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The Role of Nuclear Serine Proteases in the Degradation of Newly Synthesized Histones and Ribosomal Proteins[1]

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To examine whether serine proteases of rat liver chromatin are also involved in the degradation of newly synthesized and unbound ribosomal proteins and histones, like the nuclear thiol protease which we reported previously (Tsurugi, K. & Ogata, K. (1979) *Eur. J. Biochem.* **101**, 205–213), *in vivo* experiments were carried out with serine protease inhibitor, PMSF. The following results were obtained.

When normal rats received an intraperitoneal injection of PMSF (10 mg per 100 g body weight), nuclear serine proteases were inhibited almost completely for at least 90 min. PMSF did not affect the synthesis of proteins and RNAs of ribosomes and other subcellular fractions.

The effects of PMSF treatment *in vivo* on the degradation of newly synthesized ribosomal proteins and histones in regenerating rat liver pretreated with a low dose of actinomycin D, which preferentially inhibited rRNA synthesis, were examined by using the double-isotope method. It was found that PMSF treatment did not affect their degradation. On the other hand, administration of E-64, a thiol protease inhibitor, to partially hepatectomized rats inhibited the degradation of those proteins markedly. From these results, it is concluded that the nuclear thiol protease, but not serine proteases, is preferentially involved in the degradation of newly synthesized ribosomal proteins and histones which are not associated with rRNA and DNA, respectively.

When the synthesis of rRNA in rat liver was selectively inhibited by treatment with a low dose of actinomycin D for 1 h, the synthesis of ribosomal proteins was only slightly inhibited (*2*). Ribosomal proteins synthesized in excess over synthesized rRNA were found to be degraded rapidly with a half-life of 20 to 40 min (*2*). Similar results were obtained in HeLa cells by Warner (*3*).

When E-64, a thiol protease inhibitor, was administered to partially hepatectomized rats pretreated with a low dose of actinomycin D, newly synthesized ribosomal proteins were accumulated in liver nuclei. Newly synthesized histones were also accumulated in liver nuclei, especially in

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Abbreviations: PMSF, phenylmethanesulfonyl fluoride; DFP, diisopropylfluorophosphate; TCA, trichloroacetic acid; SDS, sodium dodecyl sulfate.

nuclear sap, by E-64 treatment regardless of actinomycin D treatment (*1*). From these results, we concluded that newly synthesized ribosomal proteins and histones that are not associated with rRNA and DNA, respectively, are degraded by a thiol protease in rat liver nuclei. Recently, we found a chromatin-bound thiol protease in regenerating rat liver and partially purified it (*4*).

On the other hand, several reports have been published on chromatin-bound serine proteases in rat liver (*5–9*). We have also shown that rat liver chromatin contains at least two kinds of serine proteases (*10*). One is a 25,000 dalton protease with maximal activity at pH 8 and shows high specificity for basic proteins. The other is a 15,000 dalton protease which is present as a large inactive form (Mr. 52,000) in chromatin. The conversion of the inactive form to active protease occurs at pH 10 or at high concentration of NaCl at pH 8. Although this protease also shows a significant affinity for basic proteins at pH 10, it appears to preferentially degrade hydrophobic proteins such as casein and non-histone proteins of chromatin. Considering these results, together with the extractability from chromatin, we postulated that the 25,000 dalton protease is involved in the metabolism of histones and the 15,000 dalton protease is involved in that of non-histone proteins. Furthermore, when chromatin was self-digested, only H1 histone was degraded at both pH 8 and pH 10, whereas non-histone proteins were preferentially degraded at pH 10 (*10*).

From these results, it is possible that nuclear serine proteases, especially 25,000 dalton protease, also participate in the degradation of newly synthesized and unbound ribosomal proteins and histones in rat liver. In the present experiments we examined this possibility by employing PMSF, a serine protease inhibitor, and E-64 *in vivo* and found that serine proteases do not participate in the degradation of newly synthesized and unbound ribosomal proteins and histones in rat liver, while the thiol protease is preferentially involved in it, as described previously (*1*).

MATERIALS AND METHODS

Animals—Wistar strain rats weighing 180–230 g were used. In the experiments employing radioisotopes *in vivo*, rats were partially hepatectomized according to Higgins and Anderson (*11*) and used 18 h after the operation. They were intraperitoneally injected with 55 μg of actinomycin D per 100 g body weight (*12*) and 1 h later subjected to labeling experiments.

In the experiments using rats treated with PMSF *in vivo*, 10 mg of PMSF was dissolved in 0.2 ml of a solution containing 50% absolute ethanol and 50% propylene glycol immediately before use and injected intraperitoneally into a rat.

Preparation of Subcellular Fractions—Nuclei, nucleoli, microsomes, ribosomes, and cell sap were prepared from rat liver as described previously (*1*, *13*, *14*). Procedures for the preparations of histones, ribosomal proteins and serum albumin from rat liver were described previously (*14*). Chromatin was prepared according to Amano (*15*) and Fukuda *et al.* (*16*). Chromatin thus obtained showed almost the same composition and proteolytic activities as that obtained according to Bacheler and Smith (*17*), as we reported previously (*10*).

Preparation of Component II—Component II, containing labeled ribosomal proteins and histones almost exclusively when prepared from the homogenate of rat liver pulse-labeled with radioactive amino acid (*14*), was prepared from total homogenate, nuclei and cell sap of regenerating rat liver as described previously (*1*).

Double-Isotope Method—To examine the effects of PMSF treatment on the relative amounts of newly synthesized ribosomal proteins in liver homogenate and subcellular fractions, a double-isotope method was employed as described previously (*1*, *14*, *18*). Briefly, control partially hepatectomized rats were intraperitoneally injected with 25 μCi of [^{14}C]leucine per 100 g body weight; other partially hepatectomized rats treated with PMSF received an intraperitoneal injection of 200 μCi [^{3}H]leucine per 100 g body weight. One h later, the two groups of rats were sacrificed, and liver homogenates prepared from both groups were mixed to obtain an appropriate ratio of ^{3}H to ^{14}C.

Enzyme Assay—The reaction mixture for acidic protease contained (in a total volume of 0.5 ml) about 100 μg protein of rat liver chromatin, 1 mM EDTA, 2 mM dithiothreitol, 50 mM acetate buffer, pH 5.5 and 2.5 μg of ^{3}H-labeled

ribosomal proteins as the substrate. For neutral and alkaline proteases, it contained (in 0.5 ml) the same amounts of liver chromatin, 50 mM Tris-HCl buffer, pH 8, or glycine buffer, pH 10, and 2.5 μg of ^{3}H-labeled ribosomal proteins. After the mixture had been incubated at 37°C for 8 h, the radioactivity of the 10% TCA-soluble fraction was measured as described previously (*4*).

Determination of Specific Activity of Proteins and RNA—To measure the radioactivity of RNA or protein of a subcellular fraction, an equal volume of 10% TCA was added to the fraction and the mixture was boiled for 10 min. After low-speed centrifugation, the supernatant was used to measure the specific radioactivity of RNA. For the measurement of the specific radioactivity of protein, the precipitate was washed twice with 5% TCA, and finally with acetone, then suspended in 0.1 ml of 6 M urea. A portion of the RNA or protein fraction was taken and the radioactivity was measured in a toluene-based scintillator containing 30% Triton X-100.

Protein was determined by the method of Lowry *et al.* (*19*) and RNA by the orcinol method (*20*).

To measure the radioactivity of the gel after SDS-polyacrylamide gel electrophoresis, a gel rod was cut into 1.5 mm-thick slices which were then crushed in counting vials. Next, 0.3 ml of Soluene-350 was added, the vial was left to stand at room temperature for 1 h, and the radioactivity was measured in a toluene-based scintillator.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis was carried out according to Laemmli (*21*).

RESULTS

Proteolytic Activities of Chromatins of Normal and Regenerating Rat Livers—In the present experiments, regenerating rat liver was used to obtain higher labeling of ribosomal proteins and histones, as described previously (*22*). On the other hand, since serine proteases were demonstrated in the chromatin of normal rat liver (*4*), it was necessary to examine whether the proteolytic activities were different between chromatins of normal and regenerating rat livers. Table I shows that both kinds of rat livers contain similar protease activities at pHs 5.5, 8, and 10, where maximal activities of the thiol protease and two kinds of serine proteases, respectively, were observed in normal rat liver (*10*).

TABLE I. Comparison of proteolytic activities of chromatins from normal and regenerating rat livers. The proteolytic activities of chromatins from normal and regenerating rat livers at pH 5.5, 8, and 10 were determined as described in "MATERIALS AND METHODS."

pH	Activity (cpm/mg protein)	
	Normal liver	Regenerating liver
8	1,324	1,112
10	1,126	839
5.5	1,515	1,643

Effects of PMSF on Nuclear Serine Proteases In Vivo—To clarify whether the nuclear serine proteases are involved in the degradation of newly synthesized ribosomal proteins or histones, we examined by using the double-isotope method, whether these proteins were accumulated in the liver nuclei when a serine protease inhibitor was administered to rats as in the case of the thiol protease inhibitor (*1*). As the serine protease inhibitor, we used PMSF instead of DFP, because DFP is much more toxic due to its high affinity for acetylcholine esterase (*23*), although it is more specific for serine proteases.

To test whether PMSF inhibits nuclear serine proteases *in vivo*, normal rats received intraperitoneal injections of various doses of PMSF, and were killed 1 h later. The proteolytic activities of liver nuclei were examined using ribosomal proteins as the substrate. Figure 1 shows that administration of 10 mg of PMSF per 100 g body weight inhibits 90 to 80% of the serine protease activity of control rats, whereas the thiol protease activity was not affected.

When rats were injected with 10 mg of PMSF per 100 g body weight, maximal inhibition was observed within 5 min and continued for at least 90 min (Fig. 2).

Effects of PMSF on the Synthesis of Proteins and Nucleic Acids—As PMSF is known to associate with other kinds of enzymes than serine proteases (*24, 25*), it is possible that it inhibits some enzymes or factors participating in the biosynthesis of proteins and RNAs. To test this

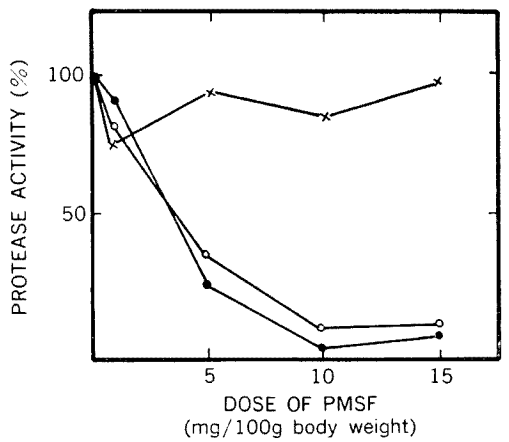

Fig. 1. Effects of PMSF treatment *in vivo* on the proteolytic activities at various pHs in rat liver chromatin. Normal rats were injected with various amounts of PMSF intraperitoneally. One h later, proteolytic activities of the chromatins were measured at various pHs as described in "MATERIALS AND METHODS." ●, the activities at pH 8; ○, pH 10; and ×, pH 5.5.

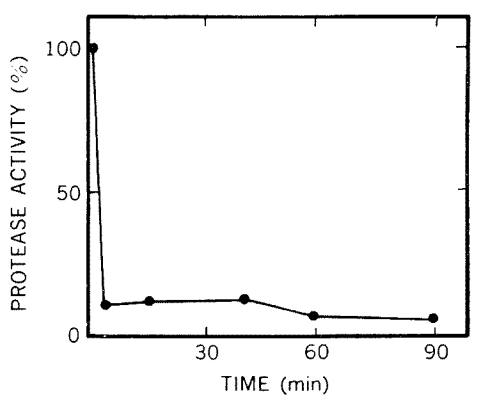

Fig. 2. Effects of PMSF treatment on the proteolytic activity of rat liver chromatin at pH 8. Normal rats were injected with 10 mg of PMSF per 100 g body weight. After various time periods, the proteolytic activities of liver chromatin were determined at pH 8.

possibility, the effects of PMSF treatment on the incorporations of [^{3}H]leucine into proteins and [^{14}C]orotic acid into RNAs were investigated. As shown in Table II, the treatment did not affect the incorporations of these labeled precursors into various proteins or RNAs tested.

Effects of PMSF on the Metabolism of Newly Synthesized Ribosomal Proteins and Histones—Partially hepatectomized rats were pretreated with a low dose of actinomycin D for 1 h. One group

TABLE II. Effects of PMSF treatment *in vivo* on the incorporation of [^{3}H]leucine and [^{14}C]orotic acid into proteins and RNAs, respectively, in rat liver. Normal rats were injected with 1 μCi of [^{14}C]orotic acid and 20 μCi of [^{3}H]leucine per 100 g body weight with and without PMSF (10 mg/100 g body weight) and labeled for 1 h. Various proteins and RNAs of subcellular fractions were prepared and their specific radioactivities were determined. The values in parentheses are percentages of the specific radioactivities of the controls.

	Specific radioactivity (dpm/mg protein or RNA)	
	Control liver	PMSF-treated liver
Proteins		
Cell sap	560 (100)	579 (103)
Microsomal	2,280	2,520 (111)
Ribosomal	891	754 (85)
Nucleolar	710	730 (103)
Histone	670	850 (123)
Albumin	607	828 (118)
RNAs		
Nuclear	24,520	31,840 (129)
Nucleolar	29,700	29,900 (102)
Ribosomal	1,674	1,780 (106)

received intraperitoneal injections of PMSF and [^{3}H]leucine, while the other group received [^{14}C]-leucine. One h later, both groups of rats were killed and liver homogenates were prepared. The [^{3}H]- and [^{14}C]leucine-labeled homogenates were mixed at an appropriate ratio of ^{3}H to ^{14}C radioactivities. One portion of the mixture was used for the preparation of component II, in which more than 90% of the proteins are ribosomal proteins and histones (*14*), and another portion was used for the preparation of subcellular fractions from which components II were prepared. Component II from liver homogenate or nuclei was subjected to SDS-polyacrylamide gel electrophoresis and the ^{3}H-to-^{14}C ratio of each gel slice was determined. The ^{3}H-to-^{14}C ratio was corrected with respect to the value, taken as 1, of this ratio in the total protein of the homogenate. Figure 3 shows that the ^{3}H-to-^{14}C ratios of all gel fractions of components II from both homogenate and nuclei are almost the same as the ratio of total

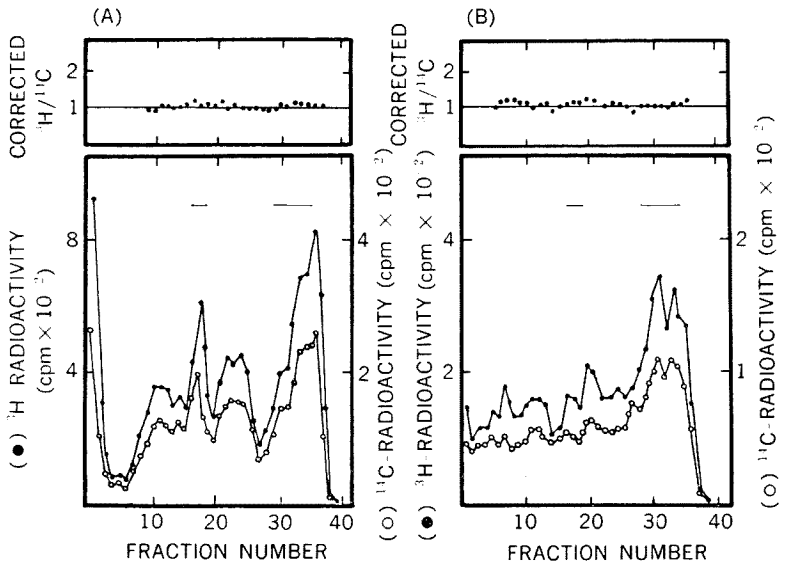

Fig. 3. Effects of PMSF treatment *in vivo* on the labeling pattern on polyacrylamide gel electrophoresis of component II from homogenate (A) and nuclei (B) of regenerating rat livers. Four partially hepatectomized rats were pretreated with a low dose of actinomycin D (55 μg/100 g body weight) for 1 h. Two of them were intraperitoneally injected with 200 μCi of [^{3}H]leucine and 10 mg PMSF per 100 g body weight and the others with 25 μCi of [^{14}C]leucine per 100 g body weight. All rats were killed 60 min later and their liver homogenates were mixed at an appropriate ratio. Component II was prepared from the total homogenate (A) or nuclei (B), and subjected to SDS-polyacrylamide gel electrophoresis. The ^{3}H-to-^{14}C radioactivity ratios of 1.5 mm gel slices were measured as described in "MATERIALS AND METHODS." The lower compartment shows the radioactivity in each gel slice. The upper compartment shows the corrected ^{3}H-to-^{14}C ratio of each gel slice. Bars in the lower compartment indicate the positions of H1 and core histones. ○, ^{3}H radioactivity; ●, ^{14}C radioactivity.

protein of the homogenate. These results suggest that serine proteases are not involved in the degradation of newly synthesized ribosomal proteins and histones.

These findings are different from the results of similar experiments using E-64, as described previously (*1*), in which the ^{3}H-to-^{14}C ratios of component II from liver homogenate or nuclei were about twice the ratio of total proteins of liver homogenate.

To rule out the possibility that PMSF treatment inhibited the transfer of newly synthesized ribosomal proteins and histones to the nucleus and, as a result, led to their accumulation in cell sap, the ^{3}H-to-^{14}C ratio of the component II of cell sap proteins was examined. Only about 1% of ^{3}H and ^{14}C radioactivities was recovered in the component II, and the corrected ^{3}H-to-^{14}C ratio was 1.03 (data not shown), which indicated negligible accumulation of those proteins in cell asp of PMSF-treated rats.

Effects of E-64 Treatment In Vivo on the Degradation Rates of Newly Synthesized Ribosomal Proteins and Histones—The results of the present and previous experiments suggest that the thiol protease was preferentially responsible for the degradation of newly synthesized and unbound ribosomal proteins and histones. To obtain further support for this view, we investigated the effects of E-64 or PMSF treatment on the degradation rates in the livers of partially hepatectomized rats pretreated with a low dose of actinomycin D. The results are shown in Fig. 4. The pulse-labeled ribosomal protein fraction (compo-

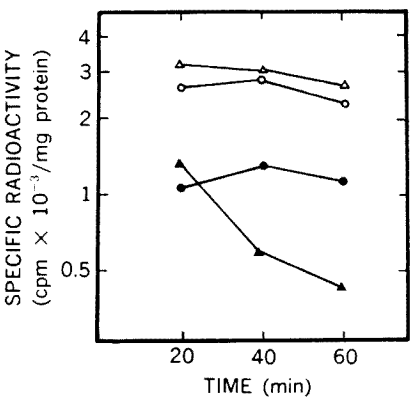

Fig. 4. Effects of E-64 and PMSF treatments on the degradation rates of total proteins and ribosomal proteins of actinomycin D-treated rat livers. Partially hepatectomized rats pretreated with a low dose of actinomycin D were intraperitoneally injected with 50 μCi of [^{3}H]leucine. After 15 min, one group was injected with 10 mg of PMSF and 5 mg of cycloheximide per 100 g body weight and the other group with 3 mg of E-64 and 5 mg of cycloheximide per 100 g body weight. At each time indicated, two rats were killed and component II were prepared from the total homogenates. The specific radioactivities of total rat liver proteins (○, △) and those of component II (●, ▲) were determined as described in "MATERIALS AND METHODS." △, ▲, PMSF treated; ○, ●, E-64 treated.

nent II) prepared from the homogenate was rapidly degraded in the livers of rats injected with PMSF with a half-life of 20 to 30 min, which is similar to that in the control regenerating rat livers described previously (*2*). On the other hand, the degradation of component II was very slow in the livers of rats injected with E-64, being similar to that of total liver proteins in the cases of both PMSF and E-64 treatments. These results support the preferential degradation of newly synthesized ribosomal proteins and histones by the thiol protease, but not by serine proteases, in the liver nuclei.

DISCUSSION

To inhibit nuclear proteases of rat liver *in vivo*, we used PMSF, which has been used for studies on the degradation of intracellular proteins in *E. coli* (*26*). As much as 10 mg of PMSF per 100 g body weight was required to inhibit nuclear serine protease when administrated intraperitoneally. This is probably because the drug is hardly soluble and is very unstable in aqueous solution (*27*). However, the inhibition starts very rapidly (within 5 min) and continues for at least 90 min. Nuclear serine proteases were found to recover about the half of the initial activity at 6 h after the injection of PMSF and almost the whole activity at 12 h. Thus, the inhibition of the unclear proteases by a single injection of PMSF was of relatively short duration. Although rats injected with this dose of PMSF showed impairment of muscular movement and other side-effects immediately after the injection, none of them died of the treatment, and RNA and protein syntheses in the liver were not inhibited.

We also tried to use another kind of serine protease inhibitor, chymostatin, to selectively inhibit serine proteases, because it is a potent inhibitor of chymotrypsin and related serine proteases, although it also inhibited, to a lesser extent, some thiol proteases such as papain (*28*) and cathepsins (*29*). However, it inhibited both the serine proteases and the thiol protease similarly *in vitro* after they had been partially purified from rat liver nuclei by gel filtration through Sepharose 6B (*4*, *10*). The concentrations of the inhibitor required for 50% inhibition of the proteases were 1 μg/ml for both serine protease A, which had maximal activity at pH 10 (*10*), and the thiol protease, and 8 μg/ml for the serine protease B, which had maximal activity at pH 8 (*10*). Therefore, chymostatin could not be used in this experiment.

The present experiments demonstrated that serine proteases in rat liver are not involved in the degradation of newly synthesized ribosomal proteins and histones, although they show high affinity for these proteins (*10*). It must be added that a large part of the newly synthesized ribosomal proteins and histones of the liver of rats treated with a low dose actinomycin D was present in the nuclear sap, where they were degraded by the thiol protease before entering chromatin (*1*). Therefore, it is possible that the serine proteases may function in the degradation of structual proteins of chromatin, including histones, in rat liver as suggested by our enzymatic studies reported previously (*10*). Recently, we found that the serine protease with a molecular weight of 25,000 was activated by DNA and preferentially degraded H1 histone in the presence of DNA (to be published).

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