# Cleavage of Membrane Secretory Component to Soluble Secretory Component Occurs on the Cell Surface of Rat Hepatocyte Monolayers

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*Abstract.* Rat liver secretory component is synthesized as an integral membrane protein (mSC) and cleaved to an 80-kD soluble form (fSC) sometime during transcellular transport from the sinusoidal to the bile canalicular plasma membrane domain of hepatocytes. We have used 24-h monolayer cultures of rat hepatocytes to characterize the conversion of mSC to fSC. Cleavage of mSC in cultured hepatocytes is inhibited by the thiol protease inhibitors leupeptin, antipain, and E-64, but not by other inhibitors, including diisopropylfluorophosphate, pepstatin, *N*-ethylmaleimide, *p*-chloromercuribenzoic acid, and chloroquine. Leupeptin-mediated inhibition of cleavage is concentration dependent and reversible. In the presence or absence of leupeptin, only 10–20% of mSC is accessible at the cell surface. To characterize the behavior of surface as opposed to intracellular mSC, cell surface mSC was labeled with $^{125}I$ by lactoperoxidase-catalyzed iodination at 4°C. Cell surface $^{125}I$-mSC was converted to extracellular fSC at 4°C in the absence of detectable internalization. Cleavage was inhibited by leupeptin and by anti-secretory component antiserum. Cleavage also occurred at 4°C after cell disruption. In contrast, $^{125}I$-mSC that had been internalized from the cell surface was not converted to fSC at 4°C in either intact or disrupted cells. Hepatocytes metabolically labeled with [$^{35}S$]cys also released small quantities of fSC into the medium at 4°C. The properties of fSC production indicate that cleavage occurs on the surface of cultured rat hepatocytes and not intracellularly. Other features of the cleavage reaction suggest that the mSC-cleaving protease is segregated from the majority of cell surface mSC, possibly within a specialized plasma membrane domain.

MEMBRANE secretory component (mSC)[1] mediates the transcytotic movement of polymeric IgA (pIgA) from the sinusoidal surface of rat hepatocytes to the bile (reviewed in references 1 and 28). After internalization from the sinusoidal plasma membrane, mSC-bound pIgA is initially found in the same endocytic compartments as the asialoglycoprotein and mannose-6-phosphate receptors and their ligands. Segregation of pIgA-mSC complexes from ligands destined for lysosomes has been reported to occur in the CURL (compartment of uncoupling receptors and ligands) (13). After leaving the CURL, pIgA is transported through the hepatocyte in uncoated vesicles that accumulate in the vicinity of the bile duct (16). pIgA, still associated with secretory component (SC), is ultimately released into the bile. Although the pIgA found in bile is intact, the molecular mass of SC has decreased from ~120 to 80 kD (20, 40, 41). This reflects cleavage near the membrane-anchoring domain of mSC, resulting in conversion of mSC from an integral membrane protein to a soluble, secreted protein (fSC) (26). fSC is also produced by rat hepatocytes in the absence of pIgA, indicating that movement of mSC to the region of the bile canaliculus and its cleavage to yield fSC do not require bound pIgA (29).

It is not known at what point cleavage of mSC to fSC takes place during transport to the bile (1, 25, 38). The absence of fSC in the blood of healthy individuals (23) suggests that cleavage rarely if ever occurs on the sinusoidal surface of hepatocytes. If the membrane-bound domain of mSC were required for segregation of mSC-pIgA complexes from other ligands and receptors, conversion of mSC to fSC is not likely to occur before or within the CURL. The reported absence of fSC in intracellular membranes (20, 42) suggests that mSC cleavage may occur at the bile canalicular surface itself or within uncoated vesicles immediately before entry of SC into the bile. Production of fSC, therefore, is likely to be a highly localized process. This suggests that the protease that cleaves mSC to fSC is either absent from or inactive within the endoplasmic reticulum, Golgi, sinusoidal plasma membrane, and endocytic vesicles through which mSC passes be-

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1. *Abbreviations used in this paper*: DFP, diisopropylfluorophosphate; E-64, L-*trans*-epoxysuccinyl-leucyl-amido(4-guanidino)butane; F12+ chase medium, Ham's F12 supplemented with 8 mM glutamine, 20 mM glucose, $2.2 \times 10^{-4}$ M cys-HCl, 100 U/ml penicillin, and 100 mg/ml streptomycin; fSC, the soluble 80-kD form of secretory component that contains the binding site for polymeric immunoglobulin A; mSC, membrane secretory component; NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoic acid; pIgA, polymeric immunoglobulin A; SC, secretory component; TLCK, tosyl-L-lysine chloromethyl ketone.

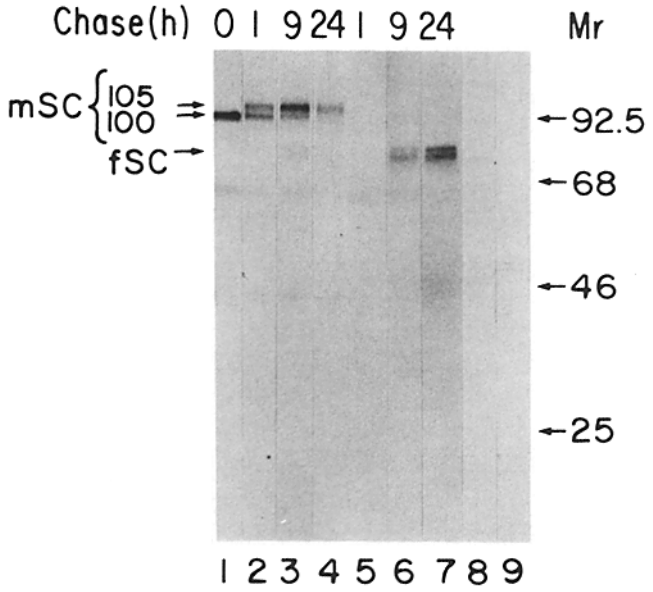


*Figure 1.* Pulse-chase analysis of metabolically labeled mSC synthesis and processing in cultured rat hepatocytes. Primary rat hepatocyte monolayer cultures were labeled with [$^{35}$S]cys for 30 min and chased in media containing excess unlabeled cysteine for up to 24 h. The media and cells were then separately boiled in SDS, immunoprecipitated with anti-SC antiserum, and analyzed by SDS PAGE and fluorography. Lanes *8* and *9* depict normal rabbit serum controls from a similar experiment. (Lanes *1–4*) Anti-SC immunoprecipitates from the lysates of cells chased for the indicated times; (lanes *5–7*) anti-SC immunoprecipitates from the media of cells chased for the indicated times; (lane *8*) cell lysate, 1-h chase, normal rabbit serum control; (lane *9*) media, 9-h chase, normal rabbit serum control.

fore its arrival at its site of cleavage in the region of the bile canaliculus.

We have used primary rat hepatocytes maintained in monolayer culture to characterize the conversion of mSC to fSC and to determine how cleavage of mSC is regulated. Hepatocyte monolayers synthesize mSC and release fSC into the culture medium in a time-dependent fashion. Conversion of mSC to fSC occurs exclusively at the surface of cultured cells, reflecting the localization of an mSC cleaving protease to the plasma membrane. The protease has only limited access to mSC at the cell surface, indicating that the protease and mSC may be segregated from each other on the plasma membrane. This raises the possibility that the mSC-cleaving protease is confined to the bile canalicular domain of both hepatocytes in culture and whole liver.

## Materials and Methods

### Materials

E-64 (L-*trans*-epoxysuccinyl-leucyl-amido(4-guanidino)butane) was purchased from Peptide Institute, Inc. (Osaka, Japan). All other inhibitors were obtained from Sigma Chemical Co. (St. Louis, MO). The compositions of the standard tissue culture media used (L15, DME) were as listed in the Gibco catalogue (Grand Island, NY). Sources of other reagents are described elsewhere (Musil, L. S., and J. U. Baenziger, manuscript submitted for publication).

### Hepatocyte Culture, Labeling, and Trypsinization

Details of the isolation and culture of the rat hepatocytes, [$^{35}$S]cys metabolic labeling of mSC, lactoperoxidase-catalyzed cell surface radioiodination, and trypsin treatment of intact cells are given elsewhere (Musil, L. S., and J. U. Baenziger, manuscript submitted for publication). All 4°C incubations were conducted on ice in a 6°C cold room with hepatocyte monolayers chilled to 4°C on ice for 10 min before the beginning of the experiment.

### Immunoprecipitation and Quantitation of SC

Experiments were terminated by addition of SDS to labeled hepatocyte monolayers and media followed by immunoprecipitation with anti-SC antiserum as detailed elsewhere (Musil, L. S., and J. U. Baenziger, manuscript submitted for publication). Immunoprecipitated mSC and fSC were separated by SDS PAGE on 10% discontinuous gels according to Laemmli (22) and visualized by either fluorography ([$^{35}$S]cys-labeled samples) (4) or autoradiography ($^{125}$I-labeled samples) on prefogged XAR-5 film. Quantitation was performed by densitometry. The effect of protease inhibitors of fSC production is presented: percent inhibition of fSC production = $[(B - A)/(B)] \times 100$, where A = (fSC)/(mSC + fSC) in the presence of inhibitor and B = (fSC)/(mSC + fSC) in the absence of inhibitor. None of the inhibitors tested caused more than a 35% decrease in the total labeled SC content of treated cells. Significant quantities of fSC were not detectable in the cell lysates under any conditions.

### Inhibitor Studies

Concentrated stock solutions of inhibitors were prepared as follows: chymostatin, 20× in 0.1 N NaOH; 1,10 phenanthroline, 1,000× in EtOH; phenylmethylsulfonyl fluoride (PMSF), 200× in isopropanol; diisopropylfluorophosphate (DFP), 1:6 in isopropanol; pepstatin, 1,000× in dimethyl sulfoxide, and *p*-chloromercuribenzoic acid (PCMB), 100× in 0.1 N NaOH. All other inhibitors were prepared as stock solutions in distilled water. Due to their lability in aqueous solutions PMSF, tosyl-L-lysine chloromethyl ketone (TLCK), DFP, and *N*-ethylmaleimide (NEM) stock solutions were prepared and diluted into media immediately before use. Hepatocyte cultures were metabolically labeled with [$^{35}$S]cys for 1 h. Unless otherwise indicated labeled cells were chased for 5 h in F12+ chase medium (Ham's F12 supplemented with 8 mM glutamine, 20 mM glucose, $2.2 \times 10^{-4}$ M cys-HCl, 100 U/ml penicillin, and 100 mg/ml streptomycin) in the presence or absence of inhibitor. The effects of agents in stock solutions, other than the inhibitors, was assessed on control cultures by addition of equivalent amounts of stock solution without inhibitor. At the end of the chase SC was immunoprecipitated from both the media and the cells as described above.

***PCMB, p-chloromercuribenzenesulfonic acid, DFP (4-h Incubation at 1.0 mM), NEM, EDTA, TLCK, and PMSF.*** Failure of nascent 100-kD [$^{35}$S]cys-labeled mSC to mature to the 105-kD form of mSC in the presence of these agents indicated that they prevented the transport of mSC from the endoplasmic reticulum to the Golgi. Monolayers labeled with [$^{35}$S]cys for 1 h were therefore incubated for 2 h in F12+ chase medium before addition of these inhibitors to allow transport of the majority of [$^{35}$S]cys mSC to the Golgi. No detectable fSC was produced during this 2-h period. Inhibitors were added and incubations continued for either 2.5 h (NEM, EDTA, and TLCK) or 4 h (PCMB, *p*-chloromercuribenzenesulfonic acid, 1.0 mM DFP, and PMSF). For DFP, PMSF, and TLCK the chase with inhibitor was carried out in F12+ chase medium. EDTA was added to cells in $Ca^{++}$- and $Mg^{++}$-free Hanks' balanced salt solution (Gibco). Chases containing NEM, PCMB, or *p*-chloromercuribenzenesulfonic acid were conducted in incubation buffer (8.0 g NaCl, 0.4 g KCl, 0.09 g $Na_2HPO_4 \cdot 7H_2O$, 0.047 g $KH_2PO_4$, 0.097 g $MgSO_4$, 0.4 g $CaCl_2 \cdot H_2O$, and 4.76 g Hepes per 1,000 ml $H_2O$ adjusted to pH 7.5 with NaOH) free of low molecular mass thiols.

***DFP (10-min Incubation).*** A modification of the procedure used by Rotundo and Fambrough to inhibit acetylcholinesterase in intact chick embryo muscle cells was used (32). Monolayers labeled with [$^{35}$S]cys for 1 h were chased for 3 h in F12+ chase medium without DFP to allow transport of labeled mSC out of the endoplasmic reticulum. The cells were then exposed to DFP at either 1.0 or 0.1 mM in L15 medium for 10 min at room temperature. DFP was subsequently removed from the cells by washing three times with L15 and then submerging the cultures in 500 ml of the same medium for 10 min before incubation at 37°C for 2 h.

***Pepstatin.*** As this inhibitor has been reported to enter cells slowly (6), hepatocytes were incubated for 10 h in pepstatin-containing DME supplemented with 15% fetal calf serum before labeling. Cells were then in-

cubated for 1 h in cys-free medium and labeled for 1 h in the presence of pepstatin. The cultures were chased for 5 h in F12+ medium supplemented with pepstatin and 15% fetal calf serum.

*Leupeptin Reversal.* Leupeptin-induced inhibition of fSC generation was reversed at either 37 or 4°C by washing the cultures eight times with 2 ml of leupeptin-free medium. Between the third and fourth washes the cells were incubated in 2 ml of this medium for 7 min to enhance dissociation of bound leupeptin.

### *Cleavage of mSC in Disrupted Hepatocytes*

Labeled hepatocyte monolayers were chilled to 4°C before being scraped into 1 ml of ice-cold 0.25 M sucrose, 10 mM Hepes, pH 7.5. The cells were disrupted by 80 strokes of a tight-fitting pestle in a 7-ml Dounce-type homogenizer (Wheaton Instruments Div., Millville, NJ) and nuclei removed by centrifugation for 10 min at 630 *g*. The samples were maintained at 4°C throughout this procedure. The postnuclear supernatant was then incubated on ice for up to 8 h. At the end of the incubation period the samples were boiled in 0.6% SDS and immunoprecipitated with anti-SC antiserum as described for the lysates from intact cells.

## *Results*

### *Synthesis, Posttranslational Processing, and Cleavage of mSC in Cultured Primary Rat Hepatocytes*

To demonstrate that cultured primary rat hepatocytes synthesize mSC and accurately cleave it to fSC, rat hepatocyte monolayers cultured for 24 h were labeled with [$^{35}$S]cys for 30 min and chased for up to 24 h as shown in Fig. 1. Using rabbit anti-rat fSC antiserum, three species were immunoprecipitated from the cultures at various times during the pulse-chase: two forms of SC migrating with $M_r$s of 100 and 105 kD were obtained from the cell lysate (lanes *1–4*) whereas a single species which migrated as a doublet of $M_r$ ~80 kD was recovered from the medium (lanes *6* and *7*). Precipitation of all three species was prevented by addition of rat bile containing fSC and did not occur when normal rabbit serum was substituted for the anti-SC antiserum (Fig. 1, lanes *8* and *9*). The species with an $M_r$ of 80 kD, immunoprecipitated from the medium, co-migrated with fSC isolated from rat bile and had the same relative molecular mass as reported for fSC produced by perfused rat liver (21). In studies described in detail elsewhere (Musil, L. S., and J. U. Baenziger, manuscript submitted for publication), we have shown that the 100-kD band represents newly synthesized mSC in the endoplasmic reticulum, which is converted to the 105-kD form of mSC by oligosaccharide processing in the Golgi. All or at least a part of the 105-kD mSC is then transported to the plasma membrane and reinternalized before being quantitatively cleaved and released into the medium as the 80-kD form of fSC. Conversion of 105-kD mSC to fSC is relatively slow ($t_{1/2}$ 13 h) in hepatocyte monolayers, in contrast to the secretion of albumin and transferrin from the same cultures ($t_{1/2}$ <1 h for albumin and between 1 and 3 h for transferrin). No significant quantities of the 80-kD species were detected in the cell lysates of cultured hepatocytes at any time. These results indicate that cultured rat hepatocytes process mSC and cleave it to fSC in a manner qualitatively similar to, albeit slower than, that of intact liver.

### *Effect of Protease Inhibitors on mSC Cleavage in Cultured Rat Hepatocytes*

A variety of protease inhibitors were examined for their effect on fSC production (Table I). Hepatocyte monolayers were labeled with [$^{35}$S]cys and the appearance of immunoprecipitable [$^{35}$S]cys-fSC in the medium monitored in the presence and absence of each inhibitor. The majority of protease inhibitors tested did not alter [$^{35}$S]cys-mSC cleavage. This included inhibitors which, under similar conditions, have been reported to decrease other forms of proteolysis in intact cells (36). Cleavage of mSC was not decreased by either $NH_4Cl$ or chloroquine, which raise the pH of acidic intracellular compartments (34, 35), implying that cleavage is not dependent on an acidic environment within intracellular vesicles. Insensitivity of biliary secretion of pIgA to chloroquine has also been reported in isolated perfused rat liver (46). Neither 1,10 phenanthroline nor pepstatin inhibited mSC cleavage, indicating that the protease is not of the metalloprotease or carboxyl classes (5, 45).

Leupeptin, antipain, aprotinin, and E-64 significantly reduced the conversion of mSC to fSC. These compounds are classified as thiol, and with the exception of E-64, serine protease inhibitors (3, 44, 45). A 10-min treatment with DFP, a potent inhibitor of serine proteases that is able to inhibit 100% of the intracellular acetylchlolinesterase activity of perfused rat liver within 5 min at 0.1 mM (15), did not effect fSC generation in cultured hepatocytes at either 0.1 or 1.0 mM. Exposure to 1 mM DFP for 4 h reduced fSC production by 60% but also inhibited transferrin secretion. Thus, extended incubation in DFP had nonspecific toxic effects on secretion and did not inhibit mSC cleavage selectively. This suggested that cleavage of mSC to fSC does not involve a serine protease. High levels of the thiol protease-specific inhibitor E-64 (3, 14) inhibited fSC production without affecting transferrin secretion, supporting the possibility that the protease responsible for the conversion of mSC to fSC was a thiol protease. However, other thiol-specific reagents such as PCMB, *p*-chloromercuribenzenesulfonic acid, and NEM (2) did not inhibit fSC generation by intact cells despite having nonspecific deleterious effects on other cellular functions such as transferrin secretion. Thiol proteases that are insensitive to some thiol-specific inhibitors such as NEM and iodoacetamide have been described (37). The protease responsible for mSC cleavage may be similar in this respect. Leupeptin and E-64 were also potent inhibitors of [$^{35}$S]cys-fSC production in metabolically labeled liver slices (not shown).

### *Leupeptin Inhibition of mSC Cleavage Is Reversible*

Leupeptin and its closely related analogue antipain were the most effective inhibitors of mSC cleavage. Immunoprecipitation of SC from media and cell lysates of [$^{35}$S]cys labeled hepatocytes incubated in the presence and absence of leupeptin is shown in Fig. 2. At $2.1 \times 10^{-4}$ M (100 μg/ml), leupeptin inhibited fSC production by 75% (Fig. 2, cf. lanes *1* and *3*) but did not reduce either albumin or transferrin secretion (not shown), indicating that processes associated with soluble protein secretion were not significantly altered. The cell-associated $^{35}$S-mSC accumulated in the presence of leupeptin was exclusively the mature 105-kD form (Fig. 2, lane *2*). Inhibition of mSC cleavage by leupeptin was concentration dependent, reaching 50% at $8.4 \times 10^{-6}$ M (see Table I) as assessed from experiments similar to those shown in Fig. 2. Inhibition of fSC production plateaued at 75% and could not be increased by exposing the cultures to leupeptin during the

*Table I. Effect of Protease Inhibitors on Conversion of mSC to fSC in Cultured Rat Hepatocytes*

| Nontoxic protease inhibitors | | | Potentially toxic protease inhibitors | | |
|---|---|---|---|---|---|
| Protease inhibitor | Concentration | Inhibition of fSC production* | Protease inhibitor | Concentration | Inhibition of fSC production* |
| | *M* | % | | *M* | % |
| Leupeptin | $1.0 \times 10^{-6}$ | 26 | TLCK | $7.0 \times 10^{-5}$ | 0 |
| | $2.1 \times 10^{-4}$ | 75 ± 3.4‡ | | | |
| | $4.0 \times 10^{-3}$ | 74 | PMSF | $1.0 \times 10^{-3}$ | 0 |
| Antipain | $2.0 \times 10^{-4}$ | 75 | DFP | $1.0 \times 10^{-4}$ | 0§ |
| | | | | $1.0 \times 10^{-3}$ | 0§ |
| | | | | $1.0 \times 10^{-3}$ | 60‖ |
| E-64 | $2.8 \times 10^{-4}$ | 20 | | | |
| | $2.1 \times 10^{-3}$ | 45 | NEM | $1.0 \times 10^{-3}$ | 0 |
| | $8.4 \times 10^{-3}$ | 64 | | | |
| | $1.9 \times 10^{-2}$ | 82 | PCMB | $1.0 \times 10^{-4}$ | −15 |
| Aprotinin | $3.2 \times 10^{-5}$ | 50 | *p*-chloromercuri-benzenesulfonic acid | $1.0 \times 10^{-3}$ | −25 |
| | $1.0 \times 10^{-4}$ | 45 | | | |
| Chymostatin | $2.4 \times 10^{-4}$ | 0 | EDTA | $1.0 \times 10^{-4}$ | 0 |
| Pepstatin | $1.5 \times 10^{-4}$ | 0 | | | |
| Chloroquine | $1.0 \times 10^{-4}$ | 0 | | | |
| $NH_4Cl$ | $2.0 \times 10^{-2}$ | 0 | | | |
| 1, 10 Phenanthroline | $4.4 \times 10^{-4}$ | 0 | | | |
| Soybean trypsin inhibitor | $3.0 \times 10^{-5}$ | 0 | | | |
| Agmatine | $1.0 \times 10^{-3}$ | 0 | | | |

* Hepatocytes were metabolically labeled with [$^{35}$S]cys for 1 h and chased in the presence or absence of inhibitor. Production of fSC was quantitated after immunoprecipitation, SDS PAGE, and fluorography. The presence of a negative number indicates enhancement of cleavage.
‡ Mean value ± standard error of the mean from six experiments.
§ Exposure to DFP for 10 min.
‖ Exposure to DFP for 4 h; transferrin secretion was also reduced by 60% under these conditions.

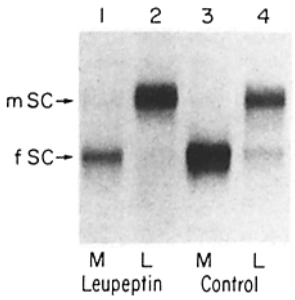


*Figure 2.* Inhibition of mSC cleavage by leupeptin. Rat hepatocytes were incubated with [$^{35}$S]cys for 1 h and then F12+ chase medium with or without leupeptin ($2.1 \times 10^{-4}$ M) for 5 h. At the end of the chase period SC was immunoprecipitated separately from the medium (*M*) and from the cell lysate (*L*) and analyzed by SDS PAGE and fluorography. (Lane *1*) Medium with leupeptin; (lane *2*) cell lysate with leupeptin; (lane *3*) medium without leupeptin; (lane *4*) cell lysate without leupeptin.

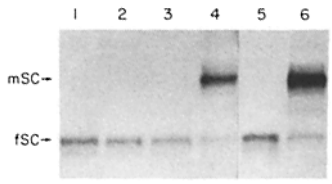


*Figure 3.* Inhibition of mSC cleavage by leupeptin is reversible. Hepatocytes were labeled with [$^{35}$S]cys for 1 h and chased for 12 h in the presence of $2.1 \times 10^{-4}$ M leupeptin. Leupeptin was removed by washing the cells eight times with inhibitor-free chase medium. The incubations were continued for 5 h at 37°C in the absence of leupeptin. At 1, 2.5, and 5 h the medium was removed and replaced with fresh chase medium. At the end of the 5-h incubation the hepatocytes were lysed. The cell lysate and individual samples of medium were immunoprecipitated with anti-SC and examined by SDS PAGE and fluorography. Hepatocytes were also labeled with [$^{35}$S]cys for 1 h and chased for 5 h in the absence of leupeptin to determine the amount of mSC cleaved to fSC in 5 h without prior exposure to leupeptin. (Lane *1*) Medium, 0–1-h chase; (lane *2*) medium, 1–2.5-h chase; (lane *3*) medium, 2.5–5-h chase; (lane *4*) cell lysate after 5 h of chase; (lane *5*) medium, 0–5-h chase without prior exposure to leupeptin; (lane *6*) cell lysate, 0–5-h chase without prior exposure to leupeptin.

labeling as well as the chase period. Thus, leupeptin inhibition of mSC cleavage is selective and concentration dependent.

Inhibition of mSC cleavage by leupeptin can be reversed by removal of leupeptin as shown in Fig. 3. Hepatocyte monolayers were labeled with [$^{35}$S]cys for 1 h and chased for 12 h in the presence of leupeptin. After washing into leupeptin-free medium, the subsequent production of fSC was monitored. Quantitation of the results shown in Fig. 3, lanes *1–4* indicated that 32% of cell-associated mSC was released into the medium as fSC during the first 1 hour after leupeptin removal. An additional 20% of the [$^{35}$S]cys-SC was cleaved to fSC between 1.0 –2.5 h of chase in leupeptin-free medium and 18% was cleaved in the following 2.5-h period. In control cells labeled for 1 h with [$^{35}$S]cys and chased for 5 h in the absence of leupeptin, only 28% of the labeled mSC was released as fSC (Fig. 3, lanes *5* and *6*). The amount of [$^{35}$S]cys-SC (mSC plus fSC) recovered from the control and leupeptin-treated cultures was approximately the

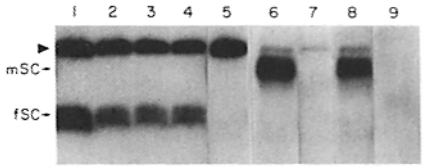


*Figure 4.* Cell surface mSC can be released as fSC at 4°C. Cell surface proteins of cultured hepatocytes were selectively labeled with $^{125}I$ by lactoperoxidase-catalyzed iodination at 4°C. Cells were washed to remove unincorporated $^{125}I$ and maintained in incubation buffer at 4°C. One plate of cells was lysed immediately after labeling (time 0). Another culture was incubated for 12 h at 4°C, during which time the medium was removed and replaced with fresh incubation buffer at 3-h intervals. A cell lysate was prepared from the hepatocytes after the 12-h incubation to determine the amount of remaining cell associated mSC. After the 12-h incubation another plate of cells was treated with trypsin before preparation of the cell lysate (lane *9*) to demonstrate that the remaining mSC was still accessible at the cell surface. All medium and lysate samples were immunoprecipitated with anti-SC and analyzed by SDS PAGE and autoradiography. Normal rabbit serum controls are shown and indicate that the high molecular mass material designated with the arrowhead in the precipitates of both media and cell lysates is nonspecific. One-third of the total medium and one-sixth of each cell lysate was used for the immunoprecipitates. The medium and cell lysate lanes are from 2- and 6-d exposures of the same gel, respectively. (Lane *1*) Medium, 0–3 h of incubation; (lane *2*) medium, 3–6 h of incubation; (lane *3*) medium, 6–9 h of incubation; (lane *4*) medium, 9–12 h of incubation; (lane *5*) medium, 0–3 h of incubation, normal rabbit serum control; (lane *6*) cell lysate, time 0; (lane *7*) cell lysate, time 0, normal rabbit serum control; (lane *8*) cell lysate after 12 h of incubation; (lane *9*) cell lysate from cells incubated for 12 h and treated with trypsin before lysis.

same. The greater extent of cleavage of mSC after leupeptin removal as compared with control cells suggested that reversal of leupeptin inhibition was rapid and that mSC had accumulated at a site within the hepatocyte that was late in the intracellular transport pathway leading to fSC generation. This site may be analogous to the transcytotic vesicles in which mSC is transported from the sinusoidal plasma membrane to the bile canaliculus in vivo (16). Since conversion of mSC to fSC after accumulation at this site is accelerated when compared with newly synthesized mSC, movement of newly synthesized mSC to its site of cleavage may be the rate-limiting step in fSC generation rather than the cleavage reaction itself.

### *Cellular Site of Surface-labeled mSC Cleavage*

The rapid reversal of leupeptin-mediated inhibition of fSC generation suggested that cleavage of mSC was occurring at a site from which leupeptin could be easily removed, such as the plasma membrane or a vesicle population in rapid equilibrium with the cell surface. Tryptic digestion of metabolically labeled hepatocytes at 4°C indicated that roughly 90% of [$^{35}S$]cys-mSC was trypsin resistant and thus intracellular. This was consistent with cell surface immunoprecipitation studies which also indicated that only a small fraction of total cellular mSC was accessible at the plasma membrane (Musil, L. S., and J. U. Baenziger, manuscript submitted for publication). mSC accumulated in the presence of leupeptin was also predominantly (~80%) intracellular. If mSC cleavage occurs intracellularly, significant amounts of intracellular [$^{35}S$]cys-fSC might be expected, especially after reversal of leupeptin inhibition. Although small quantities of cell-associated [$^{35}S$]cys-fSC were observed after leupeptin removal (Fig. 3, lane *4*) as well as in untreated cells (Fig. 3, lane *6*), this fSC was sensitive to trypsin digestion and therefore probably extracellular. The lack of intracellular fSC suggested that mSC cleavage occurs on the cell surface rather than within intracellular vesicles.

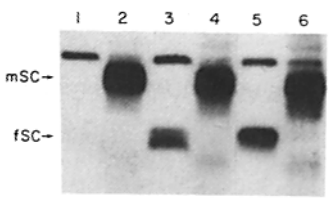


*Figure 5.* Effect of leupeptin and NEM on $^{125}I$-fSC generation at 4°C. Hepatocyte cell surface proteins were labeled with $^{125}I$ as in Fig. 4 and the labeled monolayers maintained at 4°C for 8 h in incubation buffer containing $2.1 \times 10^{-4}$ M leupeptin (lanes *1* and *2*), no additions (lanes *3* and *4*), or 125 μg/ml NEM (lanes *5* and *6*). Media (lanes *1*, *3*, and *5*) and cell lysates (lanes *2*, *4*, and *6*) were examined for SC by immunoprecipitation with anti-SC antiserum followed by SDS PAGE and autoradiography.

To examine the behavior of mSC present on the hepatocyte cell surface, plasma membrane proteins, including cell surface mSC, were selectively labeled by lactoperoxidase-catalyzed iodination with $^{125}I$ at 4°C. Immunoprecipitation of $^{125}I$-labeled SC yielded the 105-kD form of mSC (Fig. 4, lane *6*). When hepatocytes were maintained at 4°C after iodination, $^{125}I$-mSC remained completely sensitive to digestion with trypsin (Fig. 4, lane *9*), confirming the surface location of the $^{125}I$-mSC. Cleavage of mSC at the plasma membrane was assessed by maintaining the cells under conditions that prevent internalization. Hepatocyte monolayers maintained at 4°C after lactoperoxidase-catalyzed cell surface iodination were able to cleave $^{125}I$-mSC and release $^{125}I$-fSC into the medium at a slow but discernable rate, as seen in lanes *1–4* of Fig. 4. In five independent experiments, an average of 27% of the $^{125}I$-mSC was converted to fSC over an 8-h period at 4°C with little net loss of total SC (mSC plus fSC) signal during this time. The $^{125}I$-mSC that remained cell-associated after 12 h at 4°C was sensitive to trypsin digestion (Fig. 4, lane *9*), indicating that internalization was effectively prevented by incubation at 4°C even for extended periods of time. Cleavage therefore appeared to be occurring at the cell surface. The specificity of the cleavage of $^{125}I$-mSC to $^{125}I$-fSC is shown in Fig. 5. Production of $^{125}I$-mSC at 4°C was inhibited by leupeptin (Fig. 5, lane *1*) but not by NEM (Fig. 5, lane *5*) or PMSF. Since the $^{125}I$-fSC produced at 4°C had the same electrophoretic mobility as metabolically labeled fSC, and cleavage was selectively inhibited by leupeptin, the same protease appeared to be responsible for the generation of [$^{35}S$]cys-fSC at 37°C and $^{125}I$-fSC at 4°C.

Additional evidence that cleavage of $^{125}I$-mSC at 4°C was occurring on the cell surface was obtained by examining the ability of anti-SC antiserum to block $^{125}I$-fSC generation at 4°C. Anti-SC antibodies would be expected to exert any inhibitory effect by acting exclusively at the cell surface since they should not enter the cell at 4°C. Fig. 6 shows that anti-SC antiserum, but not normal rabbit serum, abolishes fSC production at 4°C.

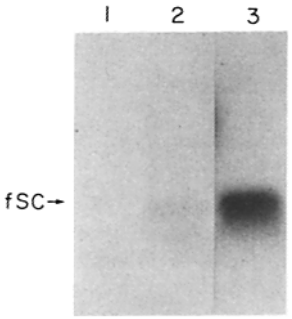


*Figure 6.* Inhibition of $^{125}$I-fSC generation at 4°C by anti-SC antiserum. Hepatocyte cell surface proteins were labeled with $^{125}$I as in Fig. 4. Anti-SC antiserum or normal rabbit serum (150 μl/ml incubation buffer) was added and the cells maintained at 4°C for 8 h. The medium was examined for SC by immunoprecipitation with additional anti-SC antiserum. Since samples were boiled in SDS before immunoprecipitation with fresh anti-SC, there is no detectable precipitation due to residual anti-SC from the initial incubation (lane *1*). (Lane *1*) Control: medium from cells incubated with anti-SC was boiled in 0.6% SDS before addition of *Staphylococcus aureus*. No additional serum was added after boiling. (Lane *2*) medium from cells incubated with anti-SC and precipitated with anti-SC; (lane *3*) medium from cells incubated with normal rabbit serum and precipitated with anti-SC.

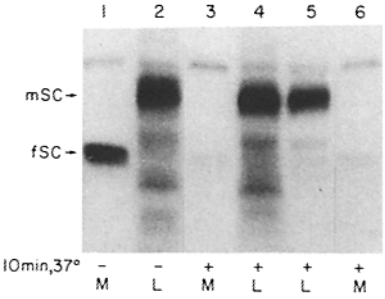


*Figure 7.* Internalized $^{125}$I-mSC is not cleaved to fSC at 4°C. Hepatocyte cell surface proteins were labeled with $^{125}$I as described in Fig. 4. After iodination cells were washed and either maintained at 4°C for 10 min or warmed to 37°C for 10 min to permit internalization of mSC present at the cell surface. This was followed by incubation for 8 h at 4°C. Media and cell lysates were examined for mSC and fSC by immunoprecipitation with anti-SC and autoradiography after SDS PAGE. Trypsin digestion of cells at 4°C was used to confirm that mSC from cells warmed to 37°C for 10 min and then maintained at 4°C was not accessible at the cell surface, i.e., was intracellular. The source of the material, medium (*M*), or cell lysate (*L*), for each immunoprecipitation is indicated. (Lane *1*) Medium from cells maintained continuously at 4°C without a warmup period; (lane *2*) cell lysate from cells maintained continuously at 4°C without a warmup period; (lane *3*) medium from cells maintained at 4°C after 10 min at 37°C; (lane *4*) cell lysate from cells maintained at 4°C after 10 min at 37°C; (lane *5*) cell lysate from cells maintained at 4°C after 10 min at 37°C and treated with trypsin at 4°C before lysis; (lane *6*) medium from 10-min incubation at 37°C.

Although most forms of intracellular transport do not continue at 4°C (17), the slow rate at which $^{125}$I-fSC was released into the medium at 4°C raised the possibility that it originated from a small fraction of $^{125}$I-mSC that continued to be internalized at this temperature. To determine if cleavage could occur intracellularly, hepatocytes were warmed to 37°C for 10 min to permit internalization of the cell surface $^{125}$I-mSC and then returned to 4°C. We had previously established that cell surface $^{125}$I-mSC became resistant to trypsin digestion after incubation at 37°C for as little as 10 min (Musil, L. S., and J. U. Baenziger, manuscript submitted for publication). As seen in Fig. 7, little $^{125}$I-fSC was produced during a subsequent 8-h incubation at 4°C (lane *3*) when compared with cells that had been maintained at 4°C for 8 h without prior incubation at 37°C (lane *1*). The small amount of $^{125}$I-fSC produced by cells that had been warmed to 37°C for 10 min may have originated from the fraction of labeled mSC that was not internalized; i.e., that which remained trypsin sensitive at the end of the 4°C incubation (cf. lanes *4* and *5* of Fig. 7). Thus, internalization prevented subsequent cleavage of mSC to fSC at 4°C, indicating that cleavage of $^{125}$I-mSC to fSC occurs exclusively on the cell surface.

Internalization of $^{125}$I-mSC may place it in an environment unfavorable for cleavage due to vesicle acidification or altered ion composition. To establish that this was not the case, cleavage of $^{125}$I-mSC originating from the cell surface and from intracellular vesicles was compared in a broken cell preparation. Cell surface mSC was labeled by lactoperoxidase-catalyzed iodination of hepatocytes at 4°C after which the cells were either maintained at 4°C or warmed to 37°C for 10 min and then returned to 4°C. The cells were then disrupted at 4°C by Dounce homogenization and a postnuclear supernatant was prepared. The postnuclear supernatants were incubated on ice for up to 8 h and then examined for the presence of mSC and fSC by immunoprecipitation and SDS PAGE as shown in Fig. 8. In postnuclear supernatants from cells that had been maintained at 4°C, virtually all of the $^{125}$I-mSC was converted to $^{125}$I-fSC (Fig. 8, lane *1*). Leupeptin inhibited fSC generation in these lysates, whereas PMSF did not (not shown). In contrast, postnuclear supernatants from hepatocytes that had been warmed to 37°C for 10 min before disruption did not produce significant amounts of $^{125}$I-mSC over the 8 h of incubation at 4°C (Fig. 8, lane *3*). Thus, the protease responsible for cleavage of mSC did not have access to internalized $^{125}$I-mSC after cell disruption. Metabolically labeled [$^{35}$S]cys-mSC, >85% of which is intracellular, was also not cleaved after cell disruption by Dounce homogenization (Fig. 8, lanes *4* and *5*), further supporting the concept that conversion of mSC to fSC is confined to the plasma membrane. Surprisingly, the extent and rate of cleavage of mSC on the plasma membrane was greater after homogenization than in intact cells. This can be

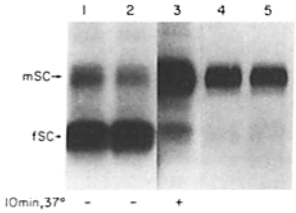


*Figure 8.* Conversion of mSC to fSC after cell disruption. Cell surface mSC was selectively labeled by lactoperoxidase-catalyzed radioiodination, or the total population of mSC was labeled metabolically with [$^{35}$S]cys for 5 h followed by a 1-h chase at 37°C. Iodinated cells were either disrupted immediately after labeling or after a 10-min incubation at 37°C. For both iodinated and metabolically labeled cells disruption was carried out by Dounce homogenization at 4°C. A postnuclear supernatant (PNS) was prepared from the homogenate and incubated for 0 or 8 h at 4°C before solubilization by boiling in 0.6% SDS and immunoprecipitation with anti-SC. (Lane *1*) $^{125}$I-mSC, no warmup period, PNS treated with SDS immediately after preparation; (lane *2*) $^{125}$I-mSC, no warmup period, PNS treated with SDS after 8-h incubation at 4°C; (lane *3*) $^{125}$I-mSC, cells incubated at 37°C for 10 min, PNS treated with SDS after 8-h incubation at 4°C; (lane *4*) [$^{35}$S]cys-mSC, PNS treated with SDS immediately after preparation; (lane *5*) [$^{35}$S]cys-mSC, PNS treated with SDS after incubation for 6 h at 4°C.

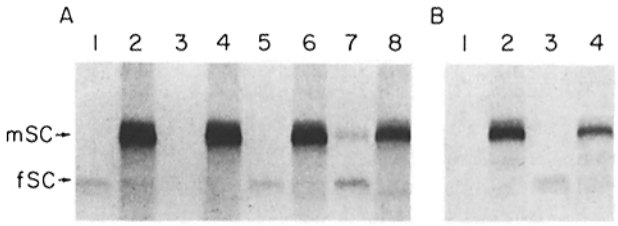


*Figure 9.* Properties of [$^{35}$S]cys-fSC generation at 4°C after leupeptin removal. Hepatocytes were labeled with [$^{35}$S]cys for 2 h and chased for 12 h in the presence of $2.1 \times 10^{-4}$ M leupeptin. The cells were then chilled to 4°C. (*A*) The cells were washed repeatedly at 4°C to remove leupeptin with the exception of one plate, which was to be maintained in leupeptin. The incubations were continued at 4°C in L15 medium without any additions or in the presence of leupeptin, TLCK, or NEM. (Lane *1*) Medium, no additions; (lane *2*) cell lysate, no additions; (lane *3*) medium, continuous incubation with $2.1 \times 10^{-4}$ M leupeptin; (lane *4*) cell lysate, continuous incubation with $2.1 \times 10^{-4}$ M leupeptin; (lane *5*) medium, 50 μg/ml TLCK; (lane *6*) cell lysate, 50 μg/ml TLCK; (lane *7*) medium, 125 μg/ml NEM; (lane *8*) cell lysate, 125 μg/ml NEM. (*B*) The cells were treated with trypsin for 50 min at 4°C, after which 2.5 mg/ml of soybean trypsin inhibitor and 50 μg/ml PMSF was added for 15 min. The inactivated trypsin solution was then removed without dislodging the monolayer, replaced with L15, and the incubation continued for 7 h at 4°C. After completion of the 4°C incubation, media and cell lysates were immediately prepared, except for one culture, which was returned to 37°C for 1 h before solubilization. Media and cell lysates were analyzed for fSC and mSC by immunoprecipitation with anti-SC and SDS PAGE. (Lane *1*) Medium from trypsinized cells maintained at 4°C; (lane *2*) cell lysate from trypsinized cells maintained at 4°C; (lane *3*) medium from trypsinized cells maintained at 4°C and then returned to 37°C for 1 h; (lane *4*) cell lysate from trypsinized cells maintained at 4°C and then returned to 37°C for 1 h.

appreciated by comparing the results in Fig. 8 with those shown in Fig. 4 obtained with intact cells maintained at 4°C. Cell surface mSC may be more accessible to cleavage after cell disruption due to a disruption of plasma membrane domains.

### *Cellular Site of Metabolically Labeled mSC Cleavage*

The evidence presented above strongly supports the hypothesis that mSC is cleaved exclusively at the cell surface; however, it is possible that iodination of surface mSC may alter its behavior. We therefore carried out experiments similar to those described for $^{125}$I-mSC using metabolically labeled mSC. Hepatocytes were labeled with [$^{35}$S]cys and chased for 11–12 h in the presence of leupeptin. The cells were chilled to 4°C, the leupeptin removed, and the incubation continued at 4°C. In the course of a 7-h incubation at 4°C, detectable levels of [$^{35}$S]cys-fSC were released into the medium (Fig. 9 *A*, lane *1*); however, the amount of fSC produced was considerably less than would have been generated at 37°C. The production of fSC was not inhibited by either TLCK or NEM (Fig. 9 *A*, lanes *5* and *7*, respectively). No fSC was produced if leupeptin was not removed (Fig. 9 *A*, lane *3*), indicating that the $^{35}$S-fSC had originated from [$^{35}$S]cys-mSC cleaved during the 4°C incubation. Conversion of [$^{35}$S]cys-mSC to fSC at 4°C was also inhibited by addition of anti-SC antiserum to the cells after leupeptin removal (not shown). Selective destruction of cell surface mSC by trypsin digestion also prevented subsequent production of fSC at 4°C after leupeptin removal (Fig. 9 *B*, lane *1*). When the trypsin-treated hepatocytes were returned to 37°C, however, fSC production resumed (Fig. 9 *B*, lane *3*). Trypsin digestion, therefore, destroyed plasma membrane mSC but did not abolish the cell's capacity to cleave mSC. Thus [$^{35}$S]cys-mSC, like $^{125}$I-mSC, is converted to fSC on the cell surface. The limited production of [$^{35}$S]cys-fSC at 4°C most likely reflects the low levels of [$^{35}$S]cys-mSC present on the plasma membrane and its accessibility to the protease mediating cleavage.

## *Discussion*

The intracellular transport and processing of SC differs from that of other hepatic cell surface receptors. Although pIgA–mSC complexes enter the cell within the same endocytic vesicles as asialoglycoproteins and lysosomal enzymes bearing mannose-6-phosphate (13), pIgA is transported to the bile rather than to the lysosome (33). Unlike the transferrin receptor and the asialoglycoprotein receptor, which are repeatedly returned to the cell surface intact and reused (39), mSC is proteolytically cleaved to a soluble form after transcellular transport of a single pIgA molecule. Previous studies had not established if the cleavage of mSC to fSC was causally related to the trafficking of SC during transcellular transport. It was possible that cleavage of mSC served as an intracellular sorting signal, directing the transport of SC to the bile canalicular domain by a distinct population of vesicles, or that transcytosis resulted in the movement of mSC to a site where cleavage to fSC could occur. In both instances it seems likely that the protease responsible for the conversion of mSC to fSC would have a distinct specificity and/or cellular location.

### *Restriction of mSC Cleavage to the Plasma Membrane*

The data we have presented indicate that cleavage of mSC to fSC occurs exclusively on the cell surface of cultured rat hepatocytes. Internalization of mSC present at the cell membrane prevents its subsequent cleavage to fSC at 4°C in either intact or disrupted hepatocytes. The most plausible explanation for this behavior is that internalization of mSC from the plasma membrane results in its segregation from the protease responsible for fSC production. This suggests that the protease is localized to the cell surface and excluded from transcytotic and other intracellular vesicles. This segregation is maintained even after cell disruption, making it highly likely that the protease is membrane-associated rather than soluble. This conclusion was supported by the lack of detectable fSC-generating activity in medium conditioned by cultured hepatocytes.

### *Distribution of mSC and the mSC-cleaving Protease on the Cell Surface*

Cleavage of cell surface $^{125}$I-mSC proceeds slowly in intact cells at 4°C. In contrast, plasma membrane $^{125}$I-mSC is rapidly and quantitatively converted to $^{125}$I-fSC when labeled monolayers are disrupted by Dounce homogenization at 4°C. This suggests that all of the mSC present on the cell surface is potentially cleavable and that protease activity is not a lim-

iting factor in fSC generation. A possible explanation for the slower rate of mSC cleavage seen with intact cells is that the majority of cell surface mSC is localized to a different region of the plasma membrane than the protease. Cleavage of mSC to fSC would then require redistribution of mSC and/or the protease such that they become colocalized in the same region(s) of the plasma membrane. At 37°C cell surface mSC is rapidly internalized and thus separated from the protease. The slow time course of fSC production at 37°C in metabolically labeled hepatocytes may reflect the time required for internalized mSC to return to a region of the plasma membrane enriched in the mSC-cleaving protease. Cell surface mSC is not internalized at 4°C. Gradual cleavage of mSC on the cell surface at 4°C may be due to incomplete segregation of mSC and protease on the cell surface, allowing a small amount of protease to gain access to mSC by lateral diffusion in the plane of the plasma membrane.

Hepatocytes maintained in monolayer culture have been shown to form distinct sinusoidal, lateral, and bile canalicular plasma membrane domains separated from each other by intercellular junctions (10, 49). Internalized pIgA–mSC complexes appear to preferentially accumulate near, and probably be secreted from, the bile canaliculus–like regions of these monolayers (12). It is possible that the protease responsible for cleavage of mSC at the plasma membrane is preferentially localized to the bile canalicular domain of cultured hepatocytes. In contrast, cell surface mSC may be located primarily in other membrane domains equivalent to the sinusoidal and/or lateral plasma membrane. As noted above, segregation of the protease and mSC on the plasma membrane may be incomplete in cultured hepatocytes. This could be due to "leaky" or partial tight junctions at the bile canalicular domain borders (11, 18, 31), allowing limited diffusion of mSC and/or the mSC-cleaving protease between membrane domains in intact cells and resulting in cleavage of cell surface mSC at 4°C. Rapid access of the protease to mSC and cleavage to fSC may occur during Dounce homogenization due to disruption of these incomplete tight junctions. Definitive localization of the mSC-cleaving protease to a specific plasma membrane domain of hepatocytes awaits the generation of antibodies against the protease with which immunofluorescence studies could be conducted.

Restriction of the mSC-cleaving protease to the bile canalicular membrane in vivo would provide a mechanism to confine secretion of fSC to the bile. Using a cell-free cleavage assay, we found that an activity that converts mSC to fSC is highly enriched in bile canalicular, but not sinusoidal plasma membranes prepared from rat liver (Musil, L. S., and J. U. Baenziger, manuscript in preparation). This would be in keeping with our hypothesized segregation of plasma membrane mSC and the mSC-converting protease in different domains.

### *Characterization of the mSC-converting Protease*

The effect of protease inhibitors on the conversion of mSC to fSC suggests that the mSC-cleaving protease is distinct from previously described, cell-associated proteases that are involved in selective posttranslational cleavage of proteins. Conversion of proalbumin to albumin in primary rat hepatocyte cultures is reportedly not affected by leupeptin (30). Processing of pro-opiocortin in lysates of rat pituitary neurointermediate lobe secretory granules is leupeptin sensitive but is also inhibited by pepstatin A and PCMB (24), which do not effect fSC production. Removal of the pro segment from proinsulin, proglucagon, and prosomatostatin in islet secretory granules is also inhibited by leupeptin; however, sensitivity to PCMB distinguishes the protease(s) involved in these reactions from the mSC-cleaving activity (8, 9). Rat liver plasma membranes have been reported to contain an insulin-degrading endoprotease (48) and a trypsin-like protease (43). The mSC-converting activity differs from the former enzyme in its insensitivity to PCMB (47) and from the latter in its resistance to DFP (43). The data in Table I indicate that the enzyme responsible for the conversion of mSC to fSC is most likely a thiol protease. The high levels of E-64 required for inhibition of fSC production and the lack of effect of PCMB and *p*-chloromercuribenzenesulfonic acid on mSC cleavage suggest, however, that the active site sulfhydryl of the protease reacts poorly with thiol-specific inhibitors when the protein is in the cell membrane.

Mostov and co-workers have recently reported expression of SC in a mouse fibroblast cell line (7) and in MDCK cells (27) transfected with a cDNA coding for rabbit mSC. Both cell types cleave the rabbit mSC to a pIgA-binding fragment that migrates with a relative molecular mass similar to that of rabbit milk fSC (~70 kD) and is recovered almost exclusively from the medium. Although the ability to convert mSC to a soluble, fSC-sized species is not restricted to cell types that normally synthesize SC, it is not known if the same protease is involved nor if cleavage in transfected cells is confined to the cell surface. It is possible that many cell types possess proteases that are capable of converting mSC to an fSC-sized fragment but which are not the same as the hepatocyte protease. Alternatively, there may be a single fSC-generating protease whose physiological substrate in cells that do not synthesize SC is unknown. In this respect the mSC-cleaving protease may resemble endopeptidase-24.11, a widely distributed cell surface peptidase which appears to have different biological functions in different tissues (reviewed in reference 19). In either case, our finding that leupeptin and E-64 inhibit fSC production in liver slices as well as in cultured hepatocytes makes it likely that the protease that converts mSC to fSC in whole liver is responsible for fSC production in cultured hepatocyte monolayers.

### *Conclusion*

We have determined that cleavage of mSC to fSC in cultured hepatocytes occurs exclusively at the cell surface. Since cleavage of mSC occurs after transcellular transport has been completed, cleavage does not appear to play a role in the intracellular trafficking or segregation of mSC from other receptors. The enzyme responsible for the conversion of mSC to fSC has the properties of a membrane-bound thiol protease and may be confined to a specialized plasma membrane domain. Production of fSC appears to be determined by the localization of the mSC-cleaving protease and not by a change in the susceptibility of cell surface mSC to cleavage.

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