Ribonuclease Activity of Rat Liver Perchloric Acid-Soluble Protein, a Potent Inhibitor of Protein Synthesis*

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Rat liver perchloric acid-soluble protein (L-PSP) is a potent inhibitor of cell-free protein synthesis; however, its mechanism of action is not known. Here we show that the protein is a unique ribonuclease and that this activity is responsible for the inhibition of translation. The addition of perchloric acid-soluble protein to a rabbit reticulocyte cell-free system at a concentration of 6.2 mM led to an almost complete inhibition of protein synthesis. The kinetics are unlike those of hemo-controlled inhibitor, a protein that acts at the initiation step. The inhibition appears to be due to an endoribonucleolytic activity of perchloric acid-soluble protein because L-PSP directly affects mRNA template activity and induces disaggregation of the reticulocyte polyribosomes into 80 S ribosomes, even in the presence of cycloheximide. These effects were observed with authentic as well as recombinant L-PSP. Analysis by thin-layer chromatography of [a-32P]UTP-labeled mRNA incubated with the protein showed production of the ribonucleoside 3'-monophosphates Ap, Gp, Up, and Cp, providing direct evidence that the protein is an endoribonuclease. When either 5' or 3'-32P-labeled 5 S rRNA was the substrate, L-PSP cleaved phosphodiester bonds only in the single-stranded regions of the molecule.

Rat liver perchloric acid-soluble protein (L-PSP) is a 136-amino acid protein that inhibits protein synthesis. Oka et al. (1) demonstrated that L-PSP, when added to a rabbit reticulocyte cell-free system, causes inhibition of a biphasic kinetic nature and also leads to the disaggregation of polysomes. This would be similar to the mode of inhibition of translation by the heme-regulated eukaryotic initiation factor 2 (2) (however, see our results below). Based on these data, it was suggested that the protein inhibits the initiation step rather than the elongation step (1).

A 14-kDa translational inhibitor protein remarkably similar to L-PSP has been characterized in human monocytes and mouse liver (3–5). A homology search revealed that these proteins belong to a new group of small proteins named the YER057c/YJGF family (3), which is of unknown physiological function. The protein sequences of these family members are highly conserved in prokaryotes (including cyanobacteria), fungi, and eukaryotes, suggesting that the proteins may be involved in a basic cellular process. Indeed, mRNA of the translational inhibitor p14.5, the human homologue of L-PSP, becomes significantly up-regulated with the induction of differentiation to macrophages (3), and the synthesis of PS from rat kidney increases from the 17th fetal day to the fourth postnatal day (4). In contrast, the expression of PS from rat kidney was down-regulated.

Recently, Schmiedeknecht et al. (7) have identified the functional promoter of the human p14.5 translational inhibitor. They reported a head-to-head orientation of p14.5 with the gene for the protein subunit hPOP1 of RNase P and with RNase MR ribonucleoproteins; the promoter region between p14.5 and hPOP1 acts as a bidirectional promoter. Because bidirectional transcription units commonly encode proteins that are different in structure but have similar biological functions (5, 8), the authors suggested that the p14.5-hPOP1 cluster may encode functionally related proteins as well.

As the first step in our endeavor to understand the physiologival role of the PSP proteins, we studied the mechanism of action of the translational inhibitor L-PSP.

EXPERIMENTAL PROCEDURES

General—The following procedures were either described or cited previously (10–12): preparation of rabbit reticulocyte lysate, sucrose density gradient analysis of polysomes, preparation of ribonucleosides, extraction of RNA with phenol and SDS, preparation of plasmid pBR322, analysis of the nucleic acids by polyacrylamide gel electrophoresis, separation of nucleotides by thin-layer chromatography, the methods used for the sequencing of 5 S RNA, and the source of materials such as [U-14C]leucine, human placental ribonuclease inhibitor (133 units/ml), [a-32P]UTP, and SP6 RNA polymerase.

Preparation of Authentic and Recombinant L-PSP—Both forms of L-PSP were purified as described previously (1, 13). Briefly, the 5% perchloric acid-soluble fraction of a rat liver lysate was loaded onto CM-Sephadex C-25, and pure L-PSP was recovered in the flow-through fraction. Recombinant PSP was expressed in Escherichia coli as a fusion protein with glutathione S-transferase using plasmid pGEX-PSP. The fusion protein was purified from the extract with glutathione-Sepharose 4B and then incubated with thrombin, and recombinant L-PSP was isolated using the same affinity column as described above. The recombinant protein was confirmed to be L-PSP by its mobility on SDS-polyacrylamide gels and by direct sequencing of the eight N-terminal amino acids. Furthermore, the recombinant protein and authentic L-PSP were immunologically identical.

Protein Synthesis Inhibition Assay—The standard cell-free protein synthesis system derived from rabbit reticulocyte lysate (10) contained the following in a final volume of 30 μl: 15 μl of micrococcal nuclease-treated lysate (containing 25 μM hemin), 25 mM Heps (pH 7.6), 2 mM dithiothreitol, 1 mM ATP, 20 μM GTP, 8 mM creatine phosphate, 1.2 μg of creatine phosphokinase, 25 μg of each of the 20 amino acids, 0.1 μM of l-[U-14C]leucine (13.3 mCi/mmol), 90 mM potassium acetate, 1 mM magnesium acetate, 0.6 mM spermidine, and 2 μg of mRNA. Endogenous globin synthesis was done under the same conditions as described above using lysate that was not treated with micrococcal nuclease.

Synthesis of [32P]mRNA—mRNA having the cap structure was syn-
incubated without or with the indicated concentration of L-PSP. In value (1). A L-PSP concentration of 1 mM almost completely abolished the capacity of the cell-free system to support protein synthesis. However, when similar experiments were done using endogenous globin mRNA, inhibition by L-PSP appeared to be about 10 times less efficient (IC50, 0.68 nM) than in experiments using exogenous capped mRNA encoding dihydrofolate reductase and all other components necessary for protein synthesis at 30 °C led to the incorporation of [14C]leucine into newly synthesized proteins. In agreement with the previous report (1), the addition of L-PSP to the system resulted in inhibition of protein synthesis in a concentration-dependent manner (Fig. 1A). The estimated IC50 (51 nM) was in the same range as the previously reported value (1). A L-PSP concentration of 1 μM almost completely abolished the capacity of the cell-free system to support protein synthesis. However, when similar experiments were done using endogenous globin mRNA, inhibition by L-PSP appeared to be about 10 times less efficient (IC50, 0.68 μM) (Fig. 1B). The observed difference in the IC50 can be ascribed to the character of the two translation systems: in the latter system, a large population of ribosomes is already engaged with globin mRNA (as polysomes) at the start of incubation, whereas practically no mRNA is associated with ribosomes in the former system. Thus, the results suggested that the lysate containing a higher proportion of polysomes was less susceptible to L-PSP.

An interesting and probably important observation here is that the kinetics of the inhibition is monophasic in both systems, rather than biphasic, as reported previously (1). In fact, careful examination of the earlier data does not reveal an inhibition lag, and the discrepancy may stem from an incorrect explanation of the kinetic data. Inhibition of translation initiation by proteins such as heme-regulated eukaryotic initiation factor 2α kinase shows biphasic kinetics (2). However, this typical biphasic shape, in which protein synthesis proceeds at the initial rate for several minutes before an abrupt decline occurs, can only be expected when the globin-synthesizing system is used because only this system has an initial rate (run-off of polysomes). Obviously, inhibitors of initiation show monophasic kinetics of translation inhibition in the exogenous mRNA programmed system, which has no initial rate because there is no initial protein synthesis. The inhibition of protein synthesis by L-PSP was not prevented by the ribonuclease inhibitor from human placenta that tightly binds and inhibits the activity of the ribonuclease A family (data not shown) (15).

In experiments that used higher amounts of mRNA than the standard reaction, the inhibitory effect on protein synthesis tended to be less. For instance, when mRNA was 20 μg instead of 2 μg in a 30 μl system, 20 nM L-PSP did not show significant inhibition (data not shown). To gain more insight into the mode of action of this small protein, we examined the effect of pre-incubation of mRNA with L-PSP. For these experiments, mRNA was first incubated with a low amount of L-PSP for a prolonged time, and then the activity of the mRNA of a small portion of the preincubation reaction mixture was tested in the standard translation system. This strategy minimizes the effect of L-PSP on the translation system because the concentration of L-PSP in the translation reaction mixture is very low. As shown in Fig. 1C, preincubation of mRNA with L-PSP results in a significant decrease of protein synthesis. Recombinant L-PSP affected the template quality of mRNA in a similar manner, making it unlikely that the observed inactivation of mRNA was due to contaminants in the L-PSP preparation. However, the activity on mRNA of authentic L-PSP differed significantly from that of recombinant PSP, as had been found previously (13). Although we cannot be certain, the difference is likely to be due to a loss of activity because of the expression of recombinant PSP in a prokaryotic system. A similar but even larger discrepancy has been reported for the human homologue of PSP, p14.5: the authentic form is more active than the recombinant form by 3 orders of magnitude (3).

Nevertheless, the results indicate that the main target of the protein is mRNA rather than the translation system. There are at least two alternative mechanisms for mRNA inactivation: 1) L-PSP might specifically modify mRNA at its 5'-untranslated region, including the cap structure (m7GpppG) that is nearly essential for the initiation reaction, or 2) the protein might degrade mRNA nucleolytically.

To test the alternative possibilities, we performed experiments using sucrose density gradient centrifugation to investigate polysome profiles in the presence of cycloheximide. Incubation of the globin synthesizing lysate system with L-PSP resulted in a disaggregation of polysomes into 80 S ribosomes (Fig. 2A), supporting the earlier result (1). The addition of the ribonuclease inhibitor at the start of the incubation did not prevent polysome disaggregation. An important feature of this
experiment was that both the incubation and the analysis were performed in the presence of a low concentration of cycloheximide at which the antibiotic freezes the elongation reaction but not initiation. Polysome disaggregation in the presence of L-PSP demonstrates that the protein does not affect the initiation process. Rather, the results strongly suggested that L-PSP disintegrates polyribosomes through fragmentation of polysomal mRNA because of ribonuclease activity.

Ribonuclease Activity of L-PSP—To obtain direct evidence, micrococcal nuclease-treated rabbit reticulocyte lysate was incubated as described above with 32P-labeled dihydrofolate reductase mRNA in the presence of both the ribonuclease inhibitor and L-PSP. After incubation for various periods of time (Fig. 2B), RNA was extracted and separated on polyacrylamide gels. Consistent with our hypothesis, the radioautograph shows intensive digestion of mRNA into small fragments in the presence of L-PSP, whereas the mRNA was fairly stable in the control sample. A similar digestion pattern of the mRNA was observed when the incubation was carried out in the absence of the ribonuclease inhibitor (data not shown). The amount of the ribonuclease inhibitor (266 units) used in Fig. 2, A and B, inhibits the activity of 1.33 µg of ribonuclease A by 50%, whereas the amount of L-PSP was 0.89 µg in a 30-µl reaction mixture. The result excludes the possibility that the inhibition by L-PSP is due to a contamination with ribonuclease A or other A-like ribonucleases, RNases known to be abundant in both animal tissues and E. coli. All of these results show that L-PSP is a ribonuclease that hydrolyzes phosphodiester bonds of RNA in an endonucleolytic fashion.

We next determined the substrate specificity of the enzyme, and the results are shown in Fig. 3A. The 32P-mRNA was incubated with L-PSP, and then the sample was separated by thin-layer chromatography. Subsequent autoradiography showed the production of the four 3' end monophosphates as identified by comparison with standard nucleotides obtained from ribonuclease T1 digests. The results are conclusive: L-PSP cleaves the phosphodiester bonds of all four nucleotides, yielding 3'-AMP, 3'-GMP, 3'-UMP, and 3'-CMP. In addition to the four discrete spots, some streaks are also seen in Fig. 3A that may represent residual small oligomers accumulating under the digestion conditions. Practically the same digestion pattern of the RNA was obtained using recombinant L-PSP (data not shown). By measuring the radioactivity of the spots, we determined the relative catalytic activity of L-PSP for the various substrates: NpA32p/U = Np/G32p/U = Np/U32p/U > Np/C32p/U (slashes indicate the sites of hydrolysis). The mechanism of cleavage probably involves 2',3'-cyclic phosphate intermediates.

Because there are nucleases that hydrolyze both RNA and DNA (16), we determined whether L-PSP has any deoxyribonuclease activity. We chose pBR322 as the substrate for the test because a single nick in the circular supercoiled DNA of the plasmid produces a change in conformation that alters its mobility in agarose gels. Prolonged incubation of the plasmid with L-PSP (2.1 µM) under conditions similar to those used for the RNA substrate did not result in any significant effect on the integrity of the plasmid (data not shown). Thus, we conclude that the protein does not have deoxyribonuclease activity.

We next determined whether L-PSP is specific for single- or double-stranded regions of RNA. We chose rat liver 5 S rRNA as substrate for the test because 1) the secondary structure is known, and 2) the RNA has a highly ordered structure (17). 5 S rRNA was labeled at its 3' end with (5'32P)ppGp or at its 5' end with (γ32P)ATP, and the sample was treated under non-denaturing conditions with either authentic PSP from rat liver or recombinant PSP. An alkaline digest and T1 digests were analyzed together with the products of the protein treatment on a 15% polyacrylamide sequencing gel (Fig. 4). Both L-PSP preparations cleaved only in single-stranded regions of the molecule. Once again, recombinant L-PSP was about four times less active than authentic L-PSP. The addition of the ribonuclease inhibitor to the reaction mixtures did not alter the digestion pattern (data not shown). The results clearly indicated that L-PSP itself is a ribonuclease with characteristics unique among liver RNases.

Effect of L-PSP on other RNAs—Other ribonucleases with unique properties are known: the cytotoxin α-sarcin and related proteins (12), colicin E3 and related bacteriocins (18), and angiogenin, which is important for angiogenesis (19). α-Sarcin is a basic 17-kDa protein produced by the mold Aspergillus giganteus. The basis of the action of the protein is an inhibition of protein synthesis caused by the inactivation of ribosomes. Although there are 5000–7000 nucleotides in ribosomes, α-sarcin hydrolyzes only a single phosphodiester bond between G4325 and A4326 of 28 S (or 23 S) rRNA. This cleavage inactivates the ribosome and is entirely responsible for the toxicity
Fig. 3. Ribonucleotide specificity of L-PSP. (32P)5rRNA (1 μg; 50,000 cpm) was incubated in 10 mM Tris-HCl (pH 7.6) and 50 mM KCl, and the 3'-labeled RNA was chromatographed on cellulose plates in two dimensions (isobutyric acid/0.5 M ammonia (5:3) for the first dimension and isopropanol/HCl/O (70:15:15) for the second dimension), and radioautographs were made. The RNA was dissolved in 50 mM Tris-HCl (pH 7.6) and 50 mM KCl, and the 3'-labeled RNA was incubated with recombinant FSP (8.5 μM for lanes 4 and 17 μM for lane 5). The 5'-labeled RNA was incubated with authentic L-PSP (2.1 μM for lane 9 and 3.5 μM for lane 10). After 20 min, the products were analyzed on polyacrylamide sequencing gels.

Fig. 4. Effect of L-PSP on 5 S rRNA. Rat liver 5 S rRNA was made radioactive at either the 5' end (gels on the right) or 3' end (gels on the left). The RNA was dissolved in 50 mM Tris-HCl (pH 7.6) and 50 mM KCl, and the 3'-labeled RNA was incubated with recombinant PSP (8.5 μM for lane 4 and 17 μM for lane 5). The 5'-labeled RNA was incubated with authentic L-PSP (2.1 μM for lane 9 and 3.5 μM for lane 10). After 20 min, the products were analyzed on polyacrylamide sequencing gels. Lanes 2 and 6, alkaline hydrolyses; lanes 1 and 7, ribonuclease T1 digest; lanes 3 and 8, incubation without the protein. ds, double-stranded regions of the nucleic acid; ss, single-stranded regions.

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with ethidium bromide (data not shown). Despite a careful examination of the bands, we did not observe any significant degradation of rRNA nor tRNA at 1.5 μM of the authentic protein (which has an IC50 of 0.68 μM). However, when similar experiments were done with higher concentrations (>2 μM) of the protein, significant fragmentation of rRNA could be observed, which supports the notion that L-PSP is a ribonuclease. These results eliminate the possibility that L-PSP inhibits cell-free protein synthesis by cleaving rRNA or tRNA. We thus conclude that L-PSP inhibits cell-free protein synthesis by cleaving mRNA. These results may have an important bearing on the physiological role of L-PSP and its related proteins.

Finally, it may be worthwhile to mention here that besides the lack of significant sequence homology with other ribonucleases, L-PSP also lacks histidine residues (1). Histidine is known to be the indispensable general acid in the catalytic activity of other ribonucleases (21, 22). Recently, another ribonuclease that lacks histidine has been reported (23): the C-terminal domain of the bacteriocin colicin E5 inhibits bacterial protein synthesis in vitro by cleaving several rRNAs at a specific site, the 3' side of the queosine nucleotide in the anticodon loop. The cytotoxin recognizes the same site even if unmodified tRNA is the substrate, in which case, cleavage occurs 5' side of the guanosine nucleotide that is at the queosine position. The proposed mechanism of cleavage involves a 2',3'-cyclic phosphate intermediate, but the exact enzymatic mechanism is unknown. A computer-aided homology search between L-PSP and the colicin E5 peptide revealed a short sequence shared by both enzymes, NDFGTV (amino acids 87–92) in L-PSP and NDFATV (positions 84–89 of the 115-amino acid C-terminal domain) in E5, but the functional significance of these hexapeptides remains to be seen.
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