CORRELATION OF RAT LIVER MEMBRANE BINDING OF POLYSOMES in vitro WITH FUNCTION OF THE COMPLEXES FORMED

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Summary

One method of destroying polysome binding sites which is reversible is mild-trypsin digestion. Unlike the original untreated sites, the trypsin altered sites when complexed with polysomes are unable to direct transfer of puromycin-discharged polypeptide to the membrane vesicle as can sites on native membranes.

Attachment of exogenous polysomes to biological membranes under in vitro conditions has been accomplished in a number of laboratories (1-10). Membrane from sources as disparate as bacteria (1,2), reticulocytes (3), and hepatic endoplasmic reticulum (4-10) have demonstrated affinity for polysomes, and it now appears that under appropriate experimental conditions the adsorptive phenomenon may be evinced in membranes of any source.

There are currently 2 bases for distinguishing among the various membrane preparations in their affinity for polysomes: The incubation conditions required for attachment, and the manner of handling puromycin-discharged nascent protein from attached polysomes. All membranes thus far examined which are classifiable as "smooth" do not accept polysomes in stable complex unless incubated at 25-37°. In contrast, microsomal membranes derived from hepatic rough endoplasmic reticulum avidly bind exogenous polysomes at 0°. However, this affinity is demonstrable only

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after displacement of endogenous polysomes from their sites of attachment (9).

Rough liver microsomes thus conditioned with ribonuclease and complexed with exogenous polysomes are able to translocate puromycin-released polypeptide from its externally situated synthetic sites to the membrane vesicles (11). This function was first described for native rough microsomes (12), and its emulation by these artificial complexes has supported their being considered reconstituted rough membranes. Membrane preparations other than ribonuclease-conditioned rough membranes will not sustain translocation. Polysomes complexed at 37° with membranes obtained from elements of hepatocyte smooth endoplasmic reticulum released their puromycin-discharged peptide exclusively into an extravesicular location (11).

Reported here are results of studies of the destruction of binding sites on rough membrane preparations conditioned for polysome adsorption with ribonuclease. The influence of physical damage to the membranes on their ability to accept polysomes is shown in Table I. Denaturation of conditioned rough membranes by boiling or by TCA precipitation caused marked reduction in polysome acceptance to a level near that observed with unconditioned rough microsomal membranes. Binding was carried out at 0° and 37°. The latter caused increased association of radioactivity with the membranes, especially with TCA-treated membranes, but this was always accompanied by a rise in the soluble radioactivity. Which polysome population, bound or unbound, contributed to this soluble activity is not clear.

Loss of conditioned rough membrane affinity for polysomes has also been observed after freeze-thawing (13), exposure jointly to pyrophosphate and citrate ions whose individual concentrations exceeded 50 mM (13), after prolonged incubation at 0° ("aging" - 8,9), and after digestion with trypsin at 3-4° (9). In the case of the latter condition, experiments were carried out with rough microsomes and conditioned rough membranes digested either sequentially with ribonuclease and trypsin or with each enzyme, separately, prior to addition

TABLE I

BINDING OF POLYSOMES TO DENATURED CONDITIONED ROUGH MEMBRANES*

Incubation Conditions	Per cent of total recovered DPM with		
	Unbound polysomes	membrane-associated polysomes	soluble, non-sedimentable DPM
R_{RN}			
0°	24.3	74.0	1.7
37°	10.4	85.2	4.4
R_{RN} boiled			
0°	91.8	7.4	0.9
37°	80.2	8.1	11.7
R_{RN} TCA			
0°	87.1	12.2	0.7
37°	67.2	25.4	7.4
R_{mic}			
0°	93.7	5.7	0.6
37°	72.3	20.8	6.9
R_{mic} boiled			
0°	93.2	6.2	6.6
37°	84.5	10.0	5.5
R_{mic} TCA			
0°	89.5	9.3	1.2
37°	77.1	16.8	6.1

*Rough microsomes (R_{mic}), polysomes labeled with tritium *in vivo* by ^{3}H-orotic acid, and postmicrosomal supernatant were prepared from rat liver as previously described (9). Conditioned rough membranes (R_{RN}) were made from R_{mic} by incubation at 0° with RNase in EDTA (9). Methods used for binding polysomes to membranes as well as the centrifugation techniques for measuring the extent of interaction have been detailed elsewhere (8,9).

In each experiment, 0.5 mg membranes (measured as protein) was mixed with 100 μg freshly made polysomes (measured as RNA) and 0.5 ml (approx. 25 mg protein) of rat liver postmicrosomal supernatant. The mixture was incubated at 0° or 37° for 2 min with shaking and layered over 1.8M sucrose for centrifugation in an SW 56 rotor (Beckman) for 10 hrs. Unbound polysomes were pelleted, and membrane-associated polysomes were obtained by collecting the tube's contents from above the dense sucrose and centrifuging the membranes into a pellet. Radioactivity of the supernatant ("soluble" DPM) and the membrane pellet was determined separately as earlier described (8,9), as was the pellet of unbound polysomes which was counted together with the 1.8M sucrose underlying the membrane layer in the SW 56 rotor tube. In each spin of the SW 56 rotor, one tube was included which contained radioactive polysomes (100 μg) and postmicrosomal supernatant but no membranes. In these tubes, an average 3% of the total recovered DPM could be obtained from the "membrane region" above the dense sucrose as sedimentable activity, while an average 0.9% was "soluble". Of the total applied radioactivity, an average 95% was recovered. Denaturation of conditioned rough membranes by boiling was accomplished at 100° for 5 min. With 5% TCA treatment, the precipitated membranes were collected at 2,000 rpm for 10 min and washed 3X with distilled water before resuspension in standard sucrose-buffer (0.44M sucrose, 50 mM Tris-HCl at pH 7.4, 25 mM KCl, and 5 mM $MgCl_2$).

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of exogenous polysomes. Mild proteolysis destroyed binding capacity on conditioned and unconditioned membranes, and this capacity was not regained by subsequent ribonuclease treatment (9).

As shown in Table 2, the trypsin-treated conditioned rough membranes may regain an ability to adsorb polysomes. Incubation of a mixture containing polysomes, trypsin-treated membranes, and a complete amino acid incorporating mixture at 37° for 7 min led to membrane association by 50.7% of the polysomes. Incubation at 3° was not as effective. Corollary experiments with ^{3}H-leucine and unlabeled polysomes established that active incorporation occurred during this period of binding. While the 50.7% polysome association with trypsin-treated membranes is considerably less than 85.2% achieved by conditioned rough membranes at 37° (Table 1), it does represent a greater than 5-fold gain over the binding capacity displayed by trypsin-treated membranes incubated in buffer at 3° (Table 2). The origin of the high level of radioactivity in the soluble fractions of these experiments appears to be the unbound polysome fraction.

Because of the amino acid incorporative capacity of the trypsin-treated membranes complexed with polysomes, it was possible to test their

TABLE 2

INTERACTION OF POLYSOMES WITH TRYPSIN-TREATED MEMBRANES*

Incubation conditions	Per cent of total recovered DPM		
	Unbound polysomes	membrane-associated polysomes	soluble, non-sedimentable radioactivity
buffer alone at 3°	80.5	9.8	9.7
buffer alone at 37°	73.5	11.9	14.6
Incorporating medium at 3°	70.3	19.7	10.0
Incorporating medium at 37°	25.2	50.7	24.1

*Tritium labeled polysomes (100 μg) and membranes (0.5 mg) were added to a complete cell-free amino acid incorporating system, as described previously (11), including postmicrosomal supernatant, 20 amino acids, and an ATP generating system in a total final volume of 1 ml. After incubation for 7 min (with shaking), cold buffer was added and the entire mixture layered over 1.8 M sucrose buffer. Centrifugation and measurement of the membrane association by the labeled polysomes were carried out as in Table 1.

ability to direct polypeptide to the membrane vesicles (Table 3). As carried out with 100 μg of reconstituted rough membranes (based on RNA), addition of puromycin to an incorporation mixture after an initial 7 min incubation caused a shift in the amount of acid precipitable ^{3}H-leucine from polysomes of the preparation to a deoxycholate-soluble fraction. 42% of the incorporated activity was released from the polysomes of the reconstituted rough membranes, and about half (55%) of this was collected in the deoxycholate soluble fraction after puromycin addition (Table 3).

In the puromycin experiments with trypsin-treated membranes (Table

TABLE 3

THE DISTRIBUTION OF ACID-PRECIPITABLE RADIOACTIVITY AFTER PUROMYCIN DISCHARGE OF POLYPEPTIDES*

	Percent (^{3}H) leucine DPM			Total precipitable
	Extravesicular supernatant	Ribosomes	Deoxycholate-soluble	DPM
Reconstituted rough membranes				
no Puromycin	10.3	80.5	8.2	2099.3
Puromycin	18.9	57.2	23.9	2432.7
Polysomes mixed with trypsin-treated membranes				
no Puromycin	36.4	50.4	13.2	2645.0
Puromycin	54.8	30.2	14.0	3051.4

*Reconstituted rough membranes (made as described in ref. 11) or 0.5 mg trypsin-digested membranes plus 100 μg unlabeled polysomes were mixed with a complete amino acid incorporation mixture (see Table 2), incubated 7 min at 37°, and 1 mM Puromycin added. Control tubes received an equal volume of buffer. Incubation was continued an additional 23 min and stopped by addition of cold buffer. The mixture was centrifuged and 1) the resulting supernatant, containing "extravesicular" material, was precipitated with 5% TCA, and 2) the resulting pellet was resuspended in buffer, treated with 0.5% deoxycholate, and centrifuged. The pellet of this last spin was considered "ribosomal" and the "deoxycholate-soluble" supernatant, the vesicular material (see ref. 11). After resuspension of the ribosomal pellet, both the suspension and the supernatant were precipitated with cold 5% TCA. Handling of the acid precipitates and scintillation counting is described in ref. 11.

3), 100 μg of unlabeled polysomes (based on RNA) and 0.5 mg of membranes were added to a complete incorporating mixture and incubated for 7 min. At the time of puromycin addition after 7 min of incubation, it was expected from the results shown in Table 2 that about 50 μg of the exogenous polysomes were bound to the membranes. This is about half the

amount of membrane-attached polysomes present in the experiments with reconstituted rough membranes. In the preparations of reconstituted rough membranes, all unattached polysomes were removed. At the time of RNA measurement and of addition to incubation tubes, only membrane-attached polysomes are present (11).

In the experiments with trypsin-treated membranes, puromycin addition caused virtually no rise in the percent DPM in the deoxycholate-soluble fraction, compared with a slightly less than 3-fold rise with the reconstituted rough membranes (Table 3). The total incorporation achieved by both systems was comparable. While with the reconstituted membranes about 44% of the discharged polypeptide was released into the extravesicular fraction, with the trypsin-treated membranes a much higher fraction (76.5%) of the incorporated radioactivity went to the extravesicular compartment. The presence of unbound polysomes in the trypsin experiments provided a possible source for some of the discharged extravesicular polypeptide. However, outnumbered 2:1 by bound polysomes, the free population seems unlikely to be the only source. The trypsin-digested membranes thus appear not to support directed transfer of puromycin-discharged polypeptide as carried out by rough microsomal and reconstituted rough membranes.

Experiments with trypsin-digested membranes in 2 ways demonstrate the influence of experimental manipulation on polysome binding. First, preparations of nonbinding rough microsomal membranes, after ribonuclease conditioning, can be converted to a preparation which accepted polysomes, yet, after trypsin digestion, the same preparation could be returned to inactivity but with a potential for recrudescence of a binding capacity on incubation with an incorporating medium. Second, all membranes thus far examined which adsorb polysomes including those treated with trypsin have allowed cell-free incorporation of amino acid by the attached polysomes (4,9,11). When compared, the rates of incorporation have appeared approximately equal in all preparations (11). The competence for incorporation

appears to be similar to adsorption in being generally permitted by a range of membrane types. Only those of ribonuclease-conditioned rough membranes show a difference in also maintaining the ability to support vectorial transport.

Thus far, only sites on ribonuclease-condition rough membranes have appeared comparable to native sites (11), principally because of their distinctive ability to support vectorial transport of peptides. Definition of all other sites as artefacts is not at present warranted, however, since, as previously postulated (10,11), some may have as yet poorly defined roles in the function of bound polysomes.

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