Vol. 90, No. 1, 1979 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 12, 1979 Pages 220-226

EFFECT OF PROTEASE INHIBITORS ON ALBUMIN SECRETION IN HEPATOMA CELLS

I. D. Algranati* and D. D. Sabatini

Department of Cell Biology, New York University Medical Center, New York, N. Y. 10016

Received July 15,1979

SUMMARY - Albumin biosynthesis and secretion have been studied in hepatoma cell cultures. The "in vivo" addition of the protease inhibitors antipain or leupeptin caused a fifty per cent reduction in the secretion of albumin without any effect on the total protein synthesis. Pulse and chase experiments in the absence or presence of protease inhibitors have also shown that these substances decrease markedly the rate of albumin secretion.

Many studies have indicated that most proteins secreted by the cells are synthesized in membrane-bound polysomes as precursors of higher molecular weight which undergo a proteolytic processing concomitant with the transfer across the membrane (1). Protein synthesis "in vitro" with reconstituted systems has clearly shown that the processing depends on the presence of membranes (2, 3). However, very little is known about the mechanisms of this proteolysis and the enzymes involved.

In order to elucidate some aspects of protein secretion and the proteolysis coupled to it, we have chosen the hepatoma cell culture as model system, and we have studied the biosynthesis and secretion of albumin. This protein is synthesized as pre-proalbumin and converted into proalbumin in the rough microsomes (4) while the nascent peptides are transferred across the membrane. The proalbumin passes from the lumen of the endoplasmic reticulum to the Golgi apparatus and the secretory granules and before reaching the extracellular environment is again proteolytically processed and converted into albumin (5, 6).

In this paper we describe the effect of various inhibitors of proteolytic enzymes on albumin secretion and synthesis.

MATERIALS AND METHODS

Sodium deoxycholate (DOC) and the protease inhibitors N-tosyl-L-lysyl chloromethane hydrochloride (TLCK), tosyl-L-phenylalanyl chloromethane (TPCK) and p-toluene sulfonyl-L-arginine methyl ester hydrochloride (TAME) were obtained from

---

* Present address: Instituto de Investigaciones Bioquímicas "Fundación Campomar", Obligado 2490, 1428 Buenos Aires, Argentina.

0006-291X/79/170220-07$01.00/0

Calbiochem. The protease inhibitors antipain and leupeptin were kindly provided by Dr. W. Troll. [^{35}S]Methionine (sp. act. 480 Ci/mmol) and [^{3}H]Leu (sp. act.15 Ci/mmol) were purchased from New England Nuclear Corp, and sodium dodecyl sulfate (SDS) from Sigma. Antiserum against rat serum albumin was raised in rabbits and obtained as described by Palmiter et al. (7).

Cell Cultures. Rat hepatoma cells FU5-C8, derived from a subclone obtained from the line FU5 (8), were grown in Ham's F_{12} medium modified as described by Coon and Weiss (9) containing 5% fetal calf serum (FCS). For labeling experiments radioactive methionine or leucine was added to cell cultures at approximately half confluency. Different aliquots from extracellular media or cell lysates were used to measure radioactive TCA-insoluble material and labeled albumin isolated by immunoprecipitation.

Immunoprecipitation and Polyacrylamide Gel Electrophoresis. Direct immunoprecipitation of albumin was performed essentially as described by Rhoads et al. (10) and Taylor and Schimke (11), using 0.1 to 1 ml samples of extracellular medium or cell lysate, 3 μg of rat serum albumin, 50 μl of antiserum containing sufficient antibody for the total precipitation of the albumin used as carrier and the detergents Triton X-100 and DOC, both at a final concentration of 1%. After incubation for 30 min at room temperature, mixtures were centrifuged through sucrose layers containing detergents (11). The precipitates were washed three times with phosphate buffered saline solution (PBS) and redissolved in SDS-containing solutions for counting, gel electrophoresis analysis or further purification by a double immunoprecipitation technique (12).

Occasionally the radioactive albumin or proalbumin was extracted from the immunoprecipitates with ethanol containing 1% TCA according to Judah and Nichols (13). Aliquots of the solubilized immunoprecipitates were counted with 5 ml Aquasol in a liquid scintillation spectrophotometer.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out as described by Maizel (14), using 10% acrylamide slab gels.

RESULTS AND DISCUSSION

Kinetics of Biosynthesis and Secretion of Albumin. We have followed the time course of biosynthesis and secretion of albumin in cultures of FU5-C8 cells by measuring the radioactive product immunoprecipitated by anti-albumin serum in cell lysates as well as in extracellular medium. In agreement with previous reports from other laboratories (5, 6, 15) we could detect albumin in the media only about 20 min after the initiation of the labeling. On the other hand, using cell lysates we were able to immunoprecipitate radioactive albumin as early as 5 to 8 min after the addition of labeled amino acid (data not shown). Although 10 to 50% of the total radioactive protein found in the extracellular medium is albumin, only one major band characterized as this protein appeared in the gel electrophoresis of the total extracellular medium (results not shown). This result suggests that albumin is the main protein secreted by FU5-C8 cells and that most of the other radioactive proteins found in the medium are probably released from some cells broken during the incubation.

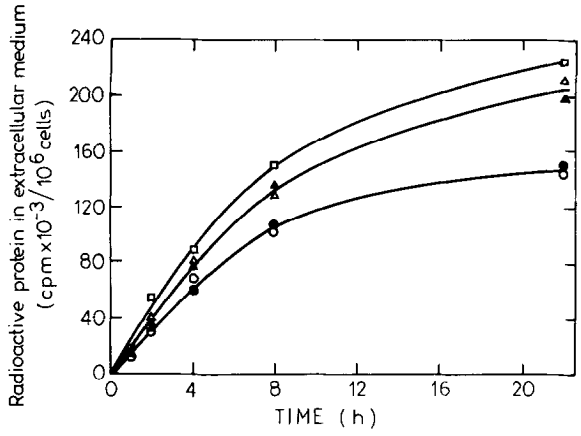


Fig. 1. Protein secretion by FU5-C8 cells in the absence and presence of protease inhibitors. Cell cultures were labeled with [^{35}S] methionine (5 μCi/ml) and 1 ml aliquots taken from the extracellular media at the indicated times were used to measure the radioactive TCA-insoluble material. The following protease inhibitors were used: △, TAME (800 μg/ml); ▲, TLCK (250 μg/ml); ○, leupeptin (0.5 mM) and ●, antipain (0.5 mM). □, control experiment without inhibitors. The values at 0 time were subtracted in each case.

In cell lysates between 0.05 to 1% of the total protein could be isolated as albumin by immunoprecipitation. The maximal relative value corresponds to the shortest pulse with labeled amino acid (namely 10 to 15 min), indicating that radioactive albumin has a rapid turnover and only accumulates intracellularly until reaching the steady state level; afterwards the secretion begins. The direct immunoprecipitation technique used in most of our experiments with the extracellular media was not specific enough to isolate pure albumin from cell lysates. To overcome this problem we have employed a double immunoprecipitation method (12).

Effect of Protease Inhibitors on Total Protein Synthesis and Albumin Secretion in Hepatoma Cells. In order to investigate whether or not the addition of inhibitors of proteolytic enzymes to the cell cultures can block the secretion of proteins into the medium and/or accumulate them or their precursors inside the cells, we have measured the amount of total proteins and albumin in extracellular medium and cell lysates obtained from cultures carried out in the absence and presence of different inhitors. Fig. 1 shows the kinetics of total radioactive protein accumulated in the medium when the cells were grown with or without the addition of protease inhibitors. The results indicate that the oligopeptides antipain and leupeptin (16) were the most effective inhibitors which did not decrease the number of viable cells. In contrast TPCK (not shown in this figure) was clearly toxic and when added to the medium many cells were lysed. Since albumin is the main secretion of the hepatoma cells, the described results could indicate that anti-

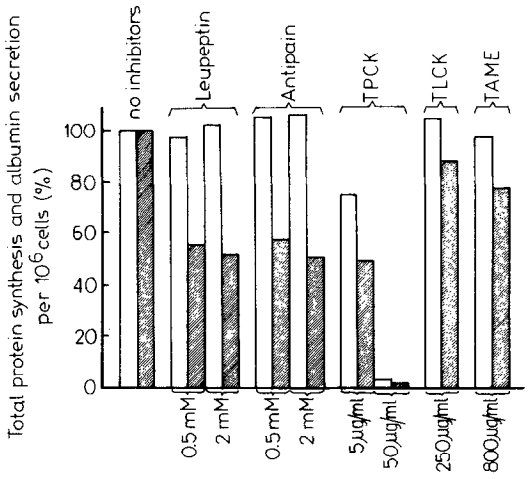
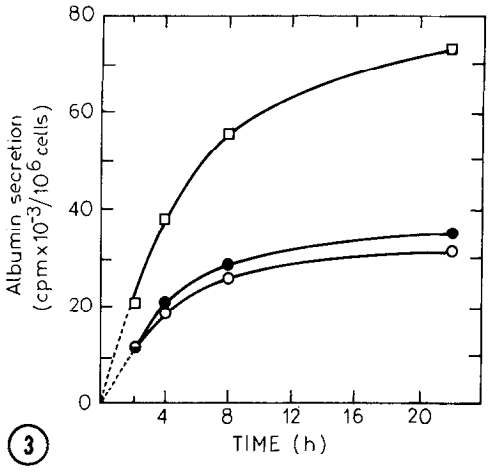


Fig. 2. Effect of protease inhibitors on protein synthesis and albumin secretion. Cell cultures with or without inhibitors (as indicated in each case) were labeled for 24 h with [^{35}S] methionine (5 μCi/ml) or [^{3}H] leucine (25 μCi/ml). Total protein synthesis was calculated by adding the TCA-insoluble radioactive material contained in cell lysate and extracellular medium. Open bars represent total protein synthesis and shaded bars albumin secretion. Values are expressed as percentages of the controls without inhibitors and represent average of triplicate experiments.

pain and leupeptin are inhibitors of albumin secretion. Fig. 2 shows the relative effects of various inhibitors on the total protein synthesis and albumin secretion after 24 h incubation. It can be seen that antipain and leupeptin are in fact potent inhibitors of albumin secretion without decreasing the total protein synthesis. The inhibition of albumin secretion was not more than 50% even at the highest concentration of inhibitors used. It is important to mention that neither albumin nor its precursor were accumulated inside the cells in the presence of inhibitors. TPCK also inhibited the secretion markedly, but at the same time it produced a significant decrease of total protein synthesis.

Effect of Protease Inhibitors on the Kinetics of Albumin Secretion. Fig. 3 shows the time course of albumin secretion from cells grown in the absence or presence of antipain or leupeptin. The level of inhibition was about the same (50%) either after 2 or 22 h of incubation, whereas the total protein synthesis was not decreased in any case (Fig. 2). Since these results correspond to albumin secretion while the biosynthesis of this labeled protein was continuously occurring inside the cell, we have carried out a pulse and chase experiment in order to measure only the secretion of radioactive albumin synthesized during a 15 min pulse. This time corresponds approximately to the normal latent period before the onset of secretion of newly synthesized albumin. The addition of a large excess of unlabeled amino acid at the end of the pulse allows to measure the secretion inde-



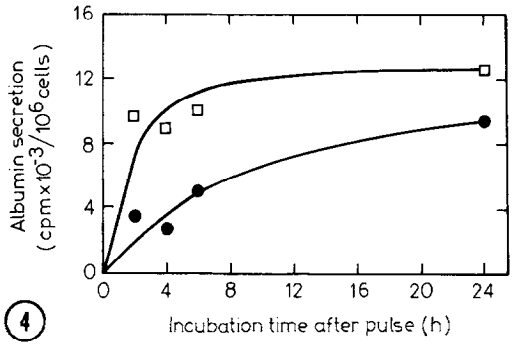


Fig. 3. Kinetics of albumin secretion by hepatoma cells grown in the absence and presence of antipain or leupeptin. Cultures were labeled with [^{35}S]methionine (5 μCi/ml). Aliquots (1 ml) taken from the extracellular media at the indicated times were used for albumin immunoprecipitation. □ , control without inhibitors; ● , antipain; ○ , leupeptin. Both inhibitors were used at 2 mM concentration.

Fig. 4. Effect of antipain on the rate of albumin secretion. Four Petri dishes containing cell cultures at about half confluency were pulsed for 15 min with [^{35}S] methionine (10 μCi/ml; 6 x 10^{-7}M) followed by the addition of unlabeled methionine at a final concentration of 1 mM and a 24 h chase. Two of the dishes also received 2 mM antipain at the end of the pulse. Albumin secretion was followed during the chase period by taking samples from the extracellular medium at different times for immunoprecipitation. Each point is the average of duplicate experiments. □ , control without inhibitors; ● , antipain.

pendently of the biosynthesis process, because the great dilution of the internal radioactive methionine pool occurs almost immediately, as demonstrated in control experiments (data not shown). Fig. 4 indicates that secretion of the albumin synthesized during the pulse was markedly slower in the presence of the protease inhibitor. Under these conditions the initial rate of secretion was about 4-fold lower than the control.

Our approach to study the mechanism of secretion by adding inhibitors of proteolytic enzymes to the cell cultures allowed us to conclude that leupeptin and antipain, which are good inhibitors of trypsin and cathepsin B (15), are able to decrease the secretion of albumin by hepatoma cells. The reduction of the secretion rate is probably due to the inhibition of the conversion of proalbumin into albumin, since it is is known that this reaction is partially blocked by leupeptin or antipain (17).

The product isolated by immunoprecipitation from extracellular medium or cell lysates was characterized by SDS-PAGE as albumin (or proalbumin, which has the same mobility), when the cultures were carried out either in the absence or in the presence of inhibitors. Moreover, amino acid sequence analyses have indicated

TABLE I: Effect of Antipain on the Secretion and Total Recovery of [^{35}S]Albumin Synthesized in a 15 min Pulse

The experiment was carried out as described in Fig. 4 and the text. Radioactive albumin was measured by the double immunoprecipitation method both at the beginning and at the end of the 24 h chase period.

Pulse (15 min) [^{35}S]Albumin (cpm)		Inhibitor addition after pulse	Chase (24 h) [^{35}S]Albumin (cpm)			Recovery after chase
Intracellular	Extracellular medium		Intracellular	Extracellular medium	Total	
84,500	0	None	7,000	84,100	91,100	108%
83,500	0	Antipain (2 mM)	5,200	67,400	72,600	87%

that albumin (and not proalbumin) was secreted in the presence of inhibitors (results not shown). These facts indicate that although antipain and leupeptin are able to slow down the secretion process, they probably do not modify the structure of the secreted albumin nor the intracellular precursor forms.

The inhibition of secretion was not complete even at high levels of inhibitors. This finding might suggest more than one kind of processing enzyme or different localizations of one enzyme in such a way that part of it cannot be reached by the inhibitor.

Proalbumin is not accumulated inside the cell when the secretion is blocked (unpublished results). This fact could mean that the product which is not secreted is degraded, since the total protein synthesis remains constant in the presence of inhibitors. However, the addition of antipain after a 15 min pulse and the subsequent recovery of most of the labeled albumin in the extracellular medium (Table I) seem to indicate that the presence of protease inhibitor does not have any significant effect on the degradation of albumin. Moreover, the rate of albumin synthesis measured in a 15 min period is not affected by the presence of antipain or leupeptin (data not shown). This finding strongly suggests that the protease inhibitors do not have a direct inhibitory effect on the albumin biosynthesis. Therefore, we favour the possibility that the albumin biosynthesis is specifically impaired when its secretion is inhibited by blocking the conversion of proalbumin into albumin.

We can speculate that in many cases the cleavage steps converting precursors into mature proteins might regulate the secretion and even the biosynthesis of secretory and membrane proteins.

In a recent report Brennan and Carrell have described a variant human proalbumin which is secreted into the serum even though it cannot be converted into albumin,

because it contains glutamine instead of arginine at the terminal amino acid residue of the propeptide segment (18). This result, supporting the idea that the cleavage of the propeptide is not necessary for the secretion, is not in agreement with our conclusions. However, the discrepancy may be more apparent than real, because the abnormal proalbumin should not be recognized by the converting enzyme/s, while in our case the proteolytic processing of normal proalbumin is inhibited by the presence of leupeptin or antipain.

Acknowledgements. We are grateful to Dr. L.A. Chasin for the FU5-C8 cell line and many helpful discussions. We thank Drs. M. Adesnik, G. Kreibich and M. Dankert for valuable suggestions and Mrs. Harriet Snitkin and Mr. William Dolan for competent technical assistance. I.D.A. acknowledges a fellowship from the John Simon Guggenheim Memorial Foundation during part of this work.

REFERENCES

1. Campbell, P. N. and Blobel G. (1976) FEBS Lett. 72, 215-226.
2. Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 835-851.
3. Blobel, G. and Dobberstein, B. (1975) J. Cell Biol. 67, 852-862.
4. Strauss, A. W., Donohue, A. M., Bennett, C. D., Rodkey, J. A. and Alberts, A. W. (1977) Proc. Natl. Acad. Sci. USA 74, 1358-1362.
5. Geller, D. M., Judah, J. D. and Nicholls, M. R. (1972) Biochem. J. 127, 865-874.
6. Judah, J. D. and Quinn, P. S. (1976) TIBS 1, 107-109.
7. Palmiter, R. D., Oka, T. and Schimke, R. T. (1971) J. Biol. Chem. 246, 724-737.
8. Schneider, J. A. and Weiss, M. C. (1971) Proc. Natl. Acad. Sci. USA 68, 127-131.
9. Coon, H. G. and Weiss, M. C. (1969) Proc. Natl. Acad. Sci. USA 62, 852-859.
10. Rhoads, R. E., McKnight, G. S. and Schimke, R. T. (1973) J. Biol. Chem. 248, 2031-2039.
11. Taylor, J. M. and Schimke, R. T. (1973) J. Biol. Chem. 248, 7661-7668.
12. Algranati, I. D. (1979) Mol. Cell. Biochem., in press.
13. Judah, J. D. and Nicholls, M. R. (1971) Biochem. J. 123, 642-648.
14. Maizel, J. V., Jr. (1971) in Methods in Virology (Maramorosh, K. and Koprowski, H., eds.) vol. 5, pp. 179-245, Academic Press, New York.
15. Glaumann, H. (1970) Biochim. Biophys. Acta 224, 206-218.
16. Umezawa, H. (1972) in Enzyme Inhibitors of Microbial Origin, pp. 16-52, University of Tokyo Press, Tokyo.
17. Judah, J. D. and Quinn, P. S. (1978) Nature 271, 384-385.
18. Brennan, S. O. and Carrell, R. W. (1978) Nature 274, 908-909.