Proteolytic Processing of Rat Liver Membrane Secretory Component

CLEAVAGE ACTIVITY IS LOCALIZED TO BILE CANALICULAR MEMBRANES

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Membrane secretory component (mSC) mediates the transcellular movement of polymeric IgA from the sinusoidal to the bile canalicular surface of rat hepatocytes. Prior to or concomitant with arrival at the bile canalicular membrane, mSC is cleaved, producing a soluble proteolytic fragment (fSC) which is released into the bile. Conversion of mSC to fSC occurs at the cell surface of cultured rat hepatocytes (Musil, L. S., and Baenziger, J. U. (1987) J. Cell Biol. 104, 1725–1733), suggesting that vectorial release of fSC into bile in vivo may reflect localization of a mSC-specific protease to bile canalicular membranes. We have established a reconstituted system to examine the process of specific cleavage of mSC to yield fSC and to characterize the protease activity responsible. A membrane fraction highly enriched for endocytic vesicles was found to contain ~90% of the [35S]Cys-mSC from metabolically labeled rat liver slices but only 5% of the cellular protein. No cleavage activity was present in these vesicles. Highly enriched bile canalicular membranes were able to mediate cleavage of metabolically labeled mSC to a fragment indistinguishable from authentic fSC. In the absence of nonionic detergent, cleavage was dependent on the presence of polyethylene glycol, presumably to mediate fusion of mSC-enriched membranes with bile canalicular membranes. Following solubilization with nonionic detergent, cleavage was no longer dependent on the addition of polyethylene glycol. Cleavage of mSC was not observed with either intact or detergent-solubilized sinusoidal, microsomal, or lysosomal membranes. We have thus identified a proteolytic activity associated with bile canalicular membranes which has the properties of a membrane protein and is likely to be responsible for production of fSC in vivo. Its highly restricted localization to the bile canalicular membrane would account for the vectorial release of fSC into the bile.

Secretory component (SC)1 is a integral membrane protein,

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1 The abbreviations used are: SC, secretory component; ASOR, asialo-orosomucoid; Endo F, endoglycosidase F; fSC, the soluble product of secretory component cleavage in vivo; mSC, membrane secretory component; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
this activity. Our results indicate that a protease specific for mSC is enriched in bile canaliculi and may account for the vectorial release of ISC into the bile.

EXPERIMENTAL PROCEDURES

Materials—L-[35S]Cys (>600 Ci/mmole) was purchased from Amer sham Corp., H215PO4 (280 Ci/ml) from ICN (Irvine, CA), and UDP-14H (11.4 Ci/mmole) from Du Pont-New England Nuclear. Heat-killed, formalin-fixed Staphylococcus aureus was provided by Dr. S. Cullen, Washington University School of Medicine. Endoglycosidase F (Endo F) was prepared according to Elder and Alexander (13) and contained both Endo F and N-glycanase activities. [35S]-Asialo-orosomucoid (ASOR) (30, 105°C for 1 h) was prepared as described previously (4). 1-Amino-2-naphthol-4-sulfonic acid (Eastman) was recrystal lized prior to use. Soybean phospholipids (type II-S) and ocyl-β- glucoside (octylglycoside) were from Sigma.

Preparation of Bile Canalicular and Sinusoidal Plasma Membranes—Membrane fractions enriched in either the bile canalicular or the sinusoidal domains of rat hepatocytes were prepared by the procedure of Inoue et al. (21, 22). Prior to homogenization, livers were perfused in situ with 30 ml of ice-cold medium A (0.25 M sucrose, 10 mM HEPES, pH 7.4, adjusted to 7.4, with solid Tris base). 1.5% sucrose bile canalicular membranes and 5-10 mg of sinusoidal membranes were obtained from four rat livers weighing 30 g, in good agreement with the yields reported by Inoue et al. (21, 22). Bile canalicular membranes (1 mg/ml) were stored in single-use aliquots at -70°C to retain full mSC-cleaving activity for up to 2 months.

Preparation of Lysosome- and Microsome-enriched Membrane Fractions—Liver membrane fractions enriched in bile or the sinusoidal domains of rat hepatocytes were prepared by the procedure of Fleischer and Kervina (15). The resulting pellet and supernatant fractions were sedimented at 100,000 X g for 20 min in a Beckman Airfuge (Beckman Instruments, Palo Alto, CA). The supernatant was removed, and the membrane pellet was mixed with a pipette tip. After 2 min at 30°C, 50 µl of a 52% (w/v) solution of polyethylene glycol (PEG 6000; Sigma) in solution A, supplemented with 0.1 mM EDTA (PEG medium) was added to each tube and the samples were thoroughly mixed with a pipette tip. The samples were placed at 37°C for 2 min and then mixed with 100 µl of "dilution medium" at 25°C (solution A supplemented with 0.1 mM EDTA, 4 mM MgCl2, and 6 mM glucose). After an additional 2 min at 30°C the samples each received 900 µl more of dilution medium and were incubated at 4°C for up to 30 min. "Mock"-fused samples were treated identically, except that PEG medium was replaced by 50 µl of dilution medium. Due to interference of PEG with recovery of proteins from the membranes, the samples were sedimented at 47,000 X g for 20 min at 4°C, and the resulting pellet and supernatant fractions were separately brought to 1.0% SDS, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride in immunoprecipitation buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, pH 7.5, and boiling for 3 min. Samples boiled for 5 min. The samples were then immunoprecipitated with anti-SC antiseraum as described previously (37). Unless otherwise noted, the anti-SC immunoprecipitates originating from the supernatant and pellet fractions of each sample were combined prior to analysis by SDS-PAGE.

Reconstitution of Membrane Secretory Component Cleavage—Pelleted membranes were brought to a volume of 35 µl with medium A (0.25 M sucrose, 10 mM HEPES, pH adjusted to 7.4, with solid Tris base), and 6 µl of a 10% solution of octylglucoside (final concentration 1.5% octylglucoside) was added. After 1 h at 4°C, the samples were sedimented at 100,000 X g for 10 min in a Beckman Airfuge (Beckman Instruments, Palo Alto, CA). The supernatant was added to a 41-µl aliquot of metabolically labeled mSC-enriched membranes (35 µg of protein) which had been solubilized in the identical manner with 1.5% octylglucoside, and the mixture was incubated at 4°C for up to 48 h. Identical results were obtained whether or not mSC-enriched membranes were sedimented at 100,000 X g for 10 min to remove insoluble material prior to combination with the supernatant from solubilized membranes being tested for protease activity. The cleavage reaction was terminated by bringing each sample to 0.6% SDS, 1 mM benzamidine, and 1 mM phenylmethylsulfonyl fluoride in immunoprecipitation buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris, 3 mM sodium azide, pH 7.6) in a final volume of 250 µl and boiling for 3 min. Samples boiled in SDS were then diluted and immunoprecipitated with anti-SC antiseraum as described for the reconstituted system using intact membranes and PEG.

SDS Gel Electrophoresis of Immunoprecipitates—Discontinuous electrophoresis was carried out on 10% polyacrylamide gels according to Laemml (26). Labeled proteins were visualized by either fluorography (7) (L-[35S]Cys-labeled proteins) or autoradiography (29,P-labeled proteins) using preflaged Kodak X-AR film. Densitometry was performed with a Hoeffer GS densitometer.
from *Staphylococcus aureus*, treated with Endo F for 13 h at 3°C, and analyzed by SDS-PAGE as previously described (37).

**RESULTS**

**Preparation and Characterization of Bile Canalicul and Sinusoidal Membranes from Rat Liver**—We previously observed that cleavage of mSC to fSC occurs exclusively at the surface of cultured rat hepatocytes and hypothesized that an mSC-specific protease might be restricted to the bile canalicul domain in vivo (36). To test this hypothesis, plasma membrane fractions enriched in either bile canalicul or sinusoidal domains were purified, and their capacity to mediate specific cleavage of mSC was compared. Characteristics of bile canalicul and sinusoidal membranes prepared by the method of Inoue et al. (21, 22) are given in Tables I and II. The fold enrichment and specific activity of plasma membrane markers in the bile canalicul fraction relative to the initial homogenate were similar to values reported by Inoue et al. (22) and to those reported by Meuer et al. (30) using a different fractionation procedure.

Unlike the bile canalicul domain, the sinusoidal domain of hepatocytes has few reliable enzymatic markers. Na⁺⁻K⁺-ATPase, the most commonly measured enzymatic activity associated with sinusoidal membranes, was not reproducibly detected using either the method of Scharschmidt et al. (45) or Kinne et al. (23). The asialoglycoprotein receptor is located in the sinusoidal and lateral domains of hepatocytes with little or no receptor in the bile canalicul domain (19, 29). Hubbard et al. (20) reported that <10% of the asialoglycoprotein-specific binding activity is associated with morphologically recognizable bile canalicul membranes prepared from rat liver which were enriched 47-fold over homogenate for alkaline phosphatase activity. The sinusoidal membranes used in this study were enriched 21-fold relative to total liver homogenate and 10-fold relative to bile canalicul membranes in asialoglycoprotein binding activity (Table I). Although the sinusoidal membrane fraction was enriched in bile canalicul markers relative to the homogenate, the enrichment of these markers was 7-10-fold greater in bile canalicul membranes than the sinusoidal membranes (Table I). Thus, the bile canalicul membranes used for the studies described below were largely free of sinusoidal membranes and vice versa. Enzyme markers for intracellular membranes including Golgi, lysosomes, mitochondria, and endoplasmic reticulum were not significantly enriched in either bile canalicul or sinusoidal membrane fractions (Table II).

**Preparation of Membranes Containing Metabolically Labeled mSC**—Since the majority of mSC present in cultured rat hepatocytes resides in intracellular membranes which are devoid of mSC cleavage activity (36), we reasoned that intracellular membranes containing metabolically labeled mSC could act as a substrate for an mSC-specific protease. A membrane fraction of low density which was highly enriched in [³⁵S]Cys-mSC but devoid of mSC-cleaving activity was therefore prepared. Rat liver slices were metabolically labeled with [³⁵S]Cys for 6 h and disrupted by Dounce homogenization, and the postnuclear supernatant was fractionated on a discontinuous sucrose density gradient according to a procedure developed by Baenziger and Fiete (3) for the fractionation of prelysosomal, endosomal vesicles from isolated rat hepatocytes. Two forms of mSC, M, 105,000 and 100,000, were present in the postnuclear supernatant (Fig. 1, lane 3) while little mSC remained in the nuclear pellet (Fig. 1, lane 4). The M, 105,000 species is the mature (terminally glycosylated) form of mSC. The minor species of M, 100,000 consists of newly synthesized, incompletely processed SC which has not yet exited the medial Golgi (37). There was a time-dependent increase in [³⁵S]Cys-fSC released into the medium (Fig. 1, lanes 2 and 8). Variable amounts of fSC, M, 80,000, were also present in the postnuclear supernatant (Fig. 1, lane 3), possibly reflecting the presence of fSC in bile canaliculi at the time of homogenization. More than 85% of the M, 105,000 species of mSC present in the postnuclear supernatant was recovered in membranes collected at the 15-33% sucrose interface (Fig. 1, lane 5). mSC present in membranes obtained at the 33-39% interface (Fig. 1, lane 6) and 39-66% interface (Fig. 1, lane 7) contained greater proportions of incompletely processed mSC (M, 100,000). The latter fractions were previously shown to be enriched for Golgi and endoplasmic reticulum markers relative to the vesicles from the 15-33% interface (3). Five percent of the protein from the homogenate was recovered at the 15-33% interface, resulting in a 17-fold enrichment of [³⁵S]Cys-labeled mSC. The membranes recovered at the 15-33% sucrose interface, designated the mSC-enriched fraction, were used as a substrate source in mSC cleavage assays without further purification.

There was no evidence of proteolysis of [³⁵S]Cys-mSC during the preparation of mSC-enriched membranes (Fig. 1, compare lanes 3 and 5), or during incubation of purified mSC-enriched membranes at 4°C for up to 20 h (Fig. 1, compare lanes 10 and 11). Incubation of mSC-enriched membranes at 37°C for 8 h resulted in the appearance of a [³⁵S]Cys-mSC cleavage fragment of M, 92,500 but not in the production of significant amounts of a genuine fSC species of M, 80,000 (Fig. 1, lane 12). Since cleavage of mSC to produce fSC on the surface of intact hepatocytes can proceed at 4°C (36), the M, 92,500 product was viewed as a nonspecific cleavage fragment. In the reconstitution system described below, incubations were carried out at 4 rather than 37°C to prevent formation of the nonspecific M, 92,500 fragment. The absence of endogenous mSC-specific cleavage activity in mSC-en-

**TABLE I**

| Marker                                | Homogenate (specific activity)* | Relative enrichment<sup>b</sup> |
|---------------------------------------|--------------------------------|---------------------------------|
|                                       |                                | Bile canalicul                  | Sinusoidal                      |
| Alkaline phosphatase                  | 0.21 ± 0.08 (3)                | 34 ± 4 (3)                      | 4.9 ± 3.2 (3)                   |
| Alkaline phosphodiesterase I          | 1.43 ± 0.29 (3)                | 168 ± 67 (3)                    | 22 ± 12 (3)                     |
| γ-Glutamyltranspeptidase              | 0.12 ± 0.02 (2)                | 41 ± 3 (2)                      | 4 (1)                          |
| 5'-nucleotidase                       | 1.56 ± 0.13 (3)                | 63 ± 10 (3)                     | 8 (3)                          |
| Asialoglycoprotein receptor           | 0.025 ± 0.006 (3)              | 1.9 ± 0.24 (2)                  | 21 ± 4 (3)                     |

* Specific activities are expressed as μmol/mg/h, except for asialoglycoprotein receptor, for which the corresponding unit is μg ASOR bound per mg protein.

<sup>b</sup> Enrichment to relative homogenate. Data are presented as the mean ± S.D. with the number of determinations in parentheses.
Selective Cleavage of mSC by a Bile Canaliculare Protease—

Rat liver subcellular fractions enriched for bile canalicular, sinusoidal, lysosomal, and microsomal membranes were examined to determine if they could support the cleavage of $[^{35}S]$Cys-mSC in mSC-enriched membranes to fSC. Incubation of intact or hypotonically lysed bile canalicular membranes or other membrane fractions with mSC-enriched membranes did not result in detectable conversion of $[^{35}S]$Cys-mSC to fSC (not shown). This suggested that if bile canalicular or other membranes contained a protease capable of cleaving mSC, this activity might require colocalization in either the same membrane or membrane-bound compartment for cleavage to occur. We therefore adapted the PEG-mediated membrane fusion procedure of Schramm (46) to fuse $[^{35}S]$Cys-labeled mSC-enriched vesicles with purified membrane fractions prepared from rat liver, in order to assay for colocalization-dependent cleavage.

The ability of bile canalicular membranes to mediate cleavage of mSC in the PEG-mediated membrane fusion assay was tested under different conditions (Fig. 2). When mSC-enriched membranes were subjected to all the manipulations utilized for the fusion assay but the PEG was replaced with dilution medium, $[^{35}S]$Cys-SC was recovered as uncleaved $[^{35}S]$Cys-mSC (Fig. 2, compare lanes 1 and 2). Treatment of mSC-enriched membranes as above, followed by incubation in the presence of bile canalicular membranes but without PEG, also did not result in cleavage (Fig. 2, lane 8). Incubation of mSC-enriched membranes with an equal amount of bile canalicular membranes in the presence of PEG (Fig 2, lane 4) resulted in quantitative conversion of mSC to a fragment of $M_r$ 80,000 which comigrated with the upper band of fSC produced by metabolically labeled liver slices (Fig. 2, lane 5). In the absence of added bile canalicular membranes, incubation of mSC-enriched membranes in the presence of PEG

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**TABLE II**

| Marker                  | Relative enrichment* |
|-------------------------|----------------------|
|                         | Bile canicular       | Sinusoidal        |
| Golgi                   | 1.9 ± 0.1 (2)        | 1.0 ± 0 (2)       |
| Galactosyltransferase   | 1.32 ± 1.15 (4)      | 1.06 ± 0.80 (3)   |
| Lysosome                | 0.86 ± 0.05 (3)      | 3.4 ± 0.5 (3)     |
| β-Galactosidase         | 2.3 ± 0.2 (3)        |                   |
| Endoplasmic reticulum  | 0.73 (1)             | 0.2 (1)           |

* Relative enrichment to homogenate. Data are presented as the mean ± S.D. with the number of determinations in parentheses.
resulted in the production of only small amounts of the M, 80,000 species (Fig. 2, lane 3). The majority of SC recovered under the latter conditions was in the form of an M, 92,500 species, the same product seen following incubation of mSC-enriched membranes at 37 °C in the absence of PEG (Fig. 1, lane 12).

Following PEG-mediated fusion of mSC-enriched and bile canalicular membranes [35S]Cys-mSC was quantitatively converted to the M, 80,000 species (Fig. 2, lanes 1 and 4). The recovery of [35S]Cys-mSC was not quantitative in the absence of efficient cleavage to the soluble species of M, 80,000. Thus, 50–75% of [35S]Cys-mSC was recovered in the form of mSC and/or SC cleavage products when mSC-enriched membranes were incubated with bile canalicular membranes in the absence of PEG (Fig. 2, lane 8) or were incubated in the absence of other membranes without or with PEG addition (Fig. 2, lanes 2 and 3, respectively). The quantitative recovery of [35S] Cys-SC as the M, 80,000 species when cleavage was complete indicated that proteolytic degradation of mSC to nonimmuno-noprecipitable forms did not account for the lower recovery of label observed when cleavage had not gone to completion. Quantitative recovery of [35S]Cys in the M, 80,000 fragment is expected since all of the Cys present in mSC resides in the soluble protein trapped within bile canalicular membrane vesicles. In addition, cleavage of mSC to the M, 92,500 species at 37 °C did not increase the proportion of mSC which was converted to the M, 80,000 species following PEG-mediated fusion of mSC-enriched and sinusoidal membranes as compared to controls (not shown).

The rate of cleavage of mSC to the M, 80,000 species after PEG-mediated membrane fusion of mSC-enriched and bile canalicular membranes was proportional to the amount of bile canalicular membrane protein added (not shown). Incubation of fused mSC-enriched and bile canalicular membranes at 37 °C increased the rate of production of the M, 80,000 species but did not result in the generation of additional cleavage forms (Fig. 2, lane 6). Since the M, 92,500 cleavage product was seen when mSC-enriched membranes were incubated in the absence of PEG at 37 °C or in the presence of PEG at 4 °C, this suggested that both mSC and the M, 92,500 species could be converted to the M, 80,000 species by the bile canalicular protease or that cleavage of mSC to the M, 80,000 species was far more rapid than cleavage to the M, 92,500 species at 37 °C in the presence of bile canalicular membranes. Conversion of mSC to the M, 80,000 species was not diminished by repeated cycles of freezing and thawing of bile canalicular membranes followed by washing to remove released material (Fig. 2, lane 7), indicating that the bile canalicular protease which mediates mSC cleavage is either a peripheral or an integral membrane protein rather than a soluble protein trapped within bile canalicular membrane vesicles. In addition, cleavage of mSC to the M, 80,000 species was dependent on PEG-mediated fusion of mSC-enriched and bile canalicular membranes (Fig. 2, lane 8), suggesting that mSC and the bile canalicular protease must be colocalized within the same lipid bilayer for cleavage to occur.

Other membrane fractions were also tested for mSC cleavage activity following PEG-mediated fusion to mSC-enriched membranes (Fig. 3). Incubation of mSC-enriched membranes with sinusoidal membranes (Fig. 3, lane 3), lysosomal membranes (Fig. 3, lane 7), microsomal membranes (Fig. 3, lane 6), or a total rat liver homogenate (Fig. 3, lane 5) in the presence of PEG did not increase the amount of the M, 80,000 species produced compared to controls (Fig. 3, lane 1), whereas incubation with bile canalicular membranes resulted in nearly quantitative conversion to the M, 80,000 species (Fig. 3, lane 2). The amount of the M, 92,500 species recovered after incubation of mSC-enriched membranes with each of these membrane fractions was also not increased over that seen when mSC-enriched membranes were incubated with PEG in the absence of other membranes (Fig. 3, lane 1). Hypotonic lysis of membranes prior to fusion, increased time of incubation (10 h), and incubation at 37 °C did not increase the proportion of mSC which was converted to the M, 80,000 species following PEG-mediated fusion of mSC-enriched and sinusoidal membranes as compared to controls (not shown).

No evidence for mSC cleavage activity in the sinusoidal membrane fraction was obtained when the amount of sinusoidal membranes included in the assay was increased 5-fold (Fig. 3, lane 4). Similar results were obtained with eight different preparations of sinusoidal membranes. The mSC-specific cleavage activity was, therefore, highly enriched in the bile canalicular membranes as compared to the sinusoidal membranes, even though both originated from the cell surface.

Characterization of the Products of mSC Cleavage in the Reconstituted System—The properties of the M, 80,000 fragment generated in the reconstituted cleavage system were compared with those of authentic fSC. The M, 80,000 fragment comigrated with the upper M, 80,000 band of fSC obtained from the medium of metabolically labeled liver slices (Fig. 2, lanes 4 and 5; Fig. 4, lanes 2 and 3) and with fSC obtained from rat bile (not shown). fSC lacks the cytosolic and membrane domains of mSC (31); therefore, the properties of the M, 80,000 fragment were examined to determine if it has the properties of a soluble rather than a membrane-associated protein. After fusion of mSC-enriched and bile canalicular membranes with PEG and incubation for 2.5 h at 4 °C, membrane-associated and soluble proteins were separated by sedimentation at 100,000 × g for 1 h. The M, 80,000 fragment was recovered predominantly in the soluble fraction (Fig. 4, lane 2), whereas mSC and the M, 92,500 fragment...
Cys-mSC-enriched and bile canalicular membranes were fused with PEG and incubated for 2.5 h at 4°C. Membrane-associated SC and soluble SC were separated by sedimentation at 100,000 x g for 1 h at 4°C. The membrane and soluble fractions were brought to 1.0% SDS, boiled, immunoprecipitated with anti-SC, and analyzed by SDS-PAGE and fluorography. Digestion with Endo F was performed after immunoprecipitation. Lane 1, membrane fraction from mSC-enriched membranes fused with bile canalicular membranes. Lane 2, soluble fraction from mSC-enriched membranes fused with bile canalicular membranes. Lane 3, authentic fSC immunoprecipitated from the medium of rat liver slices. Lane 4, soluble fraction from mSC-enriched membranes fused with bile canalicular membranes, digested with Endo F. Lane 5, authentic fSC digested with Endo F as in lane 4.

Liver homogenate did not result in the production of detectable amounts of the M, 92,500 species (Fig. 5, lane 2), even though the M, 92,500 fragment is typically produced under these conditions (Fig. 3, lane 5). Since the anti-SC antiserum utilized was raised against fSC prepared from bile, 32P-labeled SC fragments originating from the cytoplasmic tail of SC were not susceptible to immunoprecipitation with this antiserum.

The M, 80,000 cleavage product generated after PEG-mediated fusion of bile canalicular and mSC-enriched membranes therefore consists of the glycosylated extracellular domain of SC. It is electrophoretically indistinguishable from authentic fSC prior to and following enzymatic removal of Asn-linked oligosaccharides. These properties suggest that M, 80,000 species of SC produced in the reconstituted cleavage system is identical to fSC product in vivo and support the possibility that this bile canalicular protease is responsible for the conversion of mSC to fSC in vivo.

Solubilization of mSC-Cleaving Activity from Bile Canalicul Membranes—The properties of the bile canalicular protease in the reconstituted cleavage assay using intact membranes suggested that it was a membrane-associated protein. We therefore established conditions for solubilization of the mSC-cleaving protease in an active form which retains specificity for mSC. 35SCys-labeled mSC from mSC-enriched membranes was solubilized by incubation for 1 h at 4°C in 1.5% (w/v) octylglucoside. 35SCys-mSC was quantitatively recovered in the supernatant after sedimentation of octylglucoside-treated membranes at 100,000 x g, indicating complete solubilization of mSC. The bile canalicular protease active against mSC was also solubilized with 1.5% octylglucoside for 1 h at 4°C. Bile canalicular and other membrane fractions to be tested for cleavage activity were routinely sedimented at 100,000 x g after solubilization with 1.5% octylglucoside to assure that only solubilized activity would be detected.

Intact 35SCys-mSC was quantitatively recovered when octylglucoside-solubilized mSC-enriched membranes were in-
cubated for 8 h at 4 °C in the absence of other additions. In addition, there was no significant increase in the small amounts of endogenous M, 80,000 and M, 92,500 SC cleavage fragments under these conditions (Fig. 6, lanes 1 and 2). Incubation of octylglucoside-solubilized bile canaliculared membranes with solubilized mSC-enriched membranes resulted in the formation of two immunoprecipitable SC cleavage fragments of M, 92,500 and M, 80,000, and in a proportional decrease in the amount of mSC (Fig. 6, lane 3). The M, 80,000 species comigrated with the upper band of authentic fSC (Fig. 6, lane 4), indicating that the protease was effectively solubilized by 1.5% octylglucoside. Qualitatively similar results were obtained when bile canaliculared and mSC-enriched membranes were solubilized with 1.0% Triton X-100, 0.5–1.0% chololate, 1.0% octylglucoside, or 2.0% octylglucoside; however, the extent of mSC cleavage was less than that obtained with 1.5% octylglucoside. No cleavage of mSC occurred if bile canaliculared and mSC-enriched membranes were incubated together in the absence of detergent (Fig. 6, lane 6).

Following solubilization, cleavage of [35S]Cys-mSC by the bile canaliculared protease was time-, concentration-, and temperature-dependent. A time course for mSC cleavage at 4 °C is shown in Fig. 7A. Fragments of M, 80,000 and 92,500 accumulated at the similar rates during the first 8 h of incubation (Fig. 7A, lanes 1–5). Subsequently the amount of M, 92,500 fragment remained constant at ~30% of total [35S]Cys-SC, while the amount of mSC continued to decrease and the amount of the M, 80,000 species increased (Fig. 7A, lanes 6 and 7). Incubation of solubilized mSC with five different preparations of octylglucoside-solubilized bile canaliculared membranes produced similar rates and extents of mSC cleavage. There was a 0–15% net decrease in the recovery of total [35S]Cys-SC label as mSC and SC fragments, following incubation at 4 °C for up to 48 h. In contrast to the reconstitution system utilizing intact membranes and PEG, the rate of cleavage of mSC by the bile canaliculared protease was highly dilution-sensitive, following solubilization with octylglucoside (not shown). Cleavage of mSC also proceeded at a much slower rate following solubilization than when using similar amounts of intact membranes (t½, of ~30 versus 2.5 h, respectively) and may reflect loss of activity as well as dilution upon solubilization. Although cleavage of mSC was more rapid at 25 than at 4 °C (Fig. 7A, lanes 5 and 9), an additional cleavage fragment of M, 76,000, migrating slightly ahead of the lower band of authentic fSC (Fig. 7A, lane 9), was also produced. Preliminary studies examining the effect of protease inhibitors on mSC cleavage indicate that formation of the M, 76,000 species is to be due to a different protease than the one responsible for generation of the M, 80,000 fragment. Cleavage of solubilized mSC was routinely examined at 4 °C to prevent formation of the M, 76,000 species.

The M, 80,000 and M, 92,500 fragments of SC produced in the reconstituted system using 32P04-labeled mSC and intact membranes were devoid of phosphate, indicating that the phosphorylated region of the cytosolic domain of mSC had

![Fig. 6. mSC cleavage activity of octylglucoside-solubilized membranes.](image)

![Fig. 7. Time and temperature dependence of [35S]Cys-mSC cleavage.](image)
been released. When octylglucoside-solubilized $^{32}$P-PO$_4$-labeled mSC from metabolically labeled mSC-enriched membranes was incubated with octylglucoside-solubilized bile canicular protease, mSC was cleaved as indicated by the decrease in $^{32}$P-PO$_4$-labeled mSC but no phosphorylated M, 80,000 or M, 92,500 fragments were detected (Fig. 7B, lane 3). In the absence of added bile canicular protease, the majority of $^{32}$P was recovered as intact mSC after 20 h at 4°C (Fig. 7B, compare lanes 1 and 2). Thus, the M, 80,000 and the M, 92,500 fragments produced by the solubilized bile canicular protease have the same properties as the M, 80,000 and M, 92,500 species obtained in the reconstituted system using intact membranes, indicating that the mSC-cleaving protease had retained its specificity following solubilization.

The Bile Canaliculal Protease Has the Properties of an Integral Membrane Protein—Cleavage of mSC in cultured hepatocytes (36) and in the reconstituted system using intact membranes indicated that the bile canicular protease responsible for cleavage is an integral or a peripheral membrane protein. To further examine this, bile canicular membranes were subjected to eight freeze-thaw cycles and then separated into membrane and soluble fractions by centrifugation. The soluble fraction did not mediate cleavage of mSC (Fig. 8, lane 3). The membrane pellet was brought to a concentration of 1.5% octylglucoside and again sedimented at 100,000 × g. The soluble fraction obtained in the presence of octylglucoside was now able to mediate cleavage of mSC to species of 80,000 and the 80,000 or 80,000 M, pg of protein. After a 10-min incubation at 4°C, the supernatant fraction was collected, and the pellet was resuspended in medium A. After the eighth freeze-thaw cycle, the membranes were sedimented again and resuspended to the same volume as the pooled supernatant fractions. The supernatant and pellet fractions were then subjected to eight freeze-thaw cycles and then separated into membrane and soluble fractions by centrifugation. The soluble fraction did not mediate cleavage of mSC (54, 58) did not release the protease from bile canicular membranes (not shown). Thus, detergent concentrations greater than their critical micellar concentration were required for solubilization of the bile canicular protease, consistent with our previous observation that the protease is an integral membrane protein.

**FIG. 8. The bile canaliculal protease requires detergent for solubilization.** Bile canicular membranes (containing 35 μg of protein) were subjected to eight freeze-thaw cycles. Between cycles 4 and 5 the membranes were sedimented for 10 min at 100,000 × g, the supernatant fraction was collected, and the pellet was resuspended in medium A. After the eighth freeze-thaw cycle, the membranes were sedimented again and resuspended to the same volume as the pooled supernatant fractions. The supernatant and pellet fractions were then brought to a final concentration of 1.5% octylglucoside and incubated for 1 h at 4°C. Each sample was sedimented for 10 min at 100,000 × g and the soluble fraction was added to octylglucoside-solubilized mSC-enriched membranes (35 μg of protein). After a 20-h incubation at 4°C, the assays were terminated by boiling in 0.6% SDS, immunoprecipitated with anti-SC antisera, and analyzed by SDS-PAGE and fluorography. Lane 1, ISC standard immunoprecipitated from the medium of rat liver slices metabolically labeled with $^{35}$S. Lane 2, octylglucoside-treated mSC-enriched membranes incubated for 20 h at 4°C with octylglucoside-solubilized extract from bile canicular membranes not subjected to freeze-thaw. Lane 3, octylglucoside-treated mSC-enriched membranes incubated for 20 h at 4°C with the octylglucoside-solubilized supernatant fraction from bile canicular membranes subjected to eight cycles of freeze-thaw. Lane 4, octylglucoside-treated mSC-enriched membranes incubated for 20 h at 4°C with the octylglucoside-solubilized pellet obtained from bile canicular membranes subjected to eight cycles of freeze-thaw.

**DISCUSSION**

Cleavage of mSC to produce fSC occurs following internalization from the sinusoidal plasma membrane of rat hepatocytes (53). Morphological (17) and biochemical (51) evidence suggest that cleavage of internalized mSC occurs immediately prior to or concomitant with arrival at the bile canicular surface. An mSC-specific protease has, however, not been identified or characterized previously. The presence of such a protease on the bile canicular plasma membrane would provide a mechanism for insuring the vectorial release of fSC and bind dimeric IgA into the bile. Such a protease would be expected to be absent or inactive at the sinusoidal surface and within intracellular transport vesicles since there is no evidence of mSC cleavage at these sites.

In cultured rat hepatocyte monolayers, cleavage of fSC and release of fSC take place at the cell surface but not intracellularly. The proteolytic activity responsible for the generation of fSC in cultured hepatocytes is associated with the plasma membrane and is not present in the medium. Differences in the rate and extent of cleavage of cell surface mSC by viable and disrupted hepatocytes suggested that the protease-mediating mSC cleavage at the cell surface has limited access to surface mSC prior to, but not after, cell disruption (36). Although hepatocytes in culture are not fully polarized, they do form morphologically recognizable sinusoidal, lateral, and bile canicular domains (57) including intercellular channels that resemble bile canaliculi (5). A possible explanation for the cleavage behavior observed with cultured hepatocytes is that the mSC-specific protease is largely confined to plasma membrane domains resembling bile canaliculi, whereas mSC is distributed over the remaining cell surface area.

The reconstituted system for cleavage of mSC was established to allow identification and localization mSC-specific proteolytic activities in rat liver. The resistance of intracellular mSC to proteolytic cleavage following disruption of cultured hepatocytes (36) indicated that mSC is less sensitive to proteolytic degradation than previously thought (6, 24) and that a reconstituted system for mSC cleavage could be developed using a membrane fraction containing $^{35}$S-labeled mSC as a source of substrate. Since intracellular mSC in rat liver is present in endocytic transport vesicles that mediate its translocation to the bile (18, 43), prelysosomal, endocytic vesicles were used as a highly enriched source of $^{35}$S-mSC. Fractionation of isolated rat hepatocytes by this procedure yields a subpopulation of prelysosomal endocytic vesicles containing both the asialoglycoprotein receptor and endocytosed ligands (3). Whether mSC and the asialoglycoprotein receptor are present within the same endocytic vesicle or in different vesicles of similar density within this fraction has not yet been determined. The mSC-enriched fraction may be similar to the transport vesicles containing mSC which have been prepared from whole rat liver (9). The presence of more than 85% of the total $^{35}$S-mSC from liver slices in the mSC-enriched fraction is consistent with an intracellular location for the majority of mSC since only 15% of the cell surface plasma membrane is found in this fraction when it is prepared from isolated hepatocytes (3). mSC has been reported to be predominantly intracellular in whole liver as well (48). Since cleavage of mSC by mSC-enriched membranes does not occur at 4°C in the absence of exogenously added membranes, this vesicle fraction has little endogenous specific or nonspecific mSC cleavage activity.
Among the rat liver subcellular fractions tested, only bile canaliculal membranes support the cleavage of mSC in mSC-enriched membranes to generate a product with the same properties as fSC. Cleavage requires either PEG-mediated canalicular membranes support the cleavage of mSC in mSC-tested following solubilization with octylglucoside. Thus, bile vage nor are lysosomal or microsomal membranes. Total rat oidal plasma membranes are not effective in mediating clea-

fuson of intact mSC-enriched membranes with bile cana-

lular membranes indicates that mSC is cleaved at or near the same

membrane-bound and only able to mediate cleavage of mSC when colocalized within the same membrane as mSC or when both the protease and mSC have been solubilized with nonionic detergent. No mSC-specific proteolytic activi-

ties were found in other rat liver membrane fractions, sug-

gest ing that the activity associated with bile canaliculal mem-

branes is highly selective.

Comparison of authentic fSC and the M, 80,000 product generated by fusion of mSC-enriched and bile canaliculal membranes indicates that mSC is cleaved at or near the same site in the reconstituted system as in vivo, fSC produced by rat hepatocytes in culture (36, 57) and in liver (24) migrates as a closely spaced doublet of M, 80,000 and 78,000 during SDS-PAGE. Conversion of mSC to a single product M, 80,000 in the reconstituted system suggests that a separate pro-

tease(s) may be responsible for the formation of the M, 78,000 fSC species in vivo. In rat jejunum, mSC is initially cleaved to a soluble M, 80,000 form of fSC which is subsequently converted to the predominant M, 70,000 ISC form by a second cell-associated endopeptidase (2). Rat liver may contain an analogous protease which would cleave the M, 80,000 frag-

tment to the M, 78,000 fSC species in vivo; however, this protease may not be active in the reconstituted system. It is also possible that there is an alternate cleavage site on mSC which is utilized in vivo but not in vitro to generate M, 78,000 fSC directly from mSC.

An M, 92,500 cleavage fragment was also observed under a number of conditions. The properties of this fragment suggest that it arises by cleavage of the carboxyl-terminal cytoplasmic domain of mSC. This would result in a fragment which no longer contained the phosphorylated residues of the cyto-

plasmic domain, behaved as a membrane associated rather than a soluble protein, and was still susceptible to cleavage by the bile canaliculal protease to produce the M, 80,000 species. This activity cannot by itself account for the produc-

tion of fSC either in vivo or in the reconstituted system.

Although it is not possible to categorically state that the protease activity we have detected accounts for cleavage of mSC to fSC in vivo, it has the properties expected for such a protease based on our previous studies with cultured rat hepatocytes. The mSC specific activity is highly enriched in bile canaliculal membranes and is absent in other cell surface and intracellular membranes, it efficiently cleaves mSC to the appropriate soluble fragment at neutral pH, and it must be colocalized within the same membrane as mSC to effect cleavage. The localization of a highly active protease specific for mSC to the bile canaliculal plasma membrane domain would account for the presence of fSC in bile and its absence from blood. This finding represents the first description of a selective endopeptidase with a polarized cell surface distribu-

tion in liver. The properties of the mSC-cleaving protease and the mechanism of its localization to the bile canaliculal mem-

brane are of interest from the perspective of both SC-mediated trans-epithelial transport of immunoglobulins and biogenesis of polarized plasma membrane domains. The ability to solu-

bilize this protease, while retaining its specificity of mSC, should greatly facilitate its further purification and charac-

terization.

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Reconstitution of Membrane Secretory Component Cleavage