SYNTHESIS AND THE PUROMYCIN-MEDIATED RELEASE OF NASCENT POLYPEPTIDE CHAINS BY RECONSTRUCTED ROUGH ENDOPLASMIC RETICULUM FROM RAT LIVER AND BRAIN CORTEX

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1. Introduction

The mechanism by which the cell separates the synthesis of secreted proteins from the synthesis of retained proteins is not yet fully understood. Available data suggest that secretory proteins are preferentially, if not exclusively, synthesized on the membrane-bound ribosomes but the converse is not necessarily so [1,2]. The endoplasmic reticulum membranes may play an important role in the selection and the translation of the template present on the attached ribosomes [3].

In recent years a number of attempts have been made to reconstruct rough endoplasmic reticulum by reacting ribosome-free membranes and ribosomes under various conditions of incubation [4–6]. Recent evidence suggests that the reconstructed liver RER exhibits structural, and in some respects, functional properties analogous to those of authentic RER [4,7,8]. There are, however, conflicting reports on the ability of the reconstructed RER to discharge the nascent peptide chains vectorially through the ER membranes. Thus Burke and Redman [9] failed to obtain any evidence for the vectorial discharge of nascent polypeptides across the membrane in the reconstructed RER. On the other hand, Shires et al. [10] have claimed that their preparation of reconstructed RER was active in the vectorial release of the newly synthesized chains. The present investigation was undertaken to resolve this controversy by reconstructing RER from the liver, a tissue with considerable secretory activity, and from the brain which lacks an obvious secretory function. Conditions for the reconstruction of homologous and heterologous RER from the two tissues have been established and their protein synthetic activities as well as the vectorial discharge of the nascent polypeptide chains have been examined. The results demonstrate that the homologously reconstructed RER from both liver and brain are active in peptide bond formation but only the former is able to function as a secretory unit. Heterologously reconstructed RER from liver and brain failed to exhibit any appreciable degree of transmembrane discharge of the nascent chains as judged by the puromycin-mediated release of the newly synthesized polypeptides. It is also shown that the extent of ribosome attachment to the stripped membranes is independent of the source of ribosomes.

Abbreviations: ER, endoplasmic reticulum; RER, rough endoplasmic reticulum

2. Materials and methods

Male rats of the Wistar strain weighing 120–180 g were used. [^{14}C] orotic acid (40–60 mCi/mmol) was purchased from New England Nuclear (Boston, Mass.). A ^{3}H-labelled mixture of 15 amino acids was obtained from the Radiochemical Centre (Amersham, Bucks, England). Polyvinylsulphate was a product of K and K Laboratories, Inc. (Calif.). The source of the rest of the materials has been described elsewhere [4,7].

Liver ribosomes and rough membranes were prepared as described previously [4] except that polyvinyl sulphate (1–4 μg/ml) was included in the homogeni-

zation medium. Subcellular fractions from brain cerebral cortex were prepared according to Andrews and Tata [11].

RER prepared from liver or cerebral cortex was washed three times and further purified to remove lysosomal contamination [12]. The membranes were stripped of the attached ribosomes by means of lithium chloride [13]. Both liver and brain membranes were used immediately after their preparation. The RNA to protein ratio of stripped membranes from both liver and brain averaged 0.001.

The extent of ribosome attachment to stripped membranes was measured by incubating ^{14}C-labelled ribosomes with unlabelled membranes at 2°C for 60 min followed by centrifugation on a discontinuous sucrose gradient [4]. The composition of the incubation media are given in the legend to the figure and tables. The puromycin-mediated release of nascent peptide chains was accomplished essentially according to Andrews and Tata [11] except for the composition of the incubation mixture for the assay of amino acid incorporation in vitro, which has been described elsewhere [7]. Protein, RNA, phospholipids, and radioactivity were determined as described previously [7].

3. Results

In preparing different subcellular fractions from liver and brain cortex, care was taken to keep the isolation conditions as similar as could be possible without sacrificing the quality of the end result. Analysis of the isolated fractions (table 1) showed that rough ER from brain cortex had much lower figures for RNA/phospholipid compared to similar values for rough ER derived from liver. The RNA/phospholipid ratio can be taken as an index of the relative degree of packing of ribosomes on ER. The lower RNA/phospholipid ratio in brain might also be due to the presence of non-ER substances such as myelin-sheath fragments as suggested by Tata [11].

Table 2 summarizes the results of a cross-over experiment designed to study the homologous and heterologous binding of ribosomes to the conditioned membranes. It was foun that when the source of membranes was the liver, similar amount of ribosomes (about 60%) became attached to the membranes regardless of the origin of the ribosomes. On the other hand, when the brain ER was made to react with the ribosomes derived from either liver or brain, the extent of the binding was about 20% for the liver ribosomes

Table 1
The comparative compostition of the subcellular fractions from rat liver and brain cortex

Source	Fraction	RNA/Protein	RNA/Phospholipid
Liver	RER	0.15 ± 0.02	2.05 ± 0.18
	Free ribosomes	0.73 ± 0.04	18.67 ± 0.92
Brain	RER	0.10 ± 0.01	0.51 ± 0.02
	Free ribosomes	0.67 ± 0.02	6.75 ± 0.38
Liver ribosome–liver membrane complexes		0.14	–
Liver ribosomes–brain membrane complexes		0.09	–
Brain ribosomes–liver membrane complexes		0.12	–

The microsomal fractions were prepared as described in the text. The values for authentic fractions are the mean of six determinations ± SD. The values for the reconstructed RER are the mean of two or three determinations on seperate preparations.

Table 2
The homologous and heterologous attachment in vitro of ribosomes to the stripped ER

Source of membranes	% Ribosomes attached					
	Liver			Brain		
	Exp. 1	Exp. 2	Exp. 3	Exp..1	Exp. 2	Exp. 3
Liver	67	59	61	63	58	54
Brain	19	22	20	15	16	19

Ribosomes obtained from liver or brain cortex were incubated with either liver or brain endoplasmic reticulum stripped of its ribosomes and the extent of ribosome attachment was determined by analysis on a discontinuous sucrose gradient (see Materials and methods). The incubation medium for the liver system contained: 50 mM Tris–HCl (pH 7.5); 25 mM KCl and 5 mM $MgCl_2$. The conditions of incubation for brain were the same as described for the liver system except that the concentration of Mg^{2+} was raised to 10 mM.

and 16% for the brain ribosomes, suggesting that, as far as the physical binding is concerned, there is an apparent lack of discrimination on the part of membranes against the source of ribosomes.

In order to examine whether or not the ribosome–membrane complexes made in vitro mimic the authentic RER and in order to clarify the role of membranes and of ribosomes in secretion of the newly synthesized polypeptides, the puromycin-mediated release of the nascent chains was studied in authentic, homologous and heterologous RER derived from liver and brain. It has been shown that puromycin accelerates a termination process by which nascent peptide chains on membrane-bound ribosomes are segregated into the cisternal cavity of the RER from where they can be recovered only by dissolving the membrane with a detergent, such as deoxycholate [14]. One should be careful, however, in interpreting the results obtained since deoxycholate might have a nonspecific effect on the membrane [15]. The term vectorial discharge therefore has been used in a conventional sense to designate the net release of the labelled nascent polypeptides mediated by puromycin.

We chose a mixture of 15 labelled amino acids rather than a single amino acid (e.g., phenylalanine) for incorporation to achieve a more authentic labelling of the protein product and to avoid the production of a hydrophobic polypeptide which might readsorb to the membrane after its release. Initially ribosome–membrane complexes were prepared by employing ribosomes obtained by treating the liver postmitochondrial supernatant fraction with Triton X-100. The resulting preparations failed to show any significant secretory activity. In view of a possible damaging effect of the residual detergent on the membranes, it was decided to employ free ribosomes for the reconstruction of RER. The results presented in table 3 show that both the authentic as well as the reconstructed RER derived from liver behave in a strikingly different manner in the puromycin-mediated release of the newly synthesized chains from the corresponding brain preparations (cf. [11]). Whereas the liver membranes seem to provide functional binding sites for the homologous polysomes, the preparations from brain lack the ability to support vectorial discharge of the labelled peptides. The heterologous RER, composed of brain ribosomes and liver membranes did not exhibit any considerable degree of transmembrane release of the nascent chains although the extent of ribosome binding was very similar to that observed for the homologous liver preparation (table 3). This was equally true when the enzyme fractions isolated from brain were used in the amino acid incorporation mixture (results not shown). Liver ribosomes bound to brain membranes in vitro also proved to be unable to direct the vectorial release of the newly synthesized chains (table 3). When the sum of the individual effects of puromycin and of deoxycholate is subtracted from the total effect obtained by employing them in the same assay tube (see the legend to table 3), the net

Table 3
The effect of puromycin and of deoxycholate on the release of radioactive nascent polypeptide chains from free ribosome, authentic RER and from RER reconstructed from liver and brain cortex

Fraction	Source	[^{3}H] Amino acids incorporated in control (cpm/mg RNA × 10^{-2})	% Total radioactivity in supernatant after treatment with:		
			Puromycin	Deoxycholate	Puromycin followed by deoxycholate
Free ribosomes	Liver Exp 1	185	29.3 (9.3)	27.9 (9.9)	38.8 (19.8)
	Exp 2	292	45.0 (14.3)	37.5 (6.8)	50.5 (20.8)
	Brain Exp 1	201	28.1 (12.3)	24.8 (9.0)	36.9 (21.1)
	Exp 2	188	28.8 (9.6)	27.4 (8.2)	37.1 (17.9)
Authentic RER	Liver Exp 1	274	21.2 (6.0)	26.3 (11.1)	48.8 (33.6)
	Exp 2	179	23.2 (9.0)	20.4 (6.2)	40.9 (26.7)
	Brain Exp 1	186	21.7 (6.3)	21.1 (5.7)	27.5 (12.1)
	Exp 2	270	28.7 (15.2)	23.2 (9.7)	36.7 (23.2)
Homologously reconstructed RER	Liver Exp 1	210	21.6 (3.2)	28.6 (10.2)	36.5 (18.1)
	Exp 2	118	20.6 (4.4)	30.7 (14.5)	43.6 (27.4)
	Brain Exp 1	186	39.2 (7.9)	37.8 (6.5)	47.5 (16.2)
	Exp 2	149	44.4 (11.8)	45.8 (13.2)	55.3 (22.7)
Liver ribosomes–brain membrane complexes	Exp 1	263	11.6 (3.3)	31.7 (23.4)	34.3 (26.0)
	Exp 2	339	12.8 (0.2)	17.9 (5.3)	18.1 (5.5)
Brain ribosomes–liver membrane complexes	Exp 1	176	13.7 (4.1)	16.0 (6.4)	19.3 (9.7)
	Exp 2	161	12.2 (3.1)	18.2 (9.1)	22.4 (13.3)

The amino acid incorporation mixture contained ribosomal fractions from liver (40–250 μg ribosomal RNA) or from brain (45–100 μg ribosomal RNA) and 5 μCi of a tritiated mixture of 15 amino acids. The other conditions were the same as described previously [7]. The mixture was incubated for 8 min at 37°C. Puromycin, 1 mM, was then added except in the control tubes which received an equal volume of the incubation buffer. The incubation was continued for an additional 10 min and after this interval, half of the samples were treated with deoxycholate (0.5%) and the particulate fraction was separated from the supernatant fraction by differential centrifugation. The trichloroacetic acid-insoluble radioactivity was determined separately in the sediment and the supernatant fractions. The figures in the parenthesis represent the increase in ^{3}H-radioactivity compared to that in the supernatant fraction of the untreated control. The values for the net release of the labelled polypeptides mediated through puromycin can be obtained by subtracting the sum of the figures in parenthesis in columns 2 and 3 from the corresponding figure in column 4. The results summarize two experiments on different subcellular preparations.

release averages 14% for the authentic RER and about 7% for the homologously reconstructed liver RER. In some experiments the concentrations of deoxycholate was reduced to 0.05%. A significant release of labelled peptidyl-puromycin chains could still be observed although the net release decreased from that obtained when a complete dissolution of the membrane was accomplished.

4. Discussion

The results presented in this paper demonstrate that the LiCl-conditioned liver membranes are able to bind similar amounts of ribosomes derived from either liver or brain. Brain membranes, on the other hand, accept only one-third of the ribosomes that became attached to liver membranes regardless whether the source of ribosomes was brain or liver. This indicates that in so far as the physical binding is concerned it is the membrane component of RER that determines the extent of ribosome–membrane interactions in vitro. In the brain tissue the requirement of cations, such as Mg^{2+} or the polyamine spermine, for the maximum ribosome–membrane interactions is much greater than that observed for liver [16]. This could be due to a greater phospholipid content of the brain ER as compared with the liver ER (table 1). Thus if charge neutralization and/or ionic bridge formation is involved in the ribosome–membrane attachment [4] one would expect a greater requirement of cations for brain compared with liver.

On the functional level, our results show that the homologously reconstructed liver RER is able to function as a secretory unit. This observation is compatible with the results reported by Shires et al. [10] who used ribonuclease-conditioned membranes for binding ribosomes to membranes in vitro. Burke and Redman failed to observe any secretory activity in the recontructed RER [9]. The apparent discrepancy in the results could be due to the different experimental conditions employed, especially the use of detergent in the isolation of ribosomes. We found that when the detergent-treated ribosomes were used for the reconstruction of RER, the resulting preparation failed to exhibit any significant secretory activity, possibly due to the deleterious effect of the residual detergent on the membrane.

The present study delineates, for the first time, that heterologous RER reconstructed from a secretory and a non-secretory tissue is unable to discharge the newly synthesized protein vectorially across the membrane. For, although the liver membranes could bind similar amounts of liver or brain ribosomes, only the homologous liver preparation was able to function as a secretory unit. Recently Blobel and Dobberstein have reported the reconstruction of RER from reticulocytes and concluded that the information for segregation of the translational product is encoded in the mRNA [8]. The present results are not necessarily inconsistent with this conclusion. The evidence provided in this report further suggests that the vectorial discharge of proteins across the ER requires specific and co-operative interactions between polysomes and ER in the secretory tissues such as liver, since the heterologous RER composed of liver membranes and brain ribosomes failed to show any secretory activity. The observed inability of liver ribosomes attached to brain membranes to effect the vectorial discharge of proteins lends support to the idea that, in the nonsecretory tissues, the ribosome binding of membranes may have some other role than secretion of the proteins [11]. It is of interest to note that the homologous liver RER composed of isolated free ribosomes and the stripped membranes was active in the vectorial discharge of polypeptides. This may suggest that in vivo, liver ribosomes may be free to change between different topographic situations.

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