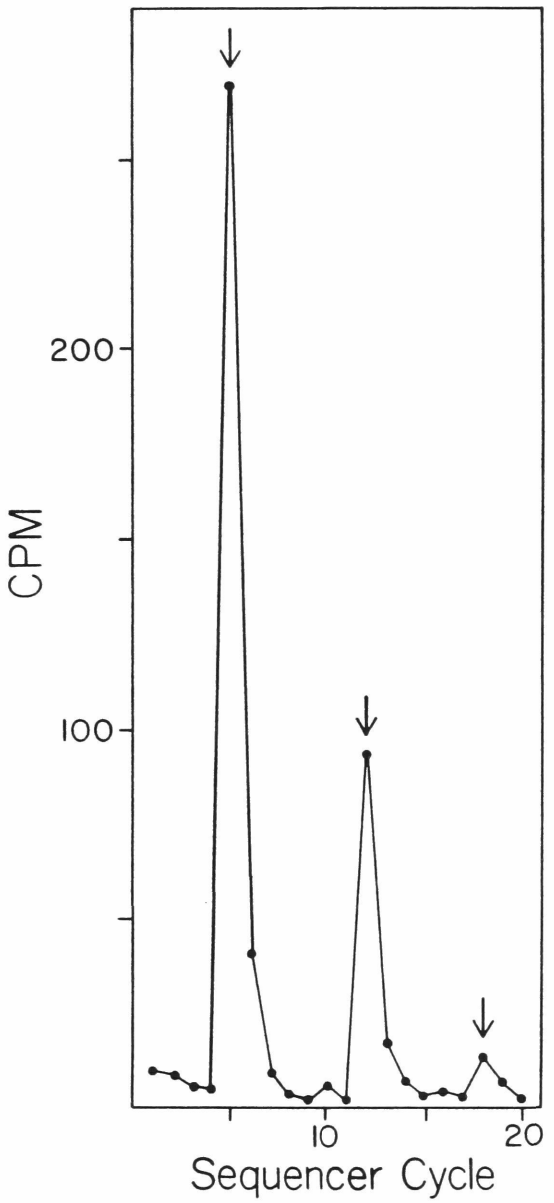
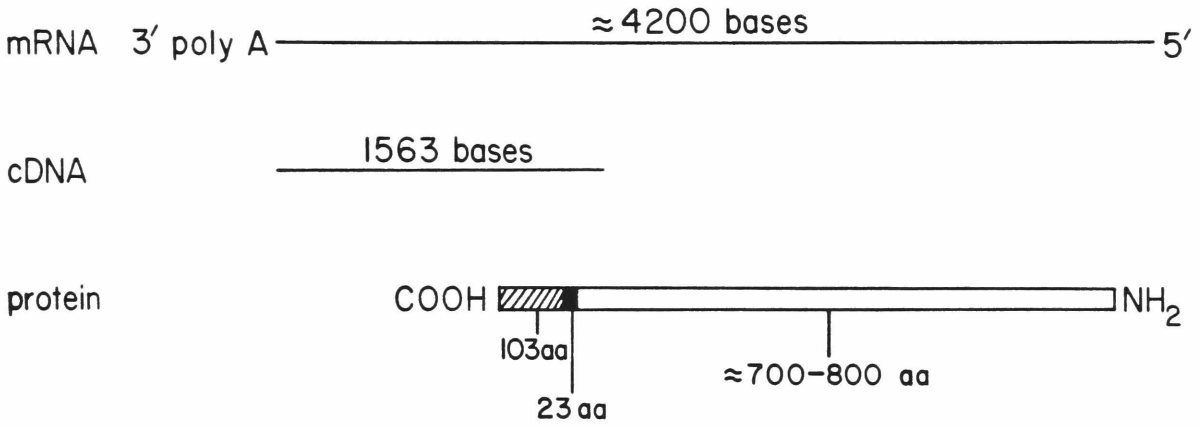

200
100
CPM
10
20
Sequencer Cycle

Figure 16.

Proposed relationship of SC mRNA, cDNA cloned in p32.23, and SC.

SC mRNA is depicted at the top, the cDNA cloned in p32.23 in the middle, and the SC precursor at the bottom. The figure represents one of the M_r =90-93,000 SC precursor molecules and its corresponding mRNA. Numbers above the mRNA and the cDNA indicate number of nucleotides. Numbers followed by "aa" below the SC protein indicate number of amino acids. The proposed cytoplasmic domain of SC is indicated by cross-hatching and the membrane spanning segment by a solid bar. The estimates of the number of nucleotides in the SC mRNA, and especially of the number of amino acids in the precursor and secreted forms of SC are only approximate. Consequently, the estimate of the size of the 5' untranslated region of the mRNA is very inexact.


mRNA 3′ poly A
≈4200 bases
5′
cDNA
1563 bases
protein
COOH
NH2
103aa
23 aa
≈700–800 aa

6. Conclusions

The central findings of this work are (i) that SC is made as a larger transmembrane precursor which has a sizable (11-15 kilodaltons) cytoplasmic, COOH-terminal domain, and (ii) that SC found in secretions is a proteolytic fragment of this precursor, derived from its ectoplasmic, pIg binding domain and containing its NH_2-terminus. These findings provide the protein structural basis for how SC can function first as a membrane receptor for pIg at the basolateral surface of the epithelial cell and later be a soluble, secreted protein.

Our data provide strong support for our proposed pathway of SC and pIg in the cell (Fig. 1): SC is made on the RER as a transmembrane protein with an 11-15 kilodalton portion remaining untranslocated across the membrane and thus exposed in the cytoplasm. The precursor passes through the Golgi apparatus. It then reaches the basolateral surface, where its ectoplasmic domain can bind pIg. We cannot rule out the possibility that the transmembrane precursor goes initially to both apical and basolateral surfaces and then is sorted to the basolateral surface, either via diffusion in the plane of the plasma membrane or via vesicular transport. The SC molecule, bound to pIg, is endocytosed and the endocytotic vesicle travels across the cell and is exocytosed at the apical surface. At some point, the ectoplasmic domain is proteolytically cleaved from the remainder of the molecule.

Several aspects of this model are unsettled. First, we have not directly localized any of these forms in the cell. On the basis of the cell-free membrane integration studies and by analogy to other membrane glycoproteins, the high-mannose form produced first in the cell-labelling experiments is probably in the RER. Similarly, the processing of the oli-

gosaccharides to the complex type probably occurs in the Golgi apparatus. However, we have not directly demonstrated that the transmembrane precursor is actually found on the basolateral surface of the cell. More importantly, we do not know where cleavage of the precursor occurs. Cleavage before endocytosis at the basolateral surface is unlikely, since otherwise SC could diffuse into the extracellular fluid and SC is not normally found in plasma. Our pulse-chase data in colon cancer cells indicated that the cleaved form of SC is found associated with the cells long before it is released into the media and this suggests that cleavage occurs inside the endocytotic vesicle. Alternatively, cleavage might occur after exocytosis and the cleaved SC could remain bound to the apical surface. However, cytochemically SC is not detected at the apical cell surface of these colon cancer-derived cells (23). In liver, on the other hand, SC and IgA are detected bound to the bile canalicular surface (18) and it is possible that cleavage in liver occurs after exocytosis.

Second, we do not know what becomes of the membrane-spanning and cytoplasmic portions of the precursor after cleavage. Our antibodies were raised against SC from secretions and we therefore do not have a handle on the remainder of the molecule. Presumably it is rapidly degraded.

Third, we do not know why rabbit SC is heterogeneous. All of the forms are transmembrane proteins with similarly sized cytoplasmic domains and all can bind pIg. Humans have only one form of SC, so the heterogeneity in rabbits may be unessential.

Fourth, although it appears that transcytosis and processing of SC may occur even in the absence of its ligand, pIg, the process may be accelerated by the binding of ligand at the basolateral surface. Isolated rat liver perfused in the absence of pIg continues to secrete SC in the bile (67).

Humans deficient in IgA have free SC in their secretions, although in this case, IgM substitutes for IgA (15).

We must also consider additional functions for SC. SC has long been thought to protect IgA from proteolytic degradation in secretions (82). In the case of the human genetic variant of IgA, IgA_2 A2m(1) [formerly designated $AM_2(+)$], which lacks heavy chain-light chain disulfide bridges, the SC also stabilizes the molecule against dissociation (83). SC might also serve to bind pIg to another receptor. Such a receptor might be present on the apical surface of duct rather than acinar cells and thus would function in keeping much of the externally exposed glandular epithelial surfaces coated with pIg. In fact, after it has been transported by hepatocytes, IgA seems to remain associated with biliary epithelium (18). Furthermore, the binding sites for SC found in rabbit mammary cells may represent binding to tubular cells and not the membranes of acinar cells (22).

Several aspects of the pathway of SC make it of general interest in studying sorting during intracellular protein traffic. In receptor mediated endocytosis (24,25) the receptor protein must localize in clathrin coated pits on the plasma membrane, and then after endocytosis travel one of several pathways, such as recycling to the plasma membrane, degradation in the lysosome, or transcellular transport (84). In the rat hepatocyte, for instance, the IgA-SC precursor complex and the asialoglycoprotein receptor are endocytosed in the same coated pit and are initially in the same endocytotic vesicle (85). (Presumably, SC and other receptors are similarly endocytosed together in liver and other tissues.) Yet, after endocytosis in a common vesicle, the various receptors and ligands follow different pathways: The asialoglycoprotein receptor recycles to the plasma membrane; its ligand dissociates from the receptor and is delivered to lysosomes and de-

graded; SC and pIg are transcytosed and discharged into bile. [Apparently, in a competing process, IgA can also bind to and be taken up by the asialoglycoprotein receptor (86)].

Polarized epithelial cells also face the problem of directing proteins to the appropriate cell surface. Certain viruses, for example, bud only from one cell surface or the other (87). The pathway for SC is unusual in that it moves first to the basolateral surface and then to the apical.

The precursor of SC is unique among receptors in that it is cleaved and its ectoplasmic domain is discharged with the ligand. It is possible that SC is continually inserted, endocytosed, and reinserted into both surfaces of the cell at random and that the unidirectionality of the transport process is established by the protease which cleaves the precursor being localized to the apical surface. However, it would be difficult to account for the speed and efficiency of pIg transport by this model. Furthermore, at least in colon cells, cleavage probably occurs in the endocytotic vesicle. Finally, this stochastic model does not account for how the differences in composition of the two surfaces are maintained.

We know very little about the mechanisms which direct membrane proteins to their appropriate locations in the cell (91). In general we assume that at least some steps in sorting occur by interaction of the membrane protein with other protein(s) in the cytoplasm. This most likely occurs via a direct interaction of the cytoplasmic tail of the membrane protein with these other cytoplasmic protein(s), although it might occur indirectly via an intermediary transmembrane protein(s) which binds to some other portion of the protein being transported. The information for sorting may be contained in one or more sorting sequences or other discrete structural features of the protein being sorted, which specify particular

steps in the process (88).

We therefore determined the structure of the cytoplasmic tail of the SC precursor and examined it for clues as to how it might function as an effector domain for guiding the molecule through the cell. As discussed in Chapter 5, except for residues 140-154, the cytoplasmic tail is very charged and hydrophilic and thus probably does not loop into the lipid bilayer, but rather is a soluble domain in the cytoplasm.

We also compared this cytoplasmic tail with those of other transmembrane proteins. A number of proteins span the membrane several times and contain large portion exposed on the cytoplasmic surface, such as the Ca^{+2} ATPase of sarcoplasmic reticulum (89), the acetylcholine receptor 66), and the erythrocyte Band 3 (90). All of these proteins function in transport of ions across the membrane and their substantial cytoplasmic domains may be involved in this process. Their cytoplasmic domains may also be involved in anchoring the proteins to the cytoskeleton, as in the case of Band 3 (90).

The SC precursor, in contrast, probably spans the membrane only once, although we cannot rule out that it spans the membrane several times. We tabulated in Fig. 17 a representative selection of other plasma membrane proteins which span the membrane once (74, 76-79). We included the only other receptor involved in endocytosis which has been sequenced, i.e. the chicken hepatic lectin, a receptor for clearance of N-acetyl-glucosamine-terminated proteins from the plasma (75). Although it has not been sequenced, the receptor for endocytosis of transferrin was included, because it has been shown by proteolysis to contain a cytoplasmic domain of about five kilodaltons (80). The striking feature is that the SC precursor tail is much larger than those of these other proteins. Furthermore, aside from

the general cluster of basic residues immediately following the membrane spanning segment, we discern no obvious sequence homology of the SC precursor tail with these other tails.

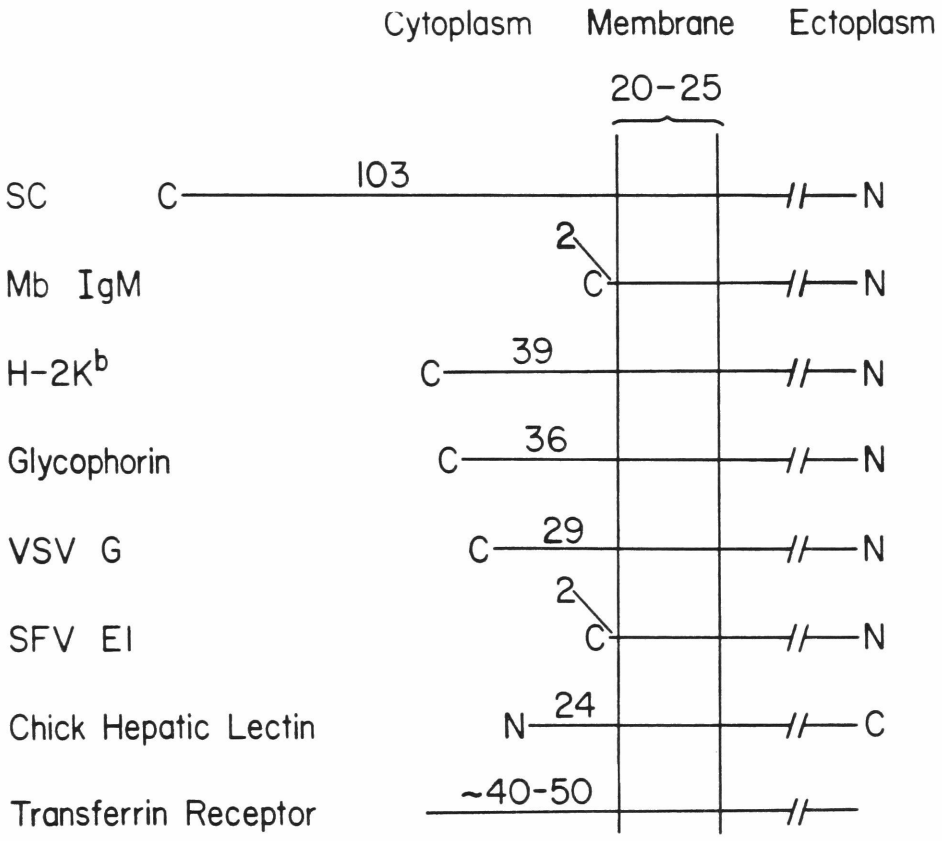
In the absence of X-ray crystallographic data, we cannot definitively compare the three-dimensional structure of these cytoplasmic domains. However, we did apply the empirical secondary structure prediction scheme of Chou and Fasman (81), which is generally 70-80% accurate, to the various tails depicted in Fig. 17. Aside from the SC precursor tail, the other tails were predicted to have little alpha helix content, which is not surprising in view of their small size. In contrast, the SC precursor tail was predicted to be mostly alpha helix (56%), as depicted in Fig. 14. Several stretches of beta sheet and beta turns were also predicted.

The large size and possible secondary structure of the tail of the SC precursor offer a tantalizing glimpse into how it may function in guiding the molecule through the cell. Since we have cloned DNA complementary to the mRNA for this portion of the molecule, it may now be possible to use recombinant DNA technology to analyze how this occurs.

Figure 17.

Comparison of cytoplasmic tails of selected proteins presumed to span the plasma membrane once.

The sizes of the cytoplasmic tails are given in number of amino acids and are drawn to scale. The ectoplasmic domains are not drawn to scale. The locations of the amino and carboxy termini with respect to the lipid bilayer are indicated by "N" and "C", respectively. Proteins are: SC, membrane-bound IgM (78), mouse histocompatibility antigen H-2K^{b} (77), human erythrocyte glycophorin (74), vesicular stomatitis glycoprotein, G (79), semliki forest virus glycoprotein E1 (76), chicken hepatic lectin (75), and transferrin receptor (80). (The transferrin receptor has not been sequenced. It is also not known where its amino and carboxy termini are located, nor even whether it spans the membrane only once.)


Cytoplasm
Membrane
Ectoplasm
20-25
SC
C
103
N
Mb IgM
2
C
N
H-2K^b
C
39
N
Glycophorin
C
36
N
VSV G
C
29
N
SFV E1
2
C
N
Chick Hepatic Lectin
N
24
C
Transferrin Receptor
~40-50

References

1. Tomasi, T.B., and Grey, H.M. (1972) Prog. Allergy 16, 81-213

2. Tomasi, T.B., Tan, E. M., Solomon, A., and Prendergast, R. A. (1965) J. Exp. Med. 121, 101-124

3. Cunningham-Rundles, C. (1978) in Immunoglobuliins (Litman, G.W., and Good, R.A., eds) pp. 155-171 (Plenum Medical, New York)

4. South, M.A., Cooper, M.D., Wollheim, F.A., Hong, R., and Good, R.A. (1966) J. Exp.Med. 123, 615-627

5. Brandtzaeg, P., and Baklien, K. (1977) in Immunology of the Gut (Porter, R., and Knight, J., eds) pp. 77-108 (Elsevier, Amsterdam)

6. Brandtzaeg, P. (1974) J. Immunol. 112, 1553-1559

7. Brown, W.R., Isobe, Y., and Nakane, P. (1976) Gastroenterology 71, 985-995

8. Brown, W.R., Isobe, K., Nakane, P., and Pacini, B. (1977) Gastroenterology 73, 1333-1339

9. Kraehenbuhl, J.P., Racine, L., and Galardy, R.E. (1975) Ann. N.Y. Acad. Sci. 254, 190-202

10. Orlans, E., Peppard, J., Reynolds, J., and Hall, J. (1978) J. Exp. Med. 147, 588-592

11. Crago, S.S., Kulhavy, R., Prince, S. J., and Mestecky, J. (1978) J. Exp. Med 147, 1832-1837

12. Brandtzaeg, P. (1978) Scand. J. Immunol 8, 39-52

13. Jackson, G.D.F., Lemâitre-Coelho, I., Vaerman, J.-P., Bazin, H., and Beckers, A. (1978) Europ. J. Immunol. 8, 123-126

14. Socken, D. J., Jeejeebhoy, K.N., Bazin, H., Underdown, B. J. (1979) J. Exp. Med. 150, 1538-1548

15. Orlans, E., Peppard, J., Fry, J. F., Hinton, R. H., and Mullock, B.M. (1979) J. Exp. Med. 150, 1577-1581

16. Fisher, M. M., Nagy B., Bazin, H., and Underdown, B. J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2008-2012

17. Lemâitre-Coelho, I., Altamivano, G. A., Barranco-Acosta, C., Meykens, R., and Vaerman, J.-P (1981) Immunology 43, 261-270

18. Takahashi, I., Nakane, P.K., and Brown, W.R. (1981) J. Immunology 128, 1181-1187

19. Renston, R.H., Jones, A. L., Christiansen, W. D., Hradek, G. T., and

Underdown, B. J. (1980) Science 208 , 1276-1278

20. Mullock, B. M., Hinton, R.H., Dobrota, M., Peppard, J., and Orlans, E. (1979) Biochim. Biophy Acta 587, 381-391

21. Mullock, B. M., Hinton, R. H., Dobrota, M., Peppard, J., and Orlans, E. (1980) Biochem. J. 190 , 819-826

22. Kühn, L. C., and Kraehenbuhl, J.-P. (1979) J. Biol. Chem. 254, 11072-11081

23. Nagura, H., Nakane, P., and Brown, W.R. (1979) J. Immunol. 123, 2359-2368

24. Goldstein, J.L., Anderson, R. G. W., and Brown, M.S. (1979) Nature 279, 679-685

25. Steinman, R. M., Mellman, I.S., Muller, W.A., and Cohn, Z.A. (1983) J. Cell Biol. 96, 1-27

26. Kuhn, L.C., and Kraehenbuhl, J.P. (1979) J. Biol. Chem. 254, 11066-11071

27. Mostov, K. E., Kraehenbuhl, J.-P., and Blobel, G. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 7257-7261

28. Mostov, K.E., and Blobel, G. (1982) J. Biol. Chem. 257, 11816-11821

29. Kobayashi, K. (1971) Immunochemistry 8, 785-800

30. Laemmli, U.K. (1970) Nature (London) 227, 680-685

31. Bonatti, S., Cancedda, R., and Blobel, G. (1979) J. Cell Biol. 80, 219-224

32. Aviv, H., and Leder, P. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 1408-1412.

33. Buel, G.N., Wickens, M.P., Payvar, F., and Schimke, R.T. (1978) J. Biol. Chem. 253, 2471-2482

34. Shields, D., and Blobel, G. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 2059-2063

35. Shields, D. and Blobel, G. (1978) J. Biol. Chem. 253, 3753-3756

36. Bonner, W. M., and Laskey, R.A. (1974) Eur. J. Biochem. 46, 83-88

37. Mostov, K.E., DeFoor, P., Fleischer, S., and Blobel, G. (1981) Nature (London) 292, 87-88

38. Huang, S. W., Fogh, J., and Hong, R. (1976) Scand. J. Immunol., 263-268

39. Erickson, A. H., Conner, G.E., and Blobel, G. (1981) J. Biol. Chem.

256, 11224-11231

40. Erickson, A.H., and Blobel, G. (1979) J. Biol. Chem. 254, 11771-11774

41. Tarentino, A.L., Trimble, R.B., and Maley, F. (1978) Methods Enzymol. 50, 574-580

42. Norgard, M.V., Tocci, M.J., and Monohan, J.J. (1980) J. Biol. Chem. 255, 7665-7672

43. Birnboim, H.C., and Doly, J. (1979) Nucleic Acids Res. 7, 1513-1523

44. Clewell, D.B., and Helinski, D.R. (1969) Proc. Natl. Acad. Sci. U.S.A. 62, 1159-1166

45. Ricciardi, R.P., Miller, J.S., and Roberts, B.E. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 4927-4931

46. Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) Molecular Cloning (Cold Spring Harbor Laboratory, Cold Spring Habor, N.Y.)

47. Rigby, P.W.J., Dieckman, M., Rhodes, C., and Berg, P. (1977) J. Molec. Biol. 113, 237-242

48. Grunstein, M. and Hogness, D. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 3961-3965

49. Derman, E., Krauter, K., Walling, L., Weinberger, C., Ray. M., and Darnell, J. (1981) Cell 23, 731-739

50. Sanger, F., Nicklen, S., and Coulson, A. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463-5467

51. Bethesda Research Laboratories (1980) M13 Cloning Dideoxy Sequencing Manual, Bethesda, MD.

52. Messing, J., Crea, R., and Seeburg, P. (1980) Nucleic Acids Res. 9, 309-321

53. Kaput, J., Goltz, S., and Blobel, G. (1982) J. Biol. Chem. 257, 15054-15058

54. Karanthanasis, S., (1982) Focus (Bethesda Research Laboratories Newsletter) 4 6-7

55. Staden, R. (1977) Nucleic Acids Res. 4, 4037-4051

56. Blobel, G., Walter, P., Chang, C.N., Goldman, B., Erickson, A., and Lingappa, V.R. (1979) Symp. Soc. Exp. Biol. 33, 9-36

57. Katz, F.N., Rothman, J.E., Lingappa, V.R., Blobel, G., and Lodish, H. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 3278-3282

58. Bonatti, S., and Blobel, G. (1979) J. Biol. Chem. 254, 12261-12264

59. Hanover, J.A., and Lennarz, W.J. (1980) J. Biol. Chem. 255, 3600-3604

60. Bielinska, M. and Boime, I. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 1768-1772

61. Toneguzzo, F., and Ghosh, H.P. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 1516-1520

62. Lingappa, V.R., Lingappa, J.R., Prasad, R., and Ebner, K. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 2338-2342

63. Underdown, B.J., and Socken, D.J. (1978) in Secretory Immunity and Infection (McGhee, J.R., Mestecky, J., and Babb, J., eds) pp. 503-511 (Plenum, New York)

64. Knight, K., Rosenzweig, M., Lichter, E.A., and Hanly, W.C. (1974) J. Immunol. 112, 877-882

65. Kühn, L.C., and Kraehenbuhl, J.-P. (1981) J. Biol. Chem. 256, 12490-12495

66. Anderson, D. J., and Blobel, G. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 5598-5602

67. Mullock, B.M., Jones, R.S., and Hinton, R.H. (1980) FEBS Lett. 113, 201-205

68. Brandtzaeg, P. (1975) Immunochemistry 12, 877-881

69. Kornfeld, R., and Kornfeld, S. (1976) Ann. Rev. Biochem. 45, 217-237

70. Cunningham-Rundles, C., Lamm, M.E., and Franklin, E.C. (1974) J. Biol. Chem. 249, 5654-5657

71. Jung, A., Sippel, A.E., Grey, M., and Schutz, G. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 5759-5763

72. Hagenbuchle, O., Borey, R., and Young, R.A. (1980) Cell 21, 179-187

73. O'Daley, and Cebra, J.J. (1971) Biochemistry 10, 3843-3850

74. Tomita, M., and Marchesi, V.T. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 2964-2968

75. Drickamer, K. (1981) J. Biol. Chem. 256, 5827-5839

76. Garoff, H., Frishauf, A., Simons, K., Lehrach, H., and Delius, H. (1980) Nature 282, 236-241

77. Coligan, J.E., Kindt, T.J., Uehara, H., Martinko, J., and Nathanson, S.G. (1981) Nature 291, 35-39

78. Rogers, J., Early, P., Carter, C., Calame, K., Bond, M., Hood, L., and Wall, R. (1980) Cell 20, 303-312

79. Rose, J.K., Welch, W.J., Sefton, B.M., Esch, F.S., and Ling, N.C. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 3884-3888

80. Schneider, C, Sutherland, R., Newman, R., and Greaves, M. (1982) J. Biol. Chem. 257, 8516-8522

81. Chou, P.Y., and Fasman, G.D. (1978) Ann. Rev. Biochem. 47, 251-276

82. Lindh, E. (1975) J. Immunol. 114, 284-286

83. Jerry, L.M., Kunkel, H.G., and Adams, L. (1972) J. Immunol. 109, 275-283

84. Rodewald, R. (1979) J. Cell Biol. 85, 18-32

85. Courtoy, P.J., Quintart, J., Limet, J.N., DeRose, C., and Baudhuin, P. (1982) J. Cell Biol. 95, 425a

86. Stockert, R.J., Kressner, M.S., Collins, J.C., Sternlieb, I., and Morrel, A. (1982) Proc. Natl. Acad. Sci. U.S.A. 79, 6229-6231

87. Rodriguez-Boulan, E. R., and Sabatini, D.D. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 5071-5075

88. Blobel, G. (1980) Proc. Natl. Acad. Sci. U.S.A. 77, 1496-1500

89. Saito, A., Wang, C., and Fleischer, S. (1978) J. Cell Biol. 79, 601-

616

90. Branton, D., Cohen, C.M., and Tyler, J. (1981) Cell 24, 24-32

91. Palade, G. (1975) Science 189, 347-358

92. Engelman, D. M., Henderson, R., McLachlan, A. D., and Wallace, B. A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77 , 2023-2027

End