EFFECT OF SPERMINE AND MAGNESIUM ON THE ATTACHMENT OF FREE RIBOSOMES TO ENDOPLASMIC RETICULUM MEMBRANES IN VITRO

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SUMMARY

A portion of the free ribosomes became attached to the isolated total endoplasmic reticulum membranes when incubated at 0°C for 60 min in buffers containing either 0.3-0.5 mM spermine or 5 mM Mg^{2+}. This attachment was negligible if both spermine and Mg^{2+} were omitted from the incubation medium. When smooth endoplasmic reticulum was tested alone, free ribosomes did not associate appreciably, whether or not spermine or Mg^{2+} was present. These results suggest that spermine and Mg^{2+} may function through forming ribosome-cation-membrane bridges. There is also an indication of the presence of ribosome binding sites on the endoplasmic reticulum membranes.

The existence of two topographic forms of ribosomes in the animal cell, viz. free and membrane-bound, is now well established (1-3). Although a number of reports have recently appeared, assigning separate functional roles to these two forms (reviewed by Campbell, 4), very little is known about the nature of the attachment of ribosomes to the endoplasmic reticulum (ER) membrane. It has been suggested that nascent proteins (5) and steroid hormones (6) may be involved in the ribosome-membrane association. In bacterial system Schlessinger et al. (7) have shown that Mg^{2+} has an effect on this attachment. The polyamines, spermine and spermidine, are constituents of all animal cells and are found in preferential association with isolated ribosomes and microsomes (8, 9). Spermine has been reported to stabilize ribosomes and bacterial cell membranes (10).

This study was undertaken to investigate whether spermine is involved in the interaction of ribosomes with ER membranes. The results suggest that both spermine and Mg^{2+} may play a role in the attachment of ribosomes to the ER membranes. Under identical ionic conditions, smooth membranes failed to associate themselves with ribosomes.

MATERIAL AND METHODS

Male rats of the Wistar strain (130-200 g) were used. They were starved for 20-24 hours before being killed by decapitation. Free ribosomes were prepared by a modification of the method of Blobel and Potter (2). The postmitochondrial supernatant fraction (20,000 x g) was centrifuged through a discontinuous gradient of 1.31 - 2 M sucrose in TKM buffer (50 mM Tris-HCl pH 7.6, 25 mM KCl, 5 mM $MgCl_2$) in a Spinco SW 50.1 rotor at 149,000 x g (average) for 24 hours. The pellet, consisting of free ribosomes, was gently rinsed three times with 1 ml of 50 mM Tris-HCl buffer, pH 7.6. The wall of the tube was carefully wiped clean with tissue and the pellets were gently resuspended in the desired buffer or stored as such at -18°C until used. In what follows, the free ribosomes thus prepared will be designated as 'ribosomes'. For the preparation of labeled ribosomes, the rat was treated intraperitoneally with 10 μC of 6-^{14}C-orotic acid in one ml of dist. water (specific act. 60.8 mC/mM; The Radiochemical Centre, Amersham, England) and sacrificed 16 hours later. Total ER membranes from rat liver were isolated essentially according to the method of Scott-Burden and Hawtrey (11), by treating the microsomes with an equal volume of 4 M LiCl. After the RNA had been pelleted by low-speed centrifugation, the membranes were isolated from the supernatant by centrifugation at 105,000 x g for 60 min. They were finally washed with 50 mM Tris-HCl buffer, pH 7.6, containing 25 mM KCl (TK) and stored at 0°C for not more than 72 hours.

Smooth endoplasmic reticulum (smooth membranes) was prepared by the CsCl method of Dallner (12), as modified by Ragnotti et al. (13). The isolated smooth membranes were washed and stored in the same way as the total endoplasmic reticulum membranes.

The ratio of RNA to protein in the ribosomes, total ER membranes, and smooth membranes was 0.59, 0.011 and 0.019 respectively.

Details of sucrose gradient analysis are described in the legends to the figures. The radioactivity in the fractions was determined by dissolving the trichloroacetic acid-insoluble material in 0.5 N NaOH and adding it to 5 ml of Cab-O-Sil gel in Bray's solution (14). The samples were counted in a Packard liquid scintillation spectrometer.

RESULTS

It has been shown (15,16) that after prolonged centrifugation, only free ribosomes are able to sediment through buffers containing 2 M sucrose, whereas membranes and membrane-bound ribosomes

fail to do so under similar conditions. This criterion has been utilized in the present work to study the attachment of ribosomes to membranes. We confirmed the reliability of the criterion by subjecting ^{14}C-labeled membranes and microsomes to sedimentation through a 16-34 % linear sucrose gradient, overlaid on a 5 ml cushion of 2 M sucrose, all prepared in TKM. Practically all of the loaded radioactivity in the membranes or microsomes could be recovered at the boundary directly above the 2 M sucrose cushion after centrifugation at 63,000 x g for 120 min.

Fig. 1 shows the results of an experiment designed to study the effect of Mg^{2+} on the association of ribosomes with ER membranes. When incubated and centrifuged in TKM, without membranes, ribosomes appear as monomers, dimers and higher aggregates, there being very little radioactivity at the boundary of 2 M sucrose. On addition of ER

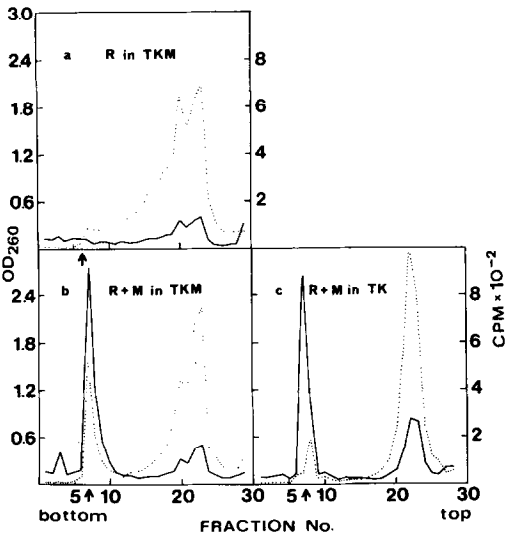

Fig. 1. Sucrose gradient analysis of free ribosomes incubated with and without ER membranes in TK or TKM. Each gradient was prepared in a buffered medium having the same ionic composition as the one used for incubation. Labeled ribosomes (0.13 mg RNA) + membranes (0.8 mg protein) in a total volume of 2 ml were incubated for 60 min at 0°C. The incubation mixture was then loaded on to 20 ml of 16-34 % linear sucrose gradient, beneath which a cushion of 5 ml of 2 M sucrose had been laid. Centrifugation was in a Spinco SW 25.1 rotor at 25,000 rpm for 120 min. One-ml fractions were collected and OD_{260} was read (———). Radioactivity (- - - - -) was determined as described in Materials and Methods. The arrow indicates the boundary between the linear gradient and the sucrose cushion. (a) Ribosomes (R) in TKM buffer. (b) Same as (a) except that total ER membranes (M) were included in the incubation. (c) Same as (b), except Mg^{2+} was omitted from the incubation medium.

membranes to the incubation mixture, a significant proportion of the ribosomes sediment close to this boundary. This peak will be designated as 'heavy peak'. Omission of Mg^{2+} from the medium resulted in a marked reduction in radioactivity in the heavy peak and an increase in the peak of single ribosomes (Fig. 1c). The high OD_{260} in the presence of membranes is not due to RNA, as shown by chemical analysis of this fraction.

The effect of spermine on the sedimentation profiles of ribosomes in the presence and absence of membranes is shown in Fig. 2. In TK + 0.3 mM spermine (Fig. 2a, 2b), the profiles were similar to those obtained when TKM was used as the medium for incubation and centrifugation (cf. Fig. 1). Increasing the concentration of spermine to 0.5 mM caused a substantial increase of radioactivity in the heavy peak, which indicates an increased association of ribosomes with membranes under the influence of spermine (Fig. 2b and 2d). A comparison of Fig. 2a and 2c shows that increasing the concentration of spermine from 0.3 to 0.5 mM affects the sedimentation of ribosomes in the absence of membranes. There is a tendency to form very large aggregates at the higher concentration of spermine. When the experiment described in Fig. 2d was repeated with a fourfold amount of membranes, while

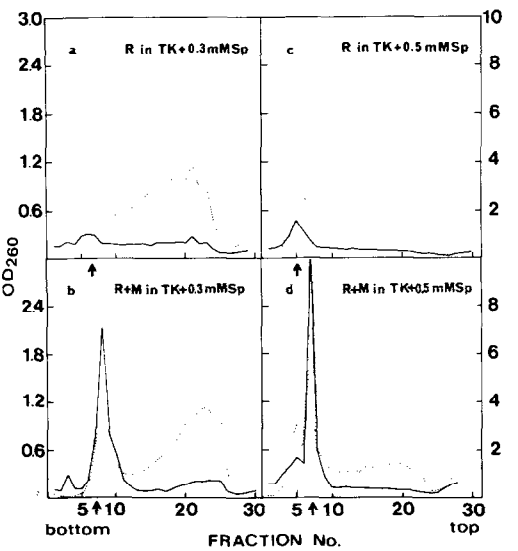

Fig. 2. Effect of spermine on the sucrose gradient profiles of free ribosomes incubated with and without ER membranes. Conditions of centrifugation etc. are identical with those given for Fig. 1. (a) Ribosomes (0.13 mg RNA) in TK + 0.3 mM spermine (Sp). (b) Same as (a) plus total ER membranes (0.8 mg protein). (c) Same as (a) except that the medium was TK + 0.5 mM spermine. (d) Same as (c) plus total ER membranes (0.8 mg protein).

keeping the concentration of ribosomes constant, almost all the radioactivity was found to be associated with the membranes (results not shown).

In the experiments described above, total endoplasmic reticulum membranes were used for the attachment of ribosomes. To see whether there was any specificity as regards the membrane fraction, isolated smooth membranes were used for the attachment instead of total ER membranes. The results are shown in Fig. 3. Neither spermine nor Mg^{2+} was effective in anchoring smooth membranes to the ribosomes. Even a fourfold increase in the amount of membranes failed to produce any effect.

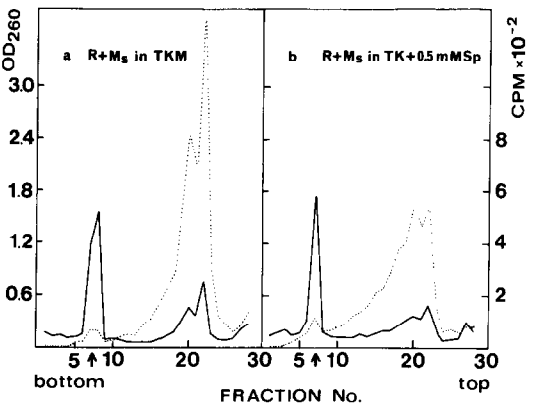

Fig. 3. Effect of spermine and Mg^{2+} on the sucrose density gradient profiles of free ribosomes incubated with smooth membranes. Conditions of centrifugation etc. are identical to those given for Fig. 1. (a) Ribosomes (0.13 mg RNA) incubated with smooth membranes (M_s, 0.91 mg protein) in the presence of TKM. (b) Same as (a) except the medium contained TK + 0.5 mM spermine.

DISCUSSION

The present results indicate that *in vitro* free ribosomes apparently can attach to the ER membranes if certain ionic conditions are met. Both Mg^{2+} and spermine promote this association, although spermine seems to be effective at lower concentrations than Mg^{2+}. Our observations on the effect of Mg^{2+} on the attachment are comparable to the results obtained by Schlessinger *et al.* (7) with bacterial ribosomes. They have shown that membrane-bound RNA decreases from 60 % of the total at 20 mM Mg^{2+} to 0 % when Mg^{2+} is omitted from the medium. In our study, it was found that spermine

can replace Mg^{2+} in anchoring ribosomes to the ER. It seems unlikely that the spermine effect is indirect, i.e. due to the release of bound Mg^{2+} from the ribosomes. This inference is derived from our recent observation that the addition of 0.25 mM Mg^{2+} (the upper limit of bound Mg^{2+} in this system) to the incubation mixture had a much smaller effect on the ribosome-membrane association as compared to 0.5 mM spermine.

ER membranes are negatively charged at physiological pH (17) and ribosomes also have an overall negative charge. It is probable that spermine and Mg^{2+} are involved in forming ribosome-cation--membrane bridges, thereby binding together the two negatively charged species. However, the possibility that other factors may be involved in the attachment cannot be ruled out by the present results. The existence of binding sites on the membranes is indicated by the observation that almost all the radioactivity of ribosomes is to be found attached when the amount of membranes is increased (cf. above). This is further borne out by the finding that isolated smooth membranes do not combine with the ribosomes, which may be explained by assuming that the binding sites for ribosomes on these membranes are in a masked or inactive state due to unknown morphological and/or biochemical factors. Our observations on the ineffectiveness of smooth membranes in combining with ribosomes are comparable with those of Süss et al. (18). It is probable that in the case of total ER the ribosomes became attached only to the rough membranes which had been stripped off the ribosomes, and not to the smooth part of the endoplasmic reticulum.

Our results indicate that polyamines may be involved in the ribosome-membrane association. In view of the significance of membranes and membrane-bound ribosomes in protein synthesis (cf. Hendler, 19), polyamines may thus perform an important physiological function in the animal cell. Whether the ribosomes bound in vitro in the presence of spermine behave like authentic bound polysomes in carrying out protein synthesis is currently being investigated in this laboratory.

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