EXTRACTION FROM FREE RIBOSOMES OF A FACTOR MEDIATING RIBOSOME DETACHMENT FROM ROUGH MICROSOMES

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SUMMARY: A salt extract of rabbit reticulocyte free monosomes or polysomes contains a factor with an activity that detaches membrane bound monosomes but not polysomes from dog pancreas rough microsomes. It is proposed that this activity, referred to as detachment factor, functions in the dissociation of membrane bound ribosomes from the microsomal membrane after each round of translation. In addition to free ribosomes, the factor is also present in a ribosome-free, high speed supernatant, the cell fractionation equivalent of the cytosol. The factor can be extracted from free ribosomes of a variety of tissues and species, and is able to function on ribosome membrane junctions homologous as well as heterologous with respect to its source.

In most eukaryotic cells, ribosomes exist either free or bound to intracellular membranes (1,2). It has been postulated recently in the so-called signal hypothesis (3,4) that the ribosome membrane junction is transient in nature and is established as a result of the translation of certain mRNA's (e.g. those for secretory proteins) which share a common feature, namely, a unique sequence of codons to the right of the initiation codon. Translation of these "signal" codons results in a unique NH_2 terminal sequence of amino acid residues on the nascent chain (referred to as signal sequence) which then triggers the formation of a functional ribosome membrane junction for transfer of these nascent chains across the membrane. It was further proposed that after completion of the nascent chain, the ribosome becomes detached from the membrane. Thus, the ribosome could cycle between the free and the membrane bound state depending on whether it is involved in the translation of mRNA's containing the characteristic signal codons.

Several aspects of the signal hypothesis have recently received strong support from work in this laboratory (4,5,6). In this paper, evidence is provided for the existence of protein associated with free ribosomes able to detach monomeric ribosomes from the microsomal membrane.

METHODS: Preparation of rough microsomes from dog pancreas has been described elsewhere (5). Rough microsomes from rat liver were prepared from a postmitochondrial supernatant as described (7).

Rabbit reticulocyte ribosomes were prepared as described (8). Reticulocyte ribosomes were fractionated into monosomes and polysomes (comprising all

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ribosomes sedimenting faster than monosomes) by sucrose gradient centrifugation under conditions which were recently used (9) for the isolation of native small ribosomal subunits.

Preparation of free ribosomes from rat liver (7) and murine myeloma MOPC 41-DL 1 (4) was as described. Preparation of free ribosomes from dog pancreas will be described elsewhere (Blobel and Scheele, unpublished).

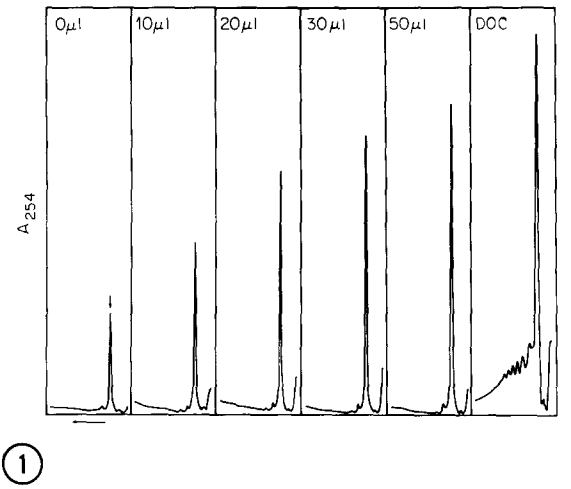
Salt Extraction of Reticulocyte Monosomes: Isolated monosomes (see above) were resuspended in ice cold double distilled water to a concentration of 200 A_{260} per ml. One volume of a concentrated salt-buffer solution was added to a final concentration of 500 mM KCl, 40 mM triethanolamine·HCl, pH 7.4, at 20°C, 5 mM $MgCl_2$, and 2 mM dithiothreitol (HSB). This suspension was incubated for 10 min at 37°C. 2-ml aliquots were layered onto 12.5 ml of 10-30% sucrose gradients in HSB. The gradients were centrifuged for 3 hr at 20°C and at 190,000 xg_{av} in the swinging bucket rotor SB 283 of the IEC centrifuge. The optical density in the gradient was monitored using an ISCO UV analyzer and ISCO fractionator. A top fraction (amounting to ~ 4 ml) comprising all material from the air-gradient interphase to just shortly before the appearance of the small ribosomal subunit peak was collected. The collected fraction was diluted to 100 mM KCl, 20 mM triethanolamine·HCl, pH 7.4, 2.5 mM $MgCl_2$, and 2 mM dithiothreitol (TKMD) and concentrated in an Amicon ultrafiltration cell using a PM 10 membrane (Amicon Corp, Lexington, Mass.). The concentrated extract is referred to as DF (abbreviated for detachment factor). DF can be stored frozen at -80°C for several months without appreciable loss of activity. The protein concentration in DF was not determined; instead it was related to the amount of monosomes from which it was extracted. 1 ml of DF was derived from 400 A_{260} of monosomes. DF from reticulocyte polysomes or free ribosomes of dog pancreas, rat liver, and murine myeloma MOPC 41-DL 1 were extracted in an identical manner.

Assay for Detachment of Ribosomes from Pancreatic Rough Microsomes: Dog pancreas rough microsomes were resuspended in TKMD (see above) to a concentration of 80-100 A_{260}/ml. 25-µl aliquots (unless otherwise specified in Figure legends) were mixed in an ice bath with TKMD or corresponding amounts of DF, and the mixture was incubated for 15 min at 37°C, cooled to 0°C, and then layered onto 12.5 ml of 10-30% sucrose gradients in 100 mM KCl, 20 mM triethanolamine·HCl, pH 7.4, and 5 mM $MgCl_2$. The gradients were centrifuged for 1 hr and 15 min (unless otherwise specified in Figure legends) at 4°C and at 190,000 xg_{av} in the SB 283 rotor (see above).

In some assays, rough microsomes from rat liver or DF from sources other than reticulocyte monosomes were used (see Figure 5).

Detergent Treatment of Pancreatic Rough Microsomes: The $MgCl_2$ concentration in suspended rough microsomes was raised to 5 mM because DOC treatment at lower $MgCl_2$ concentration resulted in a partial dissociation of ribosomes into subunits (data not shown). A solution of 10% sodium deoxycholate in water was added to the rough microsomes at 0°C to a final concentration of 1%. Subsequent analysis of the mixture by sucrose gradient centrifugation was as described above.

RESULTS AND DISCUSSION: In the proposed model for the ribosome cycle between the free and the membrane bound state (4), the postulated detachment of the ribosome could result merely from conformational changes following the release and vectorial discharge of the nascent chain. Alternatively, detachment could be mediated by protein, in analogy to the dissociation of the junction between the small and large ribosomal subunit which occurs after each round of translation and is mediated by protein constituting components of the initiation factor fraction (10). If this were the case, it could be reasoned that the putative detachment factor would become associated with the released ribosome



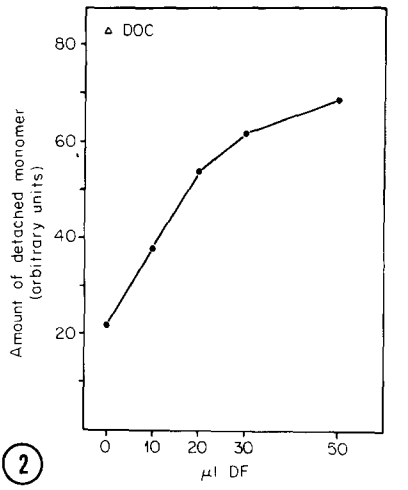


Figure 1. (Left panel) Effect of increasing amounts of DF on detachment of ribosomes from dog pancreas rough microsomes.

Rough microsomes (2.5 A_{260}) were incubated in the absence (0 μl) or presence of various amounts of DF from reticulocyte monosomes (10 μl, 20 μl, 30 μl, 50 μl) or treated with sodium deoxycholate (DOC). For details, see Methods. Direction of sedimentation is indicated by a horizontal arrow. Monosome peak is indicated by a vertical arrow.

Figure 2. (Right panel) Quantitation of the monosome peak shown in Figure 1. Area under the monosome peak was integrated and expressed in arbitrary units. Open triangle represents amount of monosomes after DOC-treatment of rough microsomes.

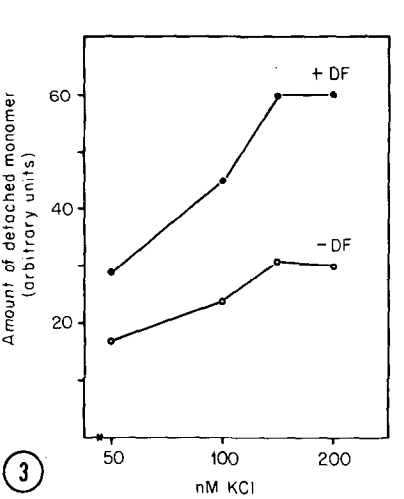
during the detachment process and would therefore be localized in the free monosome fraction. Furthermore, by similar analogy, the detachment factor may be extractable from free monosomes by high salt concentrations. In order to test these assumptions, isolated monosomes from rabbit reticulocytes were extracted with 500 mM KCl, buffer, and 5 mM $MgCl_2$; the salt extract was diluted to reduce the salt concentration to 100 mM KCl, buffer, and 2.5 mM $MgCl_2$, and was subsequently concentrated by ultrafiltration (see Methods). The extract referred to as DF was then incubated with isolated rough microsomes from dog pancreas, and release of ribosomes was monitored by sucrose gradient centrifugation under conditions whereby detached ribosomes would remain in the gradient whereas ribosomes bound to the microsomal membrane would sediment to the bottom. It can be seen from the sedimentation profiles shown in Figure 1 that some of the membrane bound ribosomes, predominantly monosomes, are detached during incubation in the absence of any added DF. However, there is a striking increase in the amount of detached monosomes following incubation with increasing amounts of DF. Figure 2 shows the results of quantitation of the amount of detached monosomes by integrating the area under the

monosome peak. There is a linear increase in the amount of detached monosomes with increasing amounts of DF, reaching a plateau at the higher concentrations of DF, results which suggest that the detachment of monosomes was complete. In order to assess the total amount of monosomes present, an equivalent amount of rough microsomes was treated with detergent to solubilize the microsomal membrane. The resulting mixture was analyzed by sucrose gradient centrifugation. From the sedimentation profile shown in Figure 1 (DOC) and from the quantitative analysis shown in Figure 2, it is evident that rough microsomes contained only slightly more monosomes than were detached by the highest concentration of DF. The balance of monosomes not detached by DF may have been generated by nucleolytic breakdown of polysomes during the detergent treatment. Alternatively, they may represent monosomes still containing nascent chains, and the presence of the latter in polysomes is presumably the reason why polysomes are not detached by DF.

It could be argued that DF contains nucleolytic activity and therefore does not affect the ribosome-membrane junction; instead, by virtue of its nucleolytic activity, it would degrade the polysomal mRNA and release those polysomal ribosomes which are localized near the 5' end of mRNA and which according to the signal hypothesis are not yet attached to the membrane (4). This possibility was ruled out, however, by an experiment in which incubation of rough microsomes with DF was followed by DOC treatment. The resulting sedimentation profile (not shown) was identical to that shown in Figure 1 (DOC), suggesting that DF does not contain nucleolytic activity and does not cause breakdown of polysomes.

That the detachment activity was due to a protein component of the salt extract was demonstrated in experiments (data not shown) in which this activity could be totally inactivated by preincubation of DF with trypsin. After preincubation, the residual trypsin activity (which by itself leads to detachment of ribosomes from the membrane (11)was inactivated by trypsin inhibitor prior to incubation in the detachment assay.

Various parameters affecting the detachment reaction were investigated. It was found that there is no monosome detachment if incubation of DF and rough microsomes is done at 0°C. Furthermore, detachment by incubation at 37°C is time dependent and is virtually complete after a 10-min incubation (data not shown). Omission of dithiothreitol in the salt extract step as well as in the incubation has no effect on detachment activity (data not shown). Figure 3 shows the results of varying the KCl concentration at a constant $MgCl_2$ concentration during the incubation of rough microsomes in the absence or presence of DF. In the absence of DF (curve - DF), increasing KCl concentrations cause enhanced detachment of monosomes from the microsomal membrane. However,under



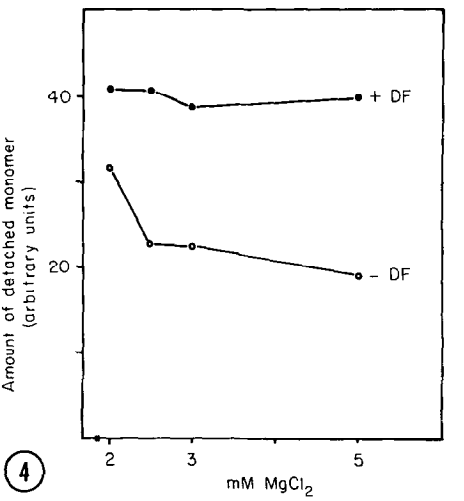


Figure 3. (Left panel) Effect of various KCl-concentrations on the detachment of monosomes.

Rough microsomes (2.2 A_{260} were incubated in 20 mM triethanolamine·HCl, pH 7.4, 2.5 mM $MgCl_2$, 2 mM dithiothreitol, and KCl concentrations as indicated on the abscissa, either in the absence (0-0) or presence (●-●) of 10 µl of DF from reticulocyte monosomes. The area under the monosome peak was integrated as in Figure 2.

Figure 4. (Right panel) Effect of various $MgCl_2$ concentrations on the detachment of monosomes from pancreatic rough microsomes.

Rough microsomes (2.5 A_{260}) were incubated in 100 mM KCl, 20 mM triethanolamine·HCl, pH 7.4, 2 mM dithiothreitol, and $MgCl_2$ concentrations as indicated on the abscissa, either in the absence (0-0) or presence (●-●) of 15 µl of DF from reticulocyte monosomes. The area under the monosome peak was integrated as in Figure 2.

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identical conditions an increase in detached monosomes is observed in the presence of DF (curve + DF). Thus, 150 mM KCl could be considered optimal for assaying monosome detachment by DF. As demonstrated in Figure 4, varying the $MgCl_2$ concentration from 2.5 to 5 mM at a constant KCl concentration has no significant effect on the detachment of monosomes either in the absence or presence of DF during the incubation of rough microsomes.

So far, all experiments were performed with DF extracted from reticulocyte monosomes. Figure 5 (upper panel, Retic) shows that a salt extract from an equivalent amount of reticulocyte polysomes is approximately as active as the monosome extract in detaching monosomes from pancreatic rough microsomes. The fact that the detachment factor extracted from reticulocyte mono- or polysomes acted upon a heterologous ribosome membrane junction suggested a widespread equivalence of the sites involved. To provide further evidence for this contention, DF was prepared from free ribosomes of rat liver, dog pancreas, and mouse murine myeloma MOPC 41 DL-1 and tested for detachment

activity on rough microsomes from rat liver and dog pancreas. It can be seen from the sedimentation profiles shown in Figure 5 that DF from various free ribosomes disassembles ribosome membrane junctions, either homologous or heterologous with respect to the source of DF.

It has been shown that adherence of initiation factors to the native small ribosomal subunit is not mediated by magnesium ions, since EDTA treatment did not dissociate the factors from the native small ribosomal subunit (12). It is noteworthy that the detachment factor cannot be extracted from free ribosomes by EDTA treatment in low salt concentrations, although such treatment results in ribosome dissociation into subunits and in the extraction of specific proteins from the large (13) as well as the small ribosomal subunit (12). As shown in Figure 5 (EDTA extract), this extract is entirely inactive in the detachment of monosomes from the microsomal membrane.

By further analogy to the initiation factors, which are found in the native small subunit fraction and to some extent in the cytosol, the localization of the detachment factor should be restricted to free ribosomes and to

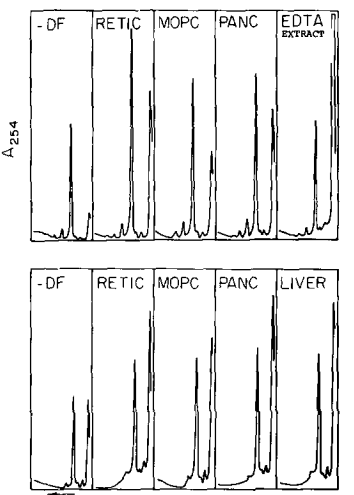

Figure 5. Effect on the detachment of monosomes from rough microsomes of dog pancreas by various DF preparations and by an EDTA extract of reticulocyte monosomes.

Shown are the sedimentation profiles of incubation mixtures containing (upper panel): pancreatic rough microsomes (5.2 A_{260}) alone (-DF) and in combination either with 25 µl of DF of rabbit reticulocyte polysomes (Retic), of murine myeloma-free ribosomes (MOPC), and of dog pancrease-free ribosomes (PANC), or with 25 µl of an EDTA extract of reticulocyte monosomes (EDTA Extract); (lower panel): liver rough microsomes (7.0 A_{260}) alone (-DF) or in combination with 25 µl of DF from free ribosomes of sources as indicated. Centrifugation was for 1 hr and 35 min (upper panel) or 1 hr and 30 min (lower panel).

the cytosol. The factor should not be present in rough microsomes (unless it is part of those polysomal ribosomes of membrane bound polysomes which are localized near the 5' end of mRNA (see above) and are as yet not attached to the membrane). This reasoning was confirmed, since it was found that a salt extract of rough microsomes does not have any detectable detachment factor activity (data not shown). The presence of the factor in the cytosol was demonstrated by incubating salt extracted reticulocyte monosomes in a ribosome-free high speed supernatant of a reticulocyte lysate, followed by reextraction of these ribosomes with salt; the extract was found to contain DF activity (data not shown).

At present it is not clear what purpose the factor mediated disassembly of the ribosome membrane junction after each round of translation would serve. However, in the signal hypothesis (4) it has been suggested that the establishment of a functional ribosome membrane junction for the transfer of the nascent chain across the membrane causes tunnel formation in the microsomal membrane, triggered by the signal sequence of the nascent chain. Detachment of the ribosomes may therefore be required as a means for eliminating this tunnel, the persistence of which otherwise could result in an undesirable equilibration of small molecules between the cytosol and the intracisternal space of the endoplasmic reticulum.

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