Biogenesis of the Polymeric IgA Receptor in Rat Hepatocytes.

I. Kinetic Studies of Its Intracellular Forms

ELIZABETH S. SZTUL, KATHRYN E. HOWELL,* and GEORGE E. PALADE
Department of Cell Biology, Yale University School of Medicine, New Haven, Connecticut 06510; and
** European Molecular Biology Laboratory, Heidelberg, Federal Republic of Germany*

ABSTRACT The polymeric IgA receptor (or secretory component [SC]) is a major biliary secretory protein in the rat. It was identified as an 80,000-mol-wt (80 K) glycoprotein by coprecipitation (with IgA) by anti-IgA antibodies (Sztul, E. S., K. E. Howell, and G. E. Palade, 1983, *J. Cell Biol.*, 97:1582–1591) and was used as antigen to raise anti-SC antibodies in rabbits. Pulse labeling with [^{35}S]cysteine in vivo, followed by the immunoprecipitation of solubilized total microsomal fractions with anti-SC sera, made possible the identification of three intracellular forms of SC (all apparently membrane proteins) and the definition of their kinetic and structural interrelations. At 5 min postinjection of [^{35}S]cysteine, a major band of M_r 105,000 was maximally labeled. This peptide lost radioactivity concomitantly with the appearance of a radioactive doublet of M_r 116,000 and 120,000 at 15–30 min postinjection. Loss of radioactivity from 116K paralleled increased labeling of the 120K peptide which appears to be the mature form of the receptor. The 105K form was sensitive to endoglycosidase H which converted it to a 96K peptide. The 116K and 120K forms were resistant to endoglycosidase H but sensitive to endoglycosidase F which converts them to 96K and 100K forms, respectively. Taken together, these findings support the following conclusions: (*a*) All rat hepatic SC forms are the products of a single gene; (*b*) all SC forms are N-glycosylated; (*c*) the 116K form is the result of the terminal glycosylation of the 105K form; and (*d*) the 120K peptide is probably produced by modifications at other sites than its complex oligosaccharide chains.

IgA in external secretions is normally associated with a glycoprotein known as secretory component (SC).[1] Recent immunoadsorption (19) and cell-free translation studies (23, 24) have shown that SC is synthesized as a larger transmembrane protein which is eventually converted to a smaller soluble entity and a membrane-associated domain. At present, it is assumed that SC is (*a*) synthesized by endoplasmic reticulum-attached polysomes (as a transmembrane protein); (*b*) transported through and modified in the Golgi complex (38); (*c*) inserted into the sinusoidal plasmalemma (26) where it acts as $(IgA)_2$ receptor (12, 21, 29, 37); (*d*) then endocytosed and transported by vesicular carriers to the bile canaliculus (26, 33, 45); and (*e*) finally, proteolytically cleaved shortly before, or upon, the fusion of the carrier vesicles with the biliary plasmalemma. The ectodomain of SC becomes a soluble protein secreted into the bile; the fate of the rest of the molecule, i.e., the hydrophobic stop transfer sequence and the hydrophilic endodomain, is currently unknown.

SC is synthesized at rates comparable to those of secretory proteins (41) and as such can be used as a convenient membrane protein marker to study the extent of overlap between intracellular pathways involved in the transport of secretory and membrane proteins from their sites of synthesis to their final functional destination. We have previously reported kinetic evidence that SC is retarded, in respect to albumin, during transport from its site of synthesis to the bile canaliculus (41). Furthermore, we have presented preliminary results

---

[1] Abbreviations used in this paper: endo F and endo H, endoglycosidases F and H, respectively; K, × 1,000 mol wt (e.g., 105K, 105,000-mol-wt [form]); NC, nitrocellulose; SC, secretory component; SRA, specific radioactivity; SUP, supernatant; TM, total microsomes.

indicating that antibodies raised against biliary SC recognize two intracellular forms of SC (M_r 94,000 and 116,000) in $[^3H]$fucose-labeled Golgi membrane preparations (41).

In this paper we document the existence of three intracellular forms of SC in rat hepatocytes and define their structural and kinetic relationships. The results obtained indicate that all three forms are successive modifications of a single gene product.

MATERIALS AND METHODS

Reagents and supplies were purchased from the following sources: general-use biochemicals, Triton X-114, phenylmethylsulfonyl fluoride, pepstatin, leupeptin, and tosyl-L-lysine chloromethyl ketone from Sigma Chemical Co. (St. Louis, MO); $[^{35}S]$cysteine (1,080 μCi/mmol) from Amersham Corp. (Arlington Heights, IL); $Na^{125}I$ and ^{125}I-labeled protein A from New England Nuclear (Boston, MA); protein A-Sepharose from Pharmacia Fine Chemicals (Piscataway, NJ); Nembutal (used for anesthesia) from Abbott Laboratories (North Chicago, IL); U-bottom microtiter plates from Falcon Labware, Div. of Becton Dickinson & Co. (Oxnard, CA); and nitrocellulose (NC) filters from Schleicher & Schuell (Keene, NH). Antibodies to rat albumin were raised in rabbits by K. Howell at the European Molecular Biology Laboratory in Heidelberg, Federal Republic of German.

Antibody Production: Bile was collected via a cannula (PE-50 tubing, Clay Adams, Parsippany, NJ) inserted into the common bile duct of anesthetized rats. Samples were treated with 10 vol of ice-cold acetone to precipitate bile proteins prior to SDS PAGE separation. After completion of electrophoresis, a gel region containing a broad band of M_r 80,000 was cut from unstained gels and prepared for injections according to Papermaster et al. (30). The identity of the antigen as SC was based on its co-precipitation with IgA, by an anti-IgA serum (41). Antibodies were produced in adult female rabbits as in reference 30 except that the antigen was injected intradermally at all times. Bleeding was from an ear artery or vein. The blood was allowed to clot, and the sera obtained were used in all subsequent experiments.

Immunochemical Tests: Enzyme-linked immunosorbent assays were performed by a modification (22) of the procedure of Rennard et al. (32); and solid-phase radioimmunoassays were carried out by the procedure of Howard et al. (16) modified as follows: 100 μl of each reagent solution was used, quenching was carried out with 12.5 mg/ml hemoglobin, and ~10,000 cpm of ^{125}I-labeled protein A were loaded per well.

Immunoprecipitations were carried out as in reference 41; the total fraction protein was kept constant, but anti-rat SC serum (rather than sheep anti-rat IgA serum) was used. The final concentration of SDS in the lysates used for immunoprecipitation was 0.4%.

Western blot overlays were performed as follows: upon completion, electrophoretograms were transferred (for 4 h at room temperature and constant 150-mA current) to NC filters which were immediately quenched in 12.5 mg/ml hemoglobin (2 h, room temperature) and then incubated overnight at room temperature with anti-SC sera (diluted 1:50 in 12.5 mg/ml hemoglobin). The filters were washed (twice for 10 min each time) with PBS, then with PBS containing 0.05% Nonidet P-40 (twice for 10 min), and finally rinsed (twice for 5 min) with PBS. Adsorbed IgGs were detected by incubating the filters in ^{125}I-labeled protein A (16×10^6 cpm in 200 ml of 12.5 mg/ml hemoglobin) for 4 h at room temperature, followed by washing (as above), drying, and autoradiography.

SDS PAGE and Fluorography: SDS PAGE and fluorography was carried out as in reference 41. Immunoprecipitated samples were processed as follows: protein A-Sepharose beads, washed as described (41), were pelleted and resuspended in 100 μl of 1M Tris-HCl, pH 6.8; 20 μl of 20% SDS and 10 μl of 0.2 M dithiothreitol were added; the mixture was boiled for 2 min, cooled, and supplemented with 20 μl of 60% sucrose containing bromophenol blue before being loaded on gels. Electrophoresis was carried out for ~12 h at room temperature at a constant current of 25 mA.

In Vivo Labeling of Animals: Preparation of Liver Cell Fractions: 160–180-gram male Sprague-Dawley rats (Charles River Breeding Laboratories Inc., Wilmington, MA) received 2 mCi of $[^{35}S]$cysteine (under ether anesthesia) by injection into the saphenous vein. At chosen intervals postinjection, the animals were anesthetized and their livers were removed for further processing. Liver lobes were placed in ice-cold 0.25 M sucrose and minced. The mince was washed free of blood and homogenized to give a ~30% (wt/vol) homogenate, which was centrifuged (7,000 *g* for 10 min) to give a supernatant (SUP 1) and a pellet, containing nuclei, mitochondria, and broken and unbroken cells (as seen by electron microscopy). SUP 1 was centrifuged (105,000 *g* for 90 min) to yield a final supernatant (SUP 2) and a total microsomal (TM) pellet, which contained all cellular particulates smaller than mitochondria. The pellet sedimenting at 7,000 *g* for 10 min accounted for 20–30% of the total radioactivity incorporated in the whole homogenate. The corresponding figures for SUP 2 and TM were ~40% and ~30%, respectively. In some experiments (see figure legends) the homogenization and subsequent isolation of TM fractions were carried out in the presence of the following protease inhibitors: 1 mM phenolmethylsulfonyl fluoride, 2 μg/ml pepstatin, 20 μg/ml leupeptin, and 1 mM tosyl-L-lysine chloromethyl ketone.

Triton X-114 Extraction: Samples of TM fractions, isolated from animals killed 30 min after injection of 2 mCi $[^{35}S]$cysteine, were immunoprecipitated with anti-SC sera, and the immunoprecipitates were either counted or processed as described by Bordier (2). Aliquots of the separated phases were re-immunoprecipitated with anti-SC sera, and the precipitates were solubilized and counted.

Trypsin Treatment: TM fractions were isolated from livers removed 30 min after injection of 2 mCi $[^{35}S]$cysteine, and 80-μl samples (~2 mg/ml protein) of resuspended fractions were treated with trypsin (final concentration: 0.5 mg/ml) at 0°C as in reference 24 in the absence or presence of 1% Triton X-100 (final concentration). At various time points, soybean trypsin inhibitor (final concentration: 0.31 mg/ml) was added; then the samples were solubilized and SC was immunoprecipitated with anti-SC antibodies.

Endoglycosidase Digestions: SC, isolated by immunoprecipitation, was digested with endo-*N*-acetylglucosaminidase H (endo H) according to Gumbiner and Kelly (14) or with endo-*N*-acetylglucosaminidase F (endo F) according to Elder and Alexander (10). Endo H was the kind gift of Dr. Robert Trimble (New York State Department of Health). Endo F was a gift from Dr. Steven Rosenzweig (Yale University).

Radioiodination: Bile proteins were radioiodinated as described (41).

Analytical Procedures: Protein was estimated using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond, CA), with BSA as the standard.

RESULTS

Antibody Characterization

ENZYME-LINKED IMMUNOSORBENT ASSAY AND RADIOIMMUNOASSAYS: The sera of immunized rabbits were surveyed for the presence of specific antibodies by enzyme-linked immunosorbent assay and radioimmunoassays. Both tests were positive when immune sera were tested against bile proteins (data not shown) and negative when preimmune sera were tested, or when rat brain microsomes were used as the primary adsorbent. Since these assays do not identify the antigen recognized by the immune antibody, Western blot overlays and immunoprecipitations were used to test for the presence of antibodies to specific bile proteins and to determine whether bile contains other cross-reaction proteins.

WESTERN BLOT IMMUNEOVERLAYS: A broad band corresponding to the original M_r 80,000 antigen and a less intense band of lower M_r (i.e., 70,000) were detected (Fig. 1 *A*, lane *4*). Longer exposures revealed additional minor bands of lower M_r, presumed to be degradation products of the 80,000-mol-wt (80K) protein. No reactive band was detected when nonimmune sera were used.

Immune sera were also tested for cross-reactivity with blood plasma proteins to check whether, during antigen excision from gels, contaminating peptides of plasma origin had been included (plasma proteins are known to be present in bile). No reactive bands were detected on NC transfers of plasma proteins[2] by overlay with the anti-SC antibody (Fig. 1 *A*, lane *2*).

IMMUNOPRECIPITATION: As shown previously (41), a major 80K band, corresponding to the original antigen, and a less intense 70K band could be immunoprecipitated from radioiodinated bile proteins (Fig. 1 *B*, lane *1*). We identified a

---

[2] The gel was intentionally overloaded to maximize the chances of detecting plasma contaminant in the original antigen preparation.

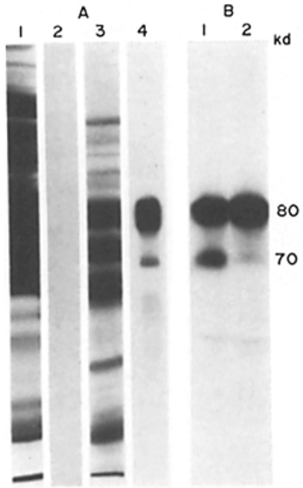

FIGURE 1 (*A*) Immune overlays with anti-SC sera of bile and blood plasma proteins. Samples of bile and blood plasma, processed through SDS PAGE (~200 μg of bile proteins or ~320 μg of plasma proteins loaded per well), were transferred to NC filters, and the latter were incubated with anti-SC serum and then with ^{125}I-labeled protein A. The dried filters were used for the autoradiographs shown in this figure. (Lane *1*) Pattern of plasma proteins stained with Coomassie Blue, gel before transfer;[2] (lane *2*) autoradiograph of the corresponding NC filter; (lane *3*) bile proteins stained with Coomassie Blue, gel before transfer; (lane *4*) autoradiograph of the corresponding NC filter. (*B*) Immunoprecipitation of bile proteins by anti-SC sera. Radioiodinated bile proteins were immunoprecipitated with either anti-SC or anti-albumin followed by anti-SC sera. The immunoprecipitates were resolved by SDS PAGE and the gels were processed for autoradiography. (Lane *1*) Bile proteins immunoprecipitated with anti-SC sera; (lane *2*) bile proteins immunoprecipitated with anti-SC serum after pretreatment with three cycles of anti-albumin serum.

major component of the 70K band as albumin,[3] on the basis of our ability to remove it by sequential incubation of labeled bile with anti-albumin antibodies. The subsequent incubation of the preadsorbed bile with anti-SC antibodies resulted in specific immunoprecipitation of the 80K peptide in amounts equivalent to those obtained without preadsorption; the levels of the 70K peptide were greatly reduced (Fig. 1 *B*, lane *2*). Since immunoverlay of bile proteins detects, in addition to the 80K antigen, a 70K band (Fig. 1 *A*, lane *4*), we assume that the 70K band detected by immunoprecipitation from preadsorbed bile represents a degradation product of the 80K band. Co-isolation of albumin during anti-SC immunoprecipitation could be due to either cross-reactivity of albumin with anti-SC antibodies or nonspecific binding of albumin to the immunosupport (albumin represents one of the major biliary proteins). Since, by immunoverlay, no reactive band was detected in electrophoretograms of serum (Fig. 1 *A*, lane *2*), we assume that the anti-SC antibody does not cross-react with albumin.

Survey of Cellular SC Forms

We have raised antibodies to rat bile SC based on the assumption that the latter represents the ectodomain (or part of it) of an originally transmembrane cellular protein (19, 23, 24); if so, secreted and membrane forms of SC might share common antigenic determinants. To test this assumption directly, we isolated TM fractions[4] under different experimental conditions (see the legend of Fig. 2), solubilized them, and separated their proteins by SDS PAGE. After electrophoresis, the gels were transferred to NC filters, and the presence of antigens, recognized by the anti-SC antibody, was checked by immunoverlay. A major band of M_r 70,000, a narrow band of M_r 105,000, and a broader band of M_r 116,000–120,000 were visible in frozen-thawed specimens (condition 1) (Fig. 2, lane *1*). The level of 70K peptide was greatly reduced in specimens processed immediately after isolation (condition 2) (Fig. 2, lane *2*), but many other peptides in the 94K–75K range were visible. To test whether these smaller peptides were degradation products of the 116K–120K band, fractions isolated in the presence of proteolytic inhibitors were processed through SDS PAGE, transferred to NC filters, and immunoverlayed with anti-SC sera. As shown in Fig. 2, lane *3*, a narrow 105K and a broad 116K–120K were the only bands detected by the antibodies; lower M_r peptides (94,000[5]–70,000) were practically absent. We conclude, therefore, that the lower M_r peptides are proteolytic fragments of the 105K and/or 116K–120K forms, the M_r 75,000–94,000 peptides observed under condition 2 being incomplete degradation products, and the 70K band seen under condition 1 representing an advanced degradation form. Consequently, all bands of M_r lower than 105,000 will not be considered in the analysis of subsequent experiments.

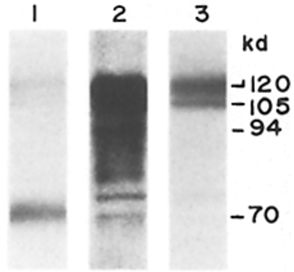

FIGURE 2 Intracellular SC forms. TM fractions were isolated in the absence or presence of protease inhibitors and assayed directly or stored frozen before analysis. TM protein samples (150 μg) were processed for SDS PAGE, the separated proteins were transferred to NC filters, and the latter were overlayed with anti-SC serum followed by ^{125}I-labeled protein A. (Lanes *1–3*) Autoradiographs of NC filters of TM proteins. (Lane *1*) TM, initially prepared without protease inhibitors, then frozen, stored, and finally thawed before analysis; (lane *2*) TM prepared without protease inhibitors and processed without freezing and thawing; (lane *3*) TM isolated in the presence of protease inhibitors.

Since TM fractions, containing both membrane and soluble (secretory) proteins, were used for the detection of intracellular SC forms, we treated these fractions with Triton X-114 to find out whether the SC precursors are membrane-integrated or soluble proteins. Triton X-114, homogenous at 0°C, has been shown to separate above 20°C into an aqueous and a detergent phase into which proteins with hydrophilic or hydrophobic properties preferentially partition (2). All SC forms (i.e., 105K and 116K–120K) were recovered in the detergent phase (Table I) indicating that they are integral membrane proteins.

These results demonstrated the presence of multiple membrane-bound antigens, which could be either the products of multiple genes or successive forms generated during the intracellular processing of a single gene product.

Pulse-Chase Conditions in Intact Animals

The presence or absence of precursor–product relationships among the different intracellular forms of SC could be investigated in vivo, if conditions approximating pulse-chase labeling could be established. Previous experiments (41) had shown that such conditions were obtained in living animals after intravenous administration of $[^3$H]fucose. To test

---

[3] In our electrophoresis system, rat albumin migrated with apparent M_r of 70,000 under reducing conditions.

[4] TM fractions contain vesicles derived from the endoplasmic reticulum, Golgi complex, and plasmalemma, in addition to vesicular carriers, that is, most if not all particulates expected to contain SC in their membranes.

[5] 94K was one of the SC forms labeled by $[^3$H]fucose and identified by immunoprecipitation in our first paper (41).

TABLE I

Triton X-114 Extraction of SC Forms

	In immunoprecipitates	Distribution
	cpm	%
Starting TM fraction	9,120	
First detergent phase	4,770	50.7
Second detergent phase	3,810	40.5
Final aqueous phase	220	8.7
Percentage recovery	96.5%	

Determinations were done in duplicate. Samples of a hepatic TM fraction isolated from a rat sacrificed 30 min after injection of 2 mCi [^{35}S]cysteine were solubilized and then immunoprecipitated with anti-SC sera. The immunoprecipitate was either counted or treated with TX-114 as in reference 2. Two detergent phases were employed (the first aqueous phase was washed with detergent) to complete extraction. SC was recovered from the separated phases by immunoprecipitation with anti-SC serum, and the radioactivity in each immunoprecipitated sample was determined. SDS PAGE analysis of the immunoprecipitates showed that the two detergent phases contained the same SC forms.

whether a similar situation could be approximated for labeled amino acids, we monitored the rate of clearance of acid-soluble radioactivity from the blood plasma following an intravenous injection of 2 mCi of [^{35}S]cysteine. 83 and 86% of the injected label were cleared from the circulation at 1 and 2 min postinjection, respectively; this value increased to 93% at 6 min postinjection and remained at that level for the duration of the experiment (data not shown).

To document more directly the degree to which pulse-chase conditions are approached within the liver cell population, we investigated the labeling kinetics of mixed hepatic proteins. Low-spin supernate (SUP 1) fractions (containing the cytosol and all membrane-bound organelles smaller than mitochondria) were prepared from animals killed at various times after the administration of 2 mCi [^{35}S]cysteine; their proteins were separated by SDS PAGE, and radiolabeled polypeptides were detected by fluorography. As shown in Fig. 3, a set of labeled proteins was detected 5 min after injection, reached specific radioactivity (SRA) peaks at 20 min postinjection, and then declined in intensity. It must be noted that whereas the rate of clearance of [^{35}S]cysteine from blood indicates a pulse of ~6 min, SRA of most cellular proteins appeared maximal at 20 min postinjection, indicating an intracellular pulse equivalent of ~20 min. Thus, there is rapid uptake of [^{35}S]cysteine into cells but considerable delay in its utilization. This finding must be taken into account in the interpretation of the kinetic data obtained in our subsequent experiments. It obliges us to rely more on the earliest time of appearance of different proteins (and on intervals between such times) than on times at which peaks of maximum SRA are detected and clearances are achieved.

Kinetics of SC Labeling

Since, with the reservations mentioned above, acceptable pulse-chase conditions can be obtained in vivo, we carried out a survey of the kinetics of labeling of the intracellular forms of SC in the hopes of elucidating whether the bands shown in Fig. 2 represent the precursor, intermediate, and final forms of a single protein, or whether all represent SC forms translated from different mRNAs. To this intent we prepared TM fractions at various times after injection of 2 mCi [^{35}S]cysteine, solubilized them, and separated SC from other proteins by immunoprecipitation. After SDS PAGE of the ensuing immunoadsorbed proteins, followed by fluorography, a number of labeled bands was detected (Fig. 4*A*).

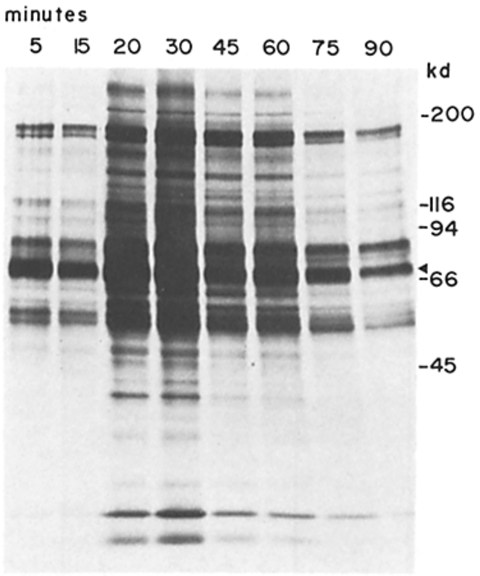

FIGURE 3 SDS PAGE analysis of radiolabeled SUP 1 proteins. SUP 1 fractions were isolated from the livers of rats sacrificed at various times after injection of 2 mCi [^{35}S]cysteine. Approximately 200 μg of fraction protein was loaded per well at each time point; the proteins were separated and the gel was processed for fluorography. Arrowhead shows the position of albumin.

At the earliest time point tested, i.e., 5 min postinjection, a major band of M_r 105,000 was already labeled. The SRA of this peptide was maximal at ~5 min after injection and decreased in intensity thereafter. The clearance of radioactivity from this protein was paralleled by the appearance at 30 min postinjection (or before) of a radioactive doublet of M_r 116,000 and 120,000. Additional experiments (see companion paper [42]) showed that the 116K form was already detected at (or before) 15 min postinjection, whereas the 120K species appeared at (or before) 30 min (Fig. 3 in the companion paper [42]). The two components of the doublet were better resolved when the immunoprecipitates were separated on a different gel (Fig. 4*B*). The 116K form was almost cleared at 45 min postinjection, at a time when the 120K peptide approached peak SRA. This last form was still detectable 90 min after injection.[6]

Since in this experiment (Fig. 4*A*) the TM fractions were isolated in the absence of proteolytic inhibitors, we assume, based on the results shown in Fig. 2, that the minor peptides of M_r 94,000 and 90,000 are proteolytic fragments generated (during our preparation procedure) from the higher M_r peptides. It should be noted that all SC forms previously detected in TM fractions by immuneoverlay (Fig. 2) were labeled during the course of our kinetic experiments in which immunoprecipitation was used to detect antigens. On the basis of these results, we conclude that the multiple bands see in Fig. 4 represent different stages in the intracellular processing of a single SC peptide.

Aliquots of TM immunoprecipitates were counted to estimate the amounts of SC present at each time point. The amounts increased up to 30 min (reflecting imperfect chase) and declined thereafter as expected from our previous data (41) which indicate that [^{35}S]cysteine-labeled SC begins to

---

[6] The kinetics of labeling have been consistently reproducible.

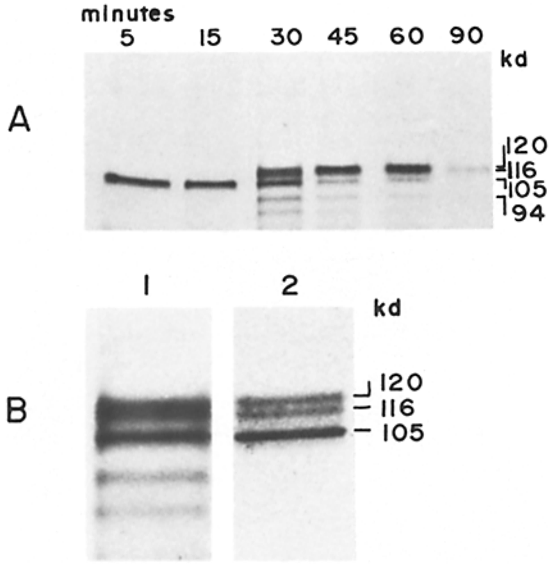

Figure 4 Appearance of radiolabeled SC in TM fractions. TM fractions were isolated, in the absence or presence of proteolytic inhibitors, from rats sacrificed at selected time intervals after an intravenous administration of 2 mCi [^{35}S]cysteine. SC polypeptides were isolated by immunoprecipitation from solubilized fractions, and aliquots of the immunoprecipitates were analyzed by SDS PAGE. (*A*) TM fractions isolated without protease inhibitors. (*B*) Lane *1*, as in *A*, lane marked *30*; Lane *2*, TM isolated in the presence of proteolytic inhibitors from rats killed 30 min postinjection of label.

appear in the bile past 35 min postinjection.

By immunoprecipitation, the first pellet contained the same three forms of SC, in the same relative proportion, as the TM fraction. The final supernate (SUP 2) contained no immunoprecipitable SC forms. These findings justify our use of TM as the appropriate preparation to study: it contains the bulk of intracellular SC, and no new or known SC form separates preferentially in the other complementary fractions.

The three SC forms were found in the same proportion at different times after injection of [^{35}S]cysteine in TM fractions prepared in the absence or presence of protease inhibitors, suggesting that no SC form is preferentially sensitive to endogenous proteases.

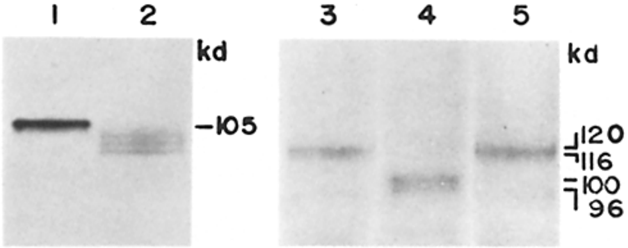

Figure 5 Sensitivity of SC forms to endoglycosidases. TM fractions were isolated from livers of rats sacrificed 15 or 30 min postinjection of 2 mCi [^{35}S]cysteine. SC was separated from the other proteins of the fraction by immunoprecipitation; one half of each immunoprecipitate was directly processed for SDS PAGE; the other half was treated with either endo H or endo F before processing for PAGE. (Lane *1*) Immunoprecipitate from TM isolated 15 min postinjection; (lane *2*) the same sample as in lane *1* after treatment with endo H; (lane *3*) immunoprecipitation from TM isolated 30 min postinjection; (lane *4*) the same sample as in lane *3* after treatment with endo F; (lane *5*) the same as in lane *3* after treatment with endo H.

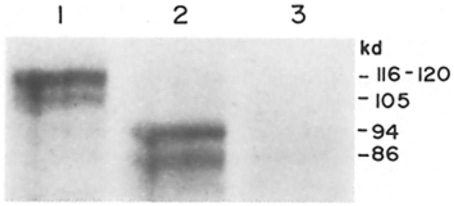

Figure 6 Trypsin cleaves the endodomain of SC forms. A TM fraction was isolated from the liver of a rat sacrificed 30 min postinjection of 2 mCi [^{35}S]cysteine. Samples were treated with trypsin in the absence or presence of 1% Triton X-100. SC was immunoprecipitated and analyzed by SDS PAGE. (Lane *1*) Immunoprecipitate from untreated microsomes; (lane *2*) immunoprecipitate from microsomes treated with trypsin; (lane*3*) immunoprecipitate from microsomes treated with detergent then by trypsin.

Endo H and Endo F Treatment of Precursor, Intermediate, and Mature SC Forms

To document whether the 105K peptide represents a newly synthesized precursor, we subjected it to endo H treatment. Since SC is known to contain N-linked complex oligosaccharide chains in man (31) and rabbit (38), its precursor is expected to be core glycosylated in the rough endoplasmic reticulum (20, 34, 44) and to be sensitive to endo H, an endoglycosidase known to cleave specifically the GlcNAc-GlcNAc bond of N-linked, mannose-rich oligosaccharide chains (46, 47). Endo H acts on the initial oligosaccharide $Glc_3Man_9GlcNAc_2$ as well as its sequentially processed forms, $Man_9GlcNAc_2$ to $Man_5GlcNAc_2$, but not on later intermediates (17). As shown in Fig. 5, lanes *1* and *2*, endo H treatment of the 105K protein generated three bands of lower M_r, indicating that this SC form is core—but not yet terminally—glycosylated. The presence of three bands is most likely due to incomplete removal of multiple oligosaccharide moieties which suggests that SC contains at least three asparagine-linked carbohydrate chains (human milk SC has been reported to contain six N-linked chains [31]). Both 116K and 120K SC forms were endo H resistant (Fig. 5, lane *5*), indicating that their high mannose core has been trimmed and that some (or all) of the terminal sugars have been added. To ascertain whether the 116K and 120K peptides represent successive forms generated during trimming of glucose and mannose residues and subsequent addition of terminal sugars, or whether they are forms produced by different modifications (e.g., addition of O-linked oligosaccharide chains [7] or sulfation [27]), we subjected both forms to endo F treatment. Endo F cleaves the GlcNAc-GlcNAc bond of glycoproteins containing high mannose or complex oligosaccharide chains (10) and can be used to determine whether the differences in mobility between 105K, 116K, and 120K peptides are due to modifications within the asp-N-linked carbohydrate portion of the molecule or involve other types of processing at different sites within the polypeptide chain. As seen in Fig. 5, lanes *3* and *4*, endo F treatment of the 116K–120K doublet resulted in two distinct bands of M_r 96,000 and 100,000, suggesting that the increase in M_r of the 120K form is not due to a different type of further processing. To test the relationship between 105K and 116K forms, we compared the M_r of the endo F-treated 116K peptide to that of the smallest peptide produced by endo H treatment of the 105K form. Both were found to be ~96K, indicating that the increase in M_r of the

116K species resulted from modifications within its N-linked oligosaccharide chains.

To provide partial evidence that all intracellular SC forms are transmembrane proteins that retain the same asymmetry of assembly during processing, TMs isolated from [^{35}S]cysteine-labeled livers were treated with trypsin. The results (Fig. 6) showed that the three forms (two of them not clearly resolved) were shortened by apparently the same mass, ~20kd, presumably as a result of the removal of an endodomain of comparable size. Similar size shifts have been observed in the rabbit liver and mammary SC (24) and in a human cell line SC (23).

DISCUSSION

A substantial body of information, obtained on various epithelial cells, hepatocytes included, has already established the role of SC as IgA receptor and transepithelial carrier (11, 21, 29), its specificity for polymeric IgA (6, 12, 37), the role of vesicular carriers in the transcellular transport of SC(IgA)$_2$ complexes (25, 45), and in the case of the liver, the discharge of a soluble form of SC (as free SC or SC(IgA)$_2$ complexes) into the bile (8). But data concerning the biosynthesis of the receptor and the kinetics of its intracellular transport and concomitant modifications are still limited and fragmentary (23, 24, 38). Our experiments, carried out in vivo on the rat liver, have addressed specific aspects on which information is incomplete or missing.

The experimental protocol involved biosynthetic labeling in vivo, in the intact animal, with intravenously administered [^{35}S]cysteine. The label was shown to be cleared relatively rapidly from the blood plasma, but more slowly from the liver. Intracellular forms of SC were immunoprecipitated from total microsomal fractions at selected intervals postinjection, separated by SDS PAGE, and detected by fluorography. This approach has the significant advantage of providing information about SC synthesis and subsequent modifications under physiological conditions in the hepatocytes of intact animals.

Our results indicate that the rat hepatocyte produces a single series of intracellular SC forms, in contradistinction to rabbit hepatocytes and mammary glandular epithelia, in which two different sets of SC were detected (18, 24). They were assumed to be the products of a single gene, but the presence of multiple genes was not ruled out (18, 24). In man, as in the rat, the presence of a single SC set was recorded (23).

The SC form detected at the earliest interval, i.e., 5 min after [^{35}S]cysteine administration, has an apparent M_r of 105,000; it is core glycosylated and sensitive to endo H treatment (which reduced its M_r to ~96,000); it has apparently three or more polymannose oligosaccharide chains (Fig. 5); it behaves like an integral membrane protein when treated with Triton X-114 (Table I); and it has an endodomain of ~20K cleavable by trypsin digestion. In all these respects, the 105K form of SC is similar to the product obtained when SC mRNAs, isolated from rabbit mammary gland cells (24) or from a human colonic carcinoma (23), were translated in vitro in heterologous cell-free systems in the presence of (pancreatic) microsomes. All these characteristics suggest, but do not prove, that 105K is the SC form co-translationally inserted into the rough endoplasmic reticulum membrane of the hepatocytes. The suggestion remains to be validated by cell fractionation experiments carried out at appropriate intervals postinjection (see companion paper [42]).

The 105K form begins to be converted into a 116K form starting at ~15 min after injection, and the conversion is largely completed by 45 min postinjection. This form is endo H resistant, but remains endo F sensitive (Fig. 5). Like 105K, 116K behaves as an integral membrane protein in a detergent-shift test, and has an endodomain of ~20K (Table I and Fig. 6). Its kinetics of labeling and its relationship to the 105K form, as well as the properties mentioned above, suggest that the 116K component is the Golgi form of SC. Based on data obtained on other membrane proteins (the G protein of vesicular stomatis virus, for instance), we assume that the conversion of 105K into 116K involves the trimming of its polymannose chains initiated in the endoplasmic reticulum (1, 17) and continued in the Golgi complex (43, 44, 48), followed by the terminal glycosylation of the trimmed chains (15, 17). Since trimming enzymes, e.g., mannosidase I and II (9, 48), and terminal glycosyltransferases (4, 9) have been localized to Golgi fractions or specific Golgi compartments (35), we assume that 116K is either in the Golgi compartments where these enzymes reside or has already moved past them. Similar kinetics for acquiring endo H resistance have been reported for other membrane proteins (3, 39, 40). As for 105K, the assumed location of 116K remains to be validated by cell fractionation experiments carried out at selected time points after [^{35}S]cysteine administration (see companion paper [42]).

Within 15 min, 116K begins to be converted to a 120K form, which, like its precursor, is resistant to endo H, sensitive to endo F, behaves like an integral membrane protein, and has an endodomain of similar (~20K) size. The conversion appears to be completed ~60 min after administration, but 120K is still present in hepatocytes up to 90 min postinjection. As shown in a previous article (41), newly synthesized SC, labeled by [^{35}S]cysteine, begins to appear in bile as a soluble, secretory protein of 80K at ~35 min postinjection, reaches its SRA peak at ~80 min, and is still detectable at ~110 min postinjection.

In the case of the plasmalemmal (3, 28, 36) and viral proteins (39) so far studied, a single intracellular precursor, presumably the endoplasmic reticulum form, has been found, but no differences have been detected between Golgi forms and plasmalemmal forms at the level of resolution obtained in one-dimensional electrophoretograms. Our results indicate that the hepatic SC may represent a rare or unique case among intrinsic plasmalemmal proteins, because it has two precursors instead of one. At present, we are investigating the nature of the increase in mass from 116kd to 120kd. We have not succeeded in labeling 120K with $^{35}SO_4^{2-}$; hence, sulfation does not seem to be responsible for this conversion. The alternatives we are still considering are: (*a*) O-glycosylation as discussed in conjunction with galactosyltransferase (40) and low-density lipoprotein receptor (7) (although human milk SC is not O-glycosylated [31]); and (*b*) phosphorylation connected perhaps with ligand binding as in the case of the receptors for the epidermal growth factor (5) and insulin (13). In recent experiments, we have succeeded in labeling the 120K form of SC after intraperitoneal injection of [^{32}P]orthophosphate. The other forms were apparently not labeled. [^{35}S]-cysteine used in parallel with the same protocol labeled all three SC forms. The type and conditions of phosphorylation of 120K remain to be investigated.

The intracellular location of the 105K and 116K forms is assumed on the basis of rather strong suggestive evidence. The evidence for the distribution of the 120K form is also suggestive, but considerably weaker. For all three forms, the compartments involved in intracellular or transcellular transport and in concurrent posttranslocational modifications remain to be identified by cell fractionation carried out at selected intervals postinjection in the framework of pulse-chase experiments of the kind used in this study. We have followed this approach and have identified by cell fractionation some of the compartments involved in the transport and processing of SC. We report the results so far obtained in the companion paper (42).

We are indebted to Pamela Ossorio for photography, Cynthia Davis, and Lynne Wootton for secretarial assistance.

This work, supported by National Institutes of Health grant GM-27303, constitutes part of a thesis submitted (by E. S. Sztul) in partial fulfillment for the degree of Doctor of Philosophy.

Received for publication 22 May 1984, and in revised form 5 November 1984.

REFERENCES

1. Bischoff, J., and R. Kornfeld. 1983. Evidence for an α-mannosidase in endoplasmic reticulum of rat liver. *J. Biol. Chem.* 258:7907–7910.
2. Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* 256:1604–1607.
3. Braell, W. A., and H. F. Lodish. 1981. Biosynthesis of the erythrocyte anion transport protein. *J. Biol. Chem.* 256:11337–11344.
4. Bretz, R., H. Bretz, and G. E. Palade. 1980. Distribution of terminal glycosyltransferases in hepatic Golgi complex fractions. *J. Cell Biol.* 84:87–101.
5. Cohen, S., G. Carpenter, and L. King, Jr. 1980. Epidermal growth factor-receptor-protein kinase interactions. *J. Biol. Chem.* 255:4834–4842.
6. Crago, S. S., R. Kulhavy, S. J. Prince, and J. Mestecky. 1978. Secretory Component on epithelial cells is a surface receptor for polymeric immunoglobulins. *J. Exp. Med.* 147:1832–1837.

7 Cummings, R. D., S. Kornfeld, W. J. Schneider, K. K. Hobgood, H. Tolleshaug, M. S. Brown, and J. L. Goldstein. 1983. Biosynthesis of N-linked and O-linked oligosaccharides of the low density lipoprotein receptor. *J. Biol. Chem.* 258:15261–15273.

8. DeLacroix, D. L., H. J. F. Hodgson, A. McPherson, C. Dive, and J. P. Vaerman. 1982. Selective transport of polymeric immunoglobulin A in bile. *J. Clin. Invest.* 70:230–241.
9. Dunphy, W. G., and J. E. Rothman. 1983. Compartmentation of asparagine-linked oligosaccharide processing in the Golgi apparatus. *J. Cell Biol.* 97:270–275.
10. Elder, J. H., and S. Alexander. 1982. Endo-β-*N*-acetylglucosaminidase F: endoglycosidase from Flavobacterium meningosepticum that cleaves both high-mannose and complex glycoproteins. *Proc. Natl. Acad. Sci. USA.* 79:4540–4544.
11. Fisher, M. M., B. Nagy, H. Barin, and B. J. Underdown. 1979. Biliary transport of IgA: role of Secretory Component. *Proc. Natl. Acad. Sci. USA.* 76:2008–2012.
12. Gebhardt, R. 1983. Primary cultures of rat hepatocytes as a model system of canalicular development, biliary secretion, and intrahepatic cholestasis. *Gastroenterology.* 84:1462–1470.
13. Grigorescu, F., M. F. White, and C. R. Kahn. 1983. Insulin binding and insulin-dependent phosphorylation of the insulin receptor solubilized from human erythrocytes. *J. Biol. Chem.* 258:13708–13716.
14. Gumbiner, B., and R. B. Kelly. 1982. Two distinct intracellular pathways transport secretory and membrane glycoproteins to the surface of pituitary tumor cells. *Cell.* 28:51–59.
15. Hanover, J. A., W. J. Lennarz, and J. D. Young. 1980. Synthesis of N- and O-linked glycopeptides in oviduct membrane preparations. *J. Biol. Chem.* 255:6713–6716.
16. Howard, F. D., J. A. Ledbetter, S. Q. Mehdi, and L. A. Herzenberg. 1980. A rapid method for the detection of antibodies to cell surface antigens: a solid phase radioimmuneassay using cell membranes. *J. Immunol. Methods.* 38:75–84.
17. Hubbard, S. C., and R. J. Ivatt. 1981. Synthesis and processing of asparagine linked oligosaccharides. *Annu. Rev. Biochem.* 50:555–583.
18. Kuhn, L. C., H. P. Kocher, W. C. Hanly, L. Cook, J-C. Jaton, and J.-P. Kraehenbuhl. 1983. Structural and genetic heterogeneity of the receptor mediating translocation of immunoglobulin A dimer antibodies across epithelia in the rabbit. *J. Biol. Chem.* 258:6653–6659.
19. Kuhn, L. C., and J. P. Kraehenbuhl. 1981. The membrane receptor for polymeric immunoglobulin is structurally related to Secretory Component. Isolation and characterization of membrane secretory component from rabbit liver and mammary gland. *J. Biol. Chem.* 256:12490–12495.
20. Lau, J. T. Y., J. K. Welpy, P. Shenbagamurthi, F. Naider, and W. J. Lennarz. 1983. Substrate recognition by oligosaccharide transferase. Inhibition of co-translational glycosylation by acceptor peptides. *J. Biol. Chem.* 258:15255–15260.
21. Lemaitre-Coelho, I., G. A. Altramirano, C. Barranco-Acosta, R. Meykens, and J. P. Vaerman. 1981. In vivo experiments involving secretory component in the rat hepatic transfer of polymeric IgA from blood to bile. *Immunology.* 43:261–270.
22. Merisko, E. M., M. G. Farquhar, and G. E. Palade. 1982. Coated vesicle isolation by immunoadsorption on *Staphylococcus aureus* cells. *J. Cell Biol.* 92:846–857.
23. Mostov, K. E., and G. Blobel. 1982. A transmembrane precursor of secretory component. The receptor for transcellular transport of polymeric immunoglobulins. *J. Biol. Chem.* 257:11816–11821.
24. Mostov, K. E., J-P. Kraehenbuhl, and G. Blobel. 1980. Receptor mediated transcellular transport of immunoglobulins: synthesis of secretory component as multiple and larger transmembrane forms. *Proc. Natl. Acad. Sci. USA.* 77:7257–7261.
25. Mullock, B. M., R. H. Hinton, M. Dobrota, J. Peppard, and E. Orlans. 1979. Endocytic vesicles in liver carry polymeric IgA from serum to bile. *Biochim. Biophys. Acta.* 587:381–391.
26. Mullock, B. M., R. H. Hinton, M. Dobrota, J. Peppard, and E. Orlans. 1980. Distribution of secretory component in hepatocytes and its mode of transfer into bile. *Biochem. J.* 190:819–826.
27. Nakamura, K., and R. W. Compans. 1977. The cellular site of sulfation of influenza viral glycoproteins. *Virology.* 79:381–392.
28. Omary, M. D., and I. S. Trowbridge. 1981. Biosynthesis of the human transferrin receptor in cultured cells. *J. Biol. Chem.* 256:12888–12892.
29. Orlans, E., J. Peppard, J. F. Fry, R. H. Hinton, and B. M. Mullock. 1979. Secretory component as the receptor for polymeric IgA on rat hepatocytes. *J. Exp. Med.* 150:1577–1581.
30. Papermaster, D. S., C. A. Converse, and M. A. Zorn. 1976. Biosynthesis and immunochemical characterization of a large protein in frog and cattle rod outer segment membranes. *Exp. Eye Res.* 23:105–116.
31. Purkayastha, S., C. V. N. Rao, and M. E. Lamm. 1979. Structure of the carbohydrate chain of free secretory component from human milk. *J. Biol. Chem.* 254:6583–6587.
32. Rennard, S. I., G. R. Martin, J. M. Foidart, and P. G. Robey. 1980. Enzyme-linked immunoassay (ELISA) for connective tissue components. *Anal. Biochem.* 104:205–214.
33. Renston, R. H., A. L. Jones, W. D. Christiansen, and G. T. Hradek. 1980. Evidence for a vesicular transport mechanism in hepatocytes for biliary secretion of Immunoglobulin A. *Science* (*Wash. DC*). 208:1276–1278.
34. Robbins, P. W., S. C. Hubbard, S. J. Turco, and D. F. Wirth. 1977. Proposal for a common oligosaccharide intermediate in the synthesis of membrane glycoproteins. *Cell.* 12:893–900.
35. Roth, J., and E. G. Berger. 1982. Immunocytochemical localization of galactosyltransferase in HeLa cells: codistribution with thiamine pyrophosphatase in trans Golgi cisternae. *J. Cell Biol.* 92:223–229.
36. Schwartz, A. L., and D. Rup. 1983. Biosynthesis of the human asialoglycoprotein receptor. *J. Biol. Chem.* 258:11249–11255.
37. Socken, D. J., K. N. Jeejeehboy, H. Bazin, and B. J. Underdown. 1979. Identification of secretory component as an IgA receptor on rat hepatocytes. *J. Exp. Med.* 50:1538–1548.
38. Solari, R., and J. P. Kraehenbuhl. 1984. Biosynthesis of the IgA antibody receptor: a model for the transepithelial sorting of a membrane glycoprotein. *Cell.* 36:61–71.
39. Strous, G. J. A. M., and H. F. Lodish. 1980. Intracellular transport of secretory and membrane proteins in rat hepatoma cells infected by vesicular stomatitis virus. *Cell.* 22:709–717.
40. Strous, G. J., P. van Kerkhof, R. Willemsen, H. J. Geuze, and E. G. Berger. 1983. Transport and topology of galactosyltransferase in endomembranes of HeLa cells. *J. Cell Biol.* 97:723–727.
41. Sztul, E. S., K. E. Howell, and G. E. Palade. 1983. Intracellular and transcellular transport of secretory component and albumin in rat hepatocytes. *J. Cell Biol.* 97:1582–1591.
42. Sztul, E. S., K. E. Howell, and G. E. Palade. 1984. Biogenesis of the polymeric IgA receptor in rat hepatocytes. II. Localization of its intracellular forms by cell fractionation studies. *J. Cell Biol.* 100:1255–1261.
43. Tabas, I., and S. Kornfeld. 1979. Purification and characterization of a rat liver Golgi mannosidase capable of processing asparagine linked oligosaccharides. *J. Biol. Chem.* 254:11655–11663.
44. Tabas, I., S. Schlesinger, and S. Kornfeld. 1978. Processing of high mannose oligosaccharides to form complex type oligosaccharides on the newly synthesized polypeptides of the vesicular stomatitis virus G protein and the IgG heavy chain. *J. Biol. Chem.* 253:716–722.
45. Takahashi, I., P. K. Nakane, and W. R. Brown. 1982. Ultrastructural events in the translocation of polymeric IgA by rat hepatocytes. *J. Immunol.* 128:1181–1187.
46. Tarentino, A. L., and F. Maley. 1974. Purification and properties of an endo-β-*N*-acetylglucosaminidase from Streptomyces griseus. *J. Biol. Chem.* 249:811–817.
47. Tarentino, A. L., T. H. Plummer, and F. Maley. 1974. The release of intact oligosaccharides from specific glycoproteins by endo-β-*N*-acetylglucosaminidase H. *J. Biol. Chem.* 249:818–824.
48. Tulsiani, D. R. P., S. C. Hubbard, P. W. Robbins, and O. Touster. 1982. α-D-Mannosidase of rat liver Golgi membranes. *J. Biol. Chem.* 257:3660–3668.