Peptidyl - tRNA hydrolase and RNase activities in cell fractions of rat liver used in *in vitro* reconstitution of rough membrane.

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ABSTRACT

Peptidyl-tRNA hydrolase and RNase activities have been studied in those fractions of rat liver, which are used in *in vitro* reconstitution of rough membrane, because these enzymes may interfere with the *in vitro* reconstitution. It was found that smooth membrane has an active peptidyl-tRNA hydrolase, while the other fractions tested, polyribosomes, rough membrane, stripped rough membrane and the post-microsomal supernatant had no, or very low, peptidyl-tRNA hydrolase activity. Polyribosomes, rough and stripped rough membrane have RNase activity; this activity could be completely inhibited by rat liver RNase inhibitor. It is shown that RNase inhibitor is an obligatory component in *in vitro* experiments, in which rough membrane is reconstituted from stripped rough membrane, ribosomes and mRNA.

INTRODUCTION

In rat liver and in other tissues of Eukaryotes, ribosomes appear in two different populations: as free polyribosomes and as ribosomes bound to the endoplasmic reticulum (1). Bound ribosomes seem to be involved in the synthesis of proteins which are to be exported from the cells, while the free polyribosomes synthesize many of the intracellular proteins (2, 3). The mode of ribosome-membrane interaction has been the subject of intense investigations in several laboratories (4-8). The results obtained from these studies support the idea that peptidyl-tRNA may play a role in the stability of the ribosome-membrane complex (9). In order to gain more information on the contribution of peptidyl-tRNA in the ribosome-membrane interaction, the influence of chemically well defined peptidyl-tRNAs on the *in vitro* reconstitution of rough membrane from its components will be investigated. Therefore, the fate of peptidyl-tRNA, when incubated with those cell fractions one might use in *in vitro* experiments on rough membrane re-

constitution, was studied. Peptidyl-tRNA can undergo enzymatic hydrolysis in two distinct ways: by RNase or by peptidyl-tRNA hydrolase. Peptidyl-tRNA hydrolase hydrolyses the ester bond linking the peptide to the tRNA, but does not attack aminoacyl-tRNA (10). Most of the peptidyl-tRNA hydrolase studies were performed with an enzyme obtained from bacterial sources (11-13). However, peptidyl-tRNA hydrolase was also found in mammalian tissues (14).

The presence of RNase during in vitro rough membrane reconstitution experiments, may cause great difficulties. RNase can attack mRNA, tRNA and other components necessary to obtain a reconstituted membrane active in protein synthesis. Therefore, we studied the RNase activities of those fractions of rat liver which are components for in vitro reconstitution experiments in the absence and in the presence of rat liver RNase inhibitor.

MATERIALS AND METHODS

[¹⁴C]phenylalanine (specific activity 495 mCi/mmole) and [¹⁴C] valine (specific activity 280 mCi/mmole) were purchased from the Radiochemical Centre (Amersham, England). Pancreatic RNase and snake venom phosphodiesterase were purchased from Sigma (USA).

Free polyribosomes (FP), rough membrane (RM) and smooth membrane (SM) from rat liver were prepared according to Czosnek and Hochberg (15). The rough membrane was stripped from its ribosomes (RMst) either by the KCl-puromycin method as described by Borgese et al. (16) or by treatment with EDTA, according to Roobol and Rabin (17). For comparison, the smooth membrane was similarly treated (SMst). 40S + 60S ribosomal subunits were prepared from free polyribosomes according to Martin and Wool (18). The purity of the different cell fractions, FP, RM, SM, RMst was checked by electron microscopy (4), buoyant density and RNA:protein ration (19). The different fractions were also tested for their amino acid incorporation capacity: the activity of the smooth and the rough membrane stripped, was less than 10% of the activity of the rough membrane fraction.

Post microsomal supernatant (PMS) was prepared by centrifugation

of the post mitochondrial supernatant for 2 hr at 105,000 xg.

Rat liver RNase inhibitor was prepared from rat liver PMS and tested for activity as described by Grikau *et al.* (20).

(Gly)₃ [¹⁴C]Val-tRNA was synthesized according to Lapidot and Rappoport (21).

Preparation of wheat germ S₂₃ fraction was done according to Marcus *et al.* (22). Globin mRNA was prepared as described by Aviv and Leder (23). TMV-tRNA was a gift from Dr. Efron, Jerusalem.

Assays both for RNase and peptidyl-tRNA hydrolase were carried out by incubating at 37° C in a final volume of 220 µl, the following: [¹⁴C]Val-tRNA (sp.ac. 934 cpm/A₂₆₀) 7000 cpm or (Gly)₃ [¹⁴C]Val-tRNA (sp.ac. 816 cpm/A₂₆₀) 9000 cpm; Tris HCl pH = 7.5, 60 mM; KCl 50 mM; DTT 1 mM; MgCl₂, 10 mM; polyribosomes, 0.13 mg protein; RM 0.08 mg protein; RMst 0.10 mg protein; SM 0.10 mg protein; SMst 0.24 mg protein; 40S + 60S 0.06 mg protein. Samples (50 µl) were placed on 3 MM Whatman filter paper discs, washed and the radioactivity was counted.

The hydrolysis products of aminoacyl- and peptidyl-tRNA were analyzed by high voltage paper electrophoresis at pH 2.5 (45 V/cm).

RESULTS AND DISCUSSION

As can be seen from Fig.1, the polyribosomes, the rough membrane and the stripped rough membrane fraction hydrolyse aminoacyl-tRNA and peptidyl-tRNA at the same, or nearly at the same, rate. The smooth membrane fraction, however, behaved differently and the hydrolysis of (Gly)₃Val-tRNA catalysed by this fraction is significantly faster than that of Val-tRNA. This difference is more pronounced after stripping of the smooth membrane. The treated smooth membrane fraction has no, or hardly any, hydrolytic activity on Val-tRNA, but is still capable of hydrolysing (Gly)₃Val-tRNA to a considerable extent. The action of the PMS fraction and the ribosomal subunits fraction (40S + 60S) on the substrates was also tested (results not shown). The 40S + 60S ribosomal fraction did not hydrolyse either aminoacyl-tRNA or peptidyl-tRNA and the PMS fraction hydrolyses both aminoacyl- and peptidyl-tRNA at a slow and practically identical rate.

Fig. 1. The hydrolysis of aminoacyl- and peptidyl-tRNA by different cell fractions.

▲-▲ [^{14}C]Val-tRNA; △-△ (Gly) $_3$ Val-tRNA in the absence of cell fraction; x-x polyribosomes; ■-■ RM; □-□ RMst; ●-● SM; ○-○ SMst;

A. Hydrolysis of [^{14}C]Val-tRNA

B. Hydrolysis of (Gly) $_3$ [^{14}C]Val-tRNA.

A possible explanation for the results obtained with the smooth membrane fraction is the existence of an enzyme activity in this fraction which can hydrolyse peptidyl-tRNA but is indifferent towards aminoacyl-tRNA, besides an activity which hydrolyses both aminoacyl and peptidyl-tRNA (such as RNase). Peptidyl-tRNA hydrolase is the most obvious candidate for such an activity. In order to base this assumption on more solid evidence, the following experiments were carried out:

The different fractions were allowed to act on the two substrates in the presence and in the absence of rat liver RNase inhibitor. The results are shown in Table I.

The RNase inhibitor inhibited the hydrolysis of both Val-tRNA and (Gly) $_3$ Val-tRNA by the different fractions nearly completely, except for the hydrolysis of (Gly) $_3$ Val-tRNA by the smooth membrane and the stripped smooth membrane fraction. These results give support to our assumption that the hydrolysis of Val-tRNA and (Gly) $_3$ Val-tRNA by the different fractions is due to the action of one or more than one RNase and the action of the RNase(s) is inhibited by the rat liver RNase inhibitor. The hydrolysis

Table I: The effect of rat liver RNase inhibitor on the hydrolysis of $[^{14}\text{C}]\text{Val}\cdot\text{tRNA}$ and $(\text{Gly})_3[^{14}\text{C}]\text{Val}\cdot\text{tRNA}$

Cell fraction	Percent of inhibition	
	$[^{14}\text{C}]\text{Val}\cdot\text{tRNA}$	$(\text{Gly})_3[^{14}\text{C}]\text{Val}\cdot\text{tRNA}$
polyribosomes	90	95
RM	85	90
RMst	90	95
SM	80	30
SMst	'a)	10

For details see Materials and Methods. RNase inhibitor when added, 20 μg protein. Incubation 15 min at 37° C

(a) The activity of SMst on $[^{14}\text{C}]\text{Val}\cdot\text{tRNA}$ was too small for exact determination, of the percent of inhibition by RNase inhibitor.

of peptidyl-tRNA by the smooth membrane fraction is partly, and that of the stripped smooth membrane is nearly solely due to the action of peptidyl-tRNA hydrolase whose action is not sensitive to the RNase inhibitor.

Additional evidence for the existence of a peptidyl-tRNA hydrolase in the smooth membrane fraction, comes from the results shown in Fig. 2. The main radioactive product of the action of the smooth membrane fraction on $(\text{Gly})_3[^{14}\text{C}]\text{Val}\cdot\text{tRNA}$ is $(\text{Gly})_3[^{14}\text{C}]\text{Val}$ and this peptide is the sole radioactive product of the action of the stripped smooth membrane on the peptidyl-tRNA. Polyribosomes, rough membrane and stripped rough membrane, produce mainly a radioactive compound which moves in an electrophoretogram with the same electrophoretic mobility as the product produced by the action of pancreatic RNase on $(\text{Gly})_3[^{14}\text{C}]\text{Val}\cdot\text{tRNA}$, namely $\text{A}\cdot[^{14}\text{C}]\text{Val}(\text{Gly})_3$.

In Fig. 2 C, the results obtained with the rough membrane fraction are shown. Very similar results were obtained when "stripped" rough membrane or polysomes were incubated with $(\text{Gly})_3[^{14}\text{C}]\text{Val}\cdot\text{tRNA}$. The small peak of $(\text{Gly})_3[^{14}\text{C}]\text{Val}$ in Fig. 2 C may be the result of some contamination of the rough membrane with smooth membrane. It is interesting to note, that 3 radioactive products could be detected after incubation of the smooth membrane with $(\text{Gly})_3[^{14}\text{C}]\text{Val}\cdot\text{tRNA}$: $(\text{Gly})_3[^{14}\text{C}]\text{Val}$, $\text{A}\cdot[^{14}\text{C}]\text{Val}(\text{Gly})_3$ and

Fig. 2. Paper electrophoresis of the hydrolysis products of peptidyl-tRNA by the different cell fractions.

- A. Treatment of $(\text{Gly})_3[^{14}\text{C}]\text{Val-tRNA}$ with pancreatic RNase
- B. Treatment of $(\text{Gly})_3[^{14}\text{C}]\text{Val-tRNA}$ with snake venom phosphodiesterase
- C. Treatment of $(\text{Gly})_3[^{14}\text{C}]\text{Val-tRNA}$ with RM*
- D. Treatment of $(\text{Gly})_3[^{14}\text{C}]\text{Val-tRNA}$ with SM
- E. Treatment of $(\text{Gly})_3[^{14}\text{C}]\text{Val-tRNA}$ with SMst.

* Similar results were obtained with polyribosomes and RMst. For details see Materials and Methods.

a product with an electrophoretic mobility like that of the product of snake venom diesterase on $(\text{Gly})_3[^{14}\text{C}]\text{Val-tRNA}$, namely $\text{pA} \cdot [^{14}\text{C}]\text{Val}(\text{Gly})_3$.

The results described can be summarized as follows: Polyribosomes, rough membrane, stripped rough membrane and smooth membrane all have RNase activity which can be suppressed by RNase inhibitors. Smooth membrane also has a peptidyl-tRNA hydrolase, which is insensitive towards the RNase inhibitor. Smooth and rough membrane differ in several aspects each from one another; in the composition of their lipids enzymatic activity and in the electrophoretic mobility of their proteins (15).

From our results it is clear that rough and smooth membranes differ also

greatly in their peptidyl-tRNA hydrolase activities. The RNase activity in the rough and the stripped rough membrane fraction may be a serious obstacle in in vitro experiments, aimed at the reconstitution of rough membranes from stripped rough membranes, polyribosomes or monosomes in the presence of messenger RNA. This obstacle is visualized in Table II. Wheat germ ribosomal system can translate exogenous messenger RNA's like globin mRNA and TMV-RNA.

It was shown by us previously that wheat germ ribosomes can bind to stripped rough membrane from rat liver (25), but the results in Table II show that the presence of this membrane fraction in the wheat germ S_{23} system completely inhibited the incorporation promoted by these two messenger RNA's. This inhibition seems to be the result of the RNase activity described here, because rat liver RNase inhibitor can overcome this inhibitory effect completely (Table II). Even the S_{23} of wheat germ itself seems to contain a RNase, which can be inhibited by rat liver RNase inhibitor. The effect of the RNase inhibitor on the translation of globin mRNA and TMV-RNA by the wheat germ S_{23} in the presence of the stripped rough membrane are striking.

Table II: The effect of rat liver RNase inhibitor on the protein synthesizing activity of wheat germ S_{23} in the presence of stripped rough membrane from rat liver.

	RNase inhibitor (units)	mRNA globin	TMV-RNA
S_{23}	0	24740	63410
S_{23}	2.0	56000	276100
RMst + S_{23}	0	314	650
RMst + S_{23}	0.4	21050	10997
RMst + S_{23}	0.8	38380	132407
RMst + S_{23}	2.0	42442	242070
RMst + S_{23}	4.0	45044	296758
RMst + S_{23}	10.0	38986	177966

The incubation mixture (22) contained in a final volume of 100 μ l the following: S_{23} fraction 15 μ l, RMst 10 μ g protein, mRNA globin 0.0015 A_{260} nm units, TMV-RNA 0.4 A_{260} nm units, [3H] leucine SpAc 38 ci/mmol 2 μ c;

These results show that RNase inhibitor has to be present in experiments aimed at the in vitro reconstruction of rough membranes from stripped rough membranes, ribosomes and exogenous messenger RNA.

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