Biogenesis of the Polymeric IgA Receptor in Rat Hepatocytes.
I. Kinetic Studies of Its Intracellular Forms

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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 co-precipitation (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 [35S]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 [35S]cysteine, a major band of Mr 105,000 was maximally labeled. This peptide lost radioactivity concomitantly with the appearance of a radioactive doublet of Mr 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). 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.
indicating that antibodies raised against biliary SC recognize two intracellular forms of SC (M, 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, leupepin, and tosyl-L-lysine chloromethyl ketone from Sigma Chemical Co. (St. Louis, MO); [35S]cysteine (1,080 μCi/mmol) from Amer shamCorp. (Arlington Heights, IL); NaCl and 125I-labeled protein A from New England Nuclear (Boston, MA); Anti-IgA serum from Southern Biotechnology Laboratories Inc., Minneapolis, MN; 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 Germany.

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 Mr, 80,000 was cut from unstained gels and prepared for injections according to Papernov et al. (30). The identity of the antigen as SC was based on its co-precipitation with IgA, by an anti-IgA, seduct rabbit antisera 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 cpn of 125I-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 125I-labeled protein A (16 × 106 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 1 ml of 0.5 M Tris-HCl, pH 6.8; 20 μl of 20% SDS and 10 μl of 0.2 M dithiothreitol were added; then the samples were solubilized and counted.

RESULTS

Antibody Characterization

ENZYME- LINKED IMMUNOSORBENT ASSAY AND RADIOMETRIC IMMUNOASSAYS: 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 IMMUNE OVERLAYS: A broad band corresponding to the original Mr, 80,000 antigen and a less intense band of lower Mr, (i.e., ~70,000) were detected (Fig. 1A, lane 4). Longer exposures revealed additional minor bands of lower Mr, presumed to be degradation products of the 80,000-100,000 (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 by overlay with the anti-SC antibody (Fig. 1A, lane 4).

COMMUNICATIONS

1. Immunoprecipitation: As shown previously (41), a major 80K band, corresponding to the original antigen, and a less intense 70K band could be immunoprecipitated from radiolabeled bile proteins (Fig. 1B, lane 1). We identified a product.
bile with anti-albumin antibodies. The subsequent incubation
band. Co-isolation of albumin during anti-SC immunoprecip-
the 80K antigen, a 70K band (Fig. 1A, lane 4), we assume
of the preadsorbed bile with anti-SC antibodies resulted in
specific immunoprecipitation of the 80K peptide in amounts
of our ability to remove it by sequential incubation of labeled
of the 70K peptide were greatly reduced (Fig. 1B, lane 2).
major component of the 70K band as albumin, 3 on the basis
we assume that the anti-SC antibody does not cross-react with
detected in electrophoretograms of serum (Fig. 1A, lane 2),
by immunoverlay, no reactive band was
immunosupport (albumin represents one of the major biliary
common antigenic determinants. To test this assumption
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 experimen-
tal 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
immunoprecipitation of bile proteins by anti-SC sera. Radioiodinated bile proteins were immunoprecipitated with either anti-SC or 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. 1B, lane 2). Since immunoprecipitation of bile proteins detects, in addition to the 80K antigen, a 70K band (Fig. 1A, 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 immunoprecipitation, no reactive band was detected in electrophoreograms of serum (Fig. 1A, lane 2), we assume that the anti-SC antibody does not cross-react with albumin.

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

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

3 94K was one of the SC forms labeled by [3H]fucose and identified
by immunoprecipitation in our first paper (41).
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 [35S]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 [35S]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 [35S]cysteine from blood indicates a pulse of ~20 min, SRA of most cellular proteins appeared maximal at ~6 min, SRA of cellular 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 [35S]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. 4A).

At the earliest time point tested, i.e., 5 min postinjection, a major band of Mr 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 Mr 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. 4B). 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. 4A) 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 Mr 94,000 and 90,000 are proteolytic fragments generated (during our preparation procedure) from the higher Mr 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 seen in Fig. 4 represent different stages in the intracellular processing of a single SC peptide.

Ailiquots 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 [35S]cysteine-labeled SC begins to
cleaved 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

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6 The kinetics of labeling have been consistently reproducible.

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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 [35S]cysteine in TM fractions prepared in the absence or presence of protease inhibitors, suggesting that no SC form is preferentially sensitive to endogenous proteases.

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 Glc3Man9GlcNAc2 as well as its sequentially processed forms, Man9GlcNAc2 to Man6GlcNAc2, 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 Mr, 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 3), 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 Mr, 96,000 and 100,000, suggesting that the increase in Mr 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 Mr 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 Mr 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 [35S]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(lGα)2 complexes (25, 45), and in the case of the liver, the discharge of a soluble form of SC (as free SC or SC(lGα) 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 [35S]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 [35S]cysteine administration, has an apparent Mr of 105,000; it is core glycosylated and sensitive to endo H treatment (which reduced its Mr 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 stomatitis virus, for instance), we assume that the conversion of 105K into 116K involves the trimming of its oligomannose 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 [35S]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 [35S]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 35S or 35SO42-, 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 [35S]orthophosphate. The other forms were apparently not labeled. [35S]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).

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