POLYSOME-BINDING CAPACITY OF MEMBRANES IN THE CYTOPLASMIC EXTRACT PREPARED FROM PHENOBARBITAL TREATED AND REGENERATING RAT LIVER

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SUMMARY

Cytoplasmic extracts from female rat liver were incubated with ^{14}C-labeled free polysomes and the binding capacity of the membrane therein was assayed by CsCl density gradient centrifugation after fixing the complex in formaldehyde. Phenobarbital treatment *in vivo* increased the binding capacity, while hepatectomy decreased it. Addition of ethanol *in vitro* into this system enhanced the binding capacity of the cytoplasmic extract from normal and phenobarbital treated rat liver but not from regenerating liver. The stimulation of binding by ethanol was inhibited by estradiol in this female system. Testosterone had no effect.

INTRODUCTION

Evidence has accumulated indicating that free and membrane-bound polysomes synthesize different kinds of protein (1,2). Free polysomes synthesize proteins for intra-cellular utilization, while membrane-bound polysomes synthesize proteins mainly destined for export and possibly incorporation into membranes. The mechanism by which polysomes interact with membranes may therefore be involved in the regulation of the pattern of proteins synthesized in each cell. We have been interested in the effect of phenobarbital treatment and partial hepatectomy on the binding capacity of membranes of liver. Both treatments appear to be associated with re-organization of membranes and alteration of membrane-polysome interaction; phenobarbital causes induction of some microsomal enzymes, most of which may be synthesized on membrane-bound polysomes, while in regenerating liver, a relatively greater proportion of proteins for intra-cellular purposes have to be accumulated.

MATERIALS AND METHODS

Female COBS, CD strain rats of 2-3 months old were used after 16-20hr starvation. The operation of partial hepatectomy was carried out according to the method of Higgins and Anderson (3). Phenobarbital (80mg/Kg) was injected intraperitoneally at 40 and 16hr before the experiment. For the uniform labeling of RNA, each rat was injected with 10μCi of [6-^{14}C]-orotic acid (42mCi/mmole, Schwarz/Mann) intraperitoneally 20hr before the experiment. Free polysomes were prepared as described previously (2), except that the post-mitochondrial fraction applied to a 1.8M-1.0M disconti-

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nuous sucrose gradient (each 1ml) was centrifuged at 40,000rpm for 2hr in a SW50.1 Spinco rotor. The pellet was suspended in a small volume of the incubation buffer (50mM HEPES*-KOH pH 7.6, 70mM KCl, 5mM $MgCl_2$, 4mM DTT**). The concentration of polysomes was determined by reading absorbancy at 260nm.

The post-mitochondrial fraction from normal or regenerating livers or livers from phenobarbital treated rats were prepared in the presence of 4mM DTT and filtered through a column of Sephadex G-25 equilibrated with the incubation buffer according to the method of Richardson et al (4). The cytoplasmic extract thus prepared was incubated with ^{14}C-labeled free polysomes in a total volume of 0.25ml at 23°C for 90min or less. Testosterone or estradiol, both dissolved in ethanol, or ethanol alone, was added in some of the reaction tubes. In some experiments, purified smooth membrane fraction was used in the incubation mixture in place of the cytoplasmic extract. The post-mitochondrial fraction was centrifuged on a 1.8M-1.0M discontinuous sucrose gradient as described above and a turbid zone above the 1.0M sucrose layer was carefully collected, diluted five fold with 0.25M sucrose solution. 3.5ml of this preparation was centrifuged on 1.5ml of 1.0M sucrose solution at 40,000rpm for 2hr in a SW50.1 rotor. A turbid zone above the 1.0M sucrose layer was taken out and used as a purified smooth membrane fraction. The protein content was determined by the method of Lowry et al (5).

After the incubation, 0.05ml of 30% formaldehyde in TEAMK (triethanolamine-HCl pH 7.8, 5mM $MgCl_2$, 25mM KCl) was added and mixed (6). The tubes were kept in ice for 20hr or more. The fixed preparation was put on a discontinuous CsCl-sucrose gradient consisting of 2ml of the heavy CsCl solution at the bottom, 2ml of the light CsCl solution in the middle and 1ml of 1M sucrose on the top, each made in 0.8% Brij 58 containing TEAMK (7). The heavy and the light CsCl solution were prepared by dissolving 0.9g and 0.45g of CsCl (optical grade, Schwarz/Mann) per ml respectively. The gradients were spun at 40,000rpm for 16hr or more in a SW50.1 rotor and then fractionated into 27 (Fig. 1) or 9 (Fig. 2-6) fractions. Trichloro-acetic acid insoluble material was collected on a glass filter disc (GF/C, Whatmann) and the radioactivity was determined by a scintillation counter.

RESULTS

We have found that formaldehyde fixation of the post-mitochondrial fraction prior to CsCl density gradient centrifugation, not only stabilizes polysomes, but also stabilizes the membrane-polysome complex, thereby permitting a good separation of free and membrane-bound polysomes. After the centri-

* HEPES; N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid
** DTT; dithiothreitol

fugation of the CsCl-sucrose gradient, the density gradient of CsCl is linear, while the sucrose layer remains on the top of the CsCl gradient and 16hr is long enough for centrifugation to equilibrate free and membrane-bound polysomes in the gradient. The evidence for this good separation is shown in Fig. 1. After fixing and CsCl centrifugation of the post-mitochondrial

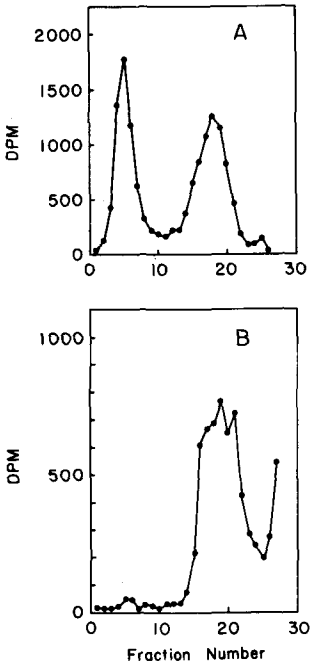

Fig. 1. CsCl density gradient analysis of free and membrane-bound polysomes.
Two rats were injected intraperitoneally with either 10μCi of [^{14}C]orotic acid for 20hr (A) or 10μCi of [methyl-^{14}C]choline for 6hr (B). 0.25ml of the post-mitochondrial fraction prepared from the liver was fixed with formaldehyde and centrifuged on a CsCl-sucrose discontinuous gradient. Each 6 drop fraction was collected from the bottom of the tube and acid insoluble radioactivity was counted.

fraction prepared from rats labeled *in vivo* for 20hr with [^{14}C]orotic acid or for 6hr with [methyl-^{14}C]choline (100μCi/0.337mg, New England Nuclear), all polysomes are found in two well separated peaks (Fig. 1-A), one of which is coincident with a heavier membrane peak (Fig. 1-B). We base our experiments on the assumption that interaction between polysomes and membranes induced *in vitro* will similarly be stabilized by formaldehyde fixation prior to CsCl density gradient centrifugation.

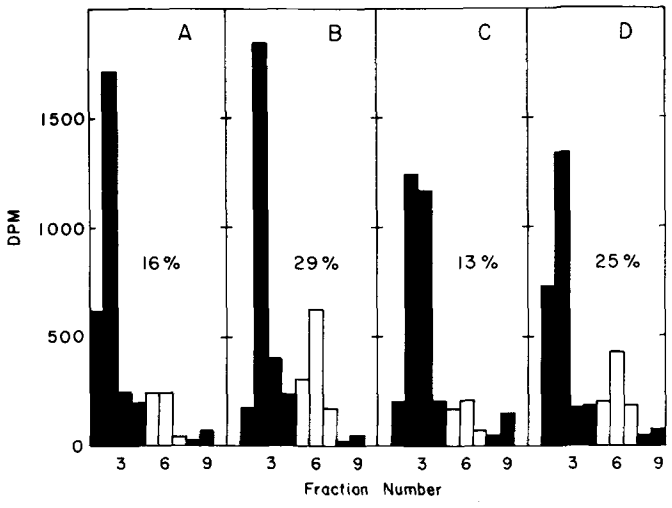

Fig. 2. Effect of testosterone, estradiol and ethanol on binding of polysomes to membranes.
Sephadex G-25 treated post-mitochondrial fraction (2mg protein) was incubated with [^{14}C]orotic acid labeled free polysomes (0.72 OD260nm unit) for 30min at 23°C in 0.25 ml, (A); without any addition, (B); with 10µl of testosterone (2mg/ml of ethanol), (C); with 10µl of estradiol (2mg/ml of ethanol), (D); with 10µl of ethanol. After incubation, each reaction mixture was fixed with formaldehyde and centrifuged on a CsCl-sucrose gradient. Each 18 drop fraction was collected and acid insoluble radioactivity was counted. The percentage of radioactivity in membrane-bound fraction was calculated and shown in the middle of each panel.

The effect of testosterone and estradiol on binding of ^{14}C-free polysomes to membranes in vitro is shown in Fig. 2. Since the steroids are dissolved in ethanol, the effect of ethanol alone was also examined. The recovery of polysomal radioactivity added to the reaction mixture was complete during the analysis. Of the total radioactivity, 15% (no addition, Fig. 2-A) or 13% (plus estradiol, Fig. 2-C) are found in the membrane-bound polysomal fraction (5th to 7th). In Fig. 2-B, stimulation of the binding by testosterone is shown, that is, 29% of the added radioactivity is found in the membrane fraction. But as shown in Fig. 2-D, ethanol alone stimulates the binding as much as testosterone. It should be mentioned here that formaldehyde fixation, which is essential to protect polysomes from degradation in high CsCl solution, does not introduce artificial binding of polysomes with membrane, since in zero time control samples, the radioactivity in the membrane-bound polysomal fraction was very low. From Fig. 2, it may be concluded that in this female system, ethanol enhances the binding capacity of membranes in the cytoplasmic extract and that estradiol inhibits this sti-

mulation while testosterone does not. In this experiment, the amount of RNA in the post-mitochondrial fraction and of radioactive RNA was about 100μg and 35μg per reaction tube respectively.

To determine the effect of phenobarbital, the post-mitochondrial fractions were prepared from normal and phenobarbital treated rat livers and incubated with ^{14}C-free polysomes. In these experiments, the final concentration of ethanol in the reaction mixture was reduced to 1.2% from 4% in the experiment shown in Fig. 2. (Later, we found that even 0.2% of ethanol stimulated the binding). When the cytoplasmic extract was prepared from the livers of phenobarbital treated rats, the binding capacity is much higher (Fig. 4) than that observed in the case of a normal liver extract

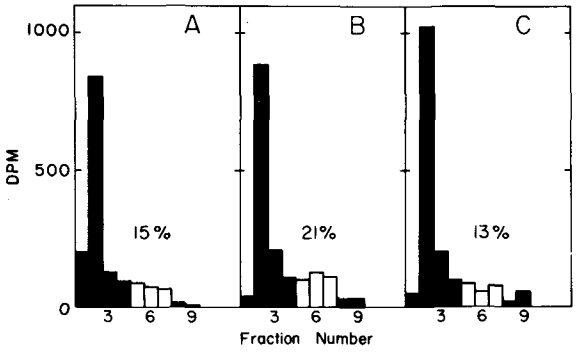

Fig. 3. Binding of polysomes to membranes in normal rat liver system. Sephadex G-25 treated post-mitochondrial fraction (2mg protein) from a normal rat liver was incubated with ^{14}Clorotic acid labeled free polysomes (0.66 OD260nm unit) for 90min, (A); without any addition, (B); with 3μl of testosterone (5mg/ml of ethanol), (C); with 3μl of estradiol (5mg/ml of ethanol). For the analysis of the reaction, see the legend of Fig. 2.

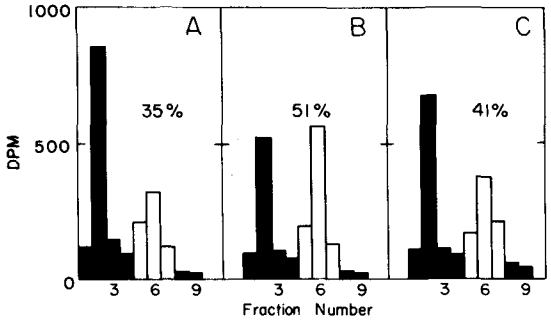

Fig. 4. Binding of polysomes to membranes in phenobarbital treated rat liver system.
Sephadex G-25 treated post-mitochondrial fraction (2mg protein) was prepared from a phenobarbital-treated rat liver. For the other experimental condition, see the legend of Fig. 3.

(Fig. 3) and stimulation by ethanol (testosterone) and its inhibition by estradiol are also observed. The enhancement of binding capacity in the post-mitochondrial fraction from phenobarbital treated rat liver might be due to both quantitative increase of polysome-accessible membranes and to qualitative change of membranes. To assess the latter possibility, smooth membranes were isolated and incubated with ^{14}C-free polysomes. As shown in Fig. 5, the smooth membrane fraction purified from a phenobarbital treated

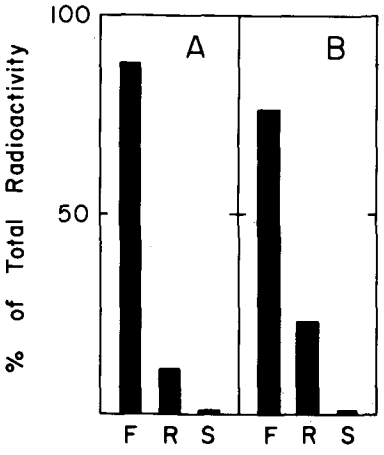

Fig. 5. Binding of polysomes to purified smooth membrane fraction prepared from a normal and phenobarbital-treated rat liver.
Purified smooth membrane fraction (0.2mg protein) from a normal (A) or a phenobarbital-treated rat liver (B) was incubated with [^{14}C]orotic acid labeled free polysomes (0.76 OD260nm unit) for 50 min. For the other experimental condition, see the legend of Fig. 3. The result of CsCl gradient analysis is summarized showing % distribution of the total radioactivity in the 1st 4th of 9 fractions (F=free polysomes), the 5th to 7th fractions (R=rough ER) and the top two fractions (S=smooth ER).

rat liver has a higher binding capacity than that from normal rat liver.

In contrast to the effect of phenobarbital, regeneration caused a reduction in binding capacity (Fig. 6-A) and stimulation by ethanol (testosterone) was not detected (Fig. 6-B) suggesting qualitative change of binding capacity in membranes of regenerating liver.

DISCUSSION

Among several *in vitro* binding system reported so far (8-13), that of Rabin's group (8-9) is characterized by the fact that sex steroid hormones appear to influence the binding efficiency, that is, activation of isolated membranes depends on estradiol in the male and on testosterone in the female. The method used by the group as an indicator of binding of poly-

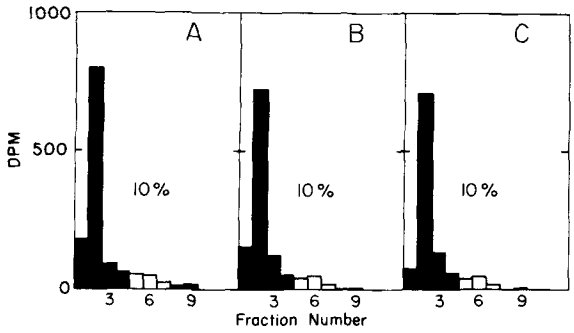

Fig. 6. Binding of polysomes to membranes in regenerating rat liver system. Sephadex G-25 treated post-mitochondrial fraction (2mg protein) was prepared from a regenerating liver. For the other experimental condition, see the legend of Fig. 3.

somes to membranes is an assay of disulfide interchange enzyme activity. It is stated that the polysome-membrane complex formed is unstable on ultracentrifugation and therefore cannot be detected directly (9). This indirect measurement of the formation of the complex makes it difficult to compare the results with those of other groups. In the studies reported here, we fixed polysome-membrane complexes with formaldehyde to stablize these structure. They were then separated by CsCl gradient centrifugation. Using this system, it was found that ethanol alone stimulated the binding of polysomes to membranes. It is unclear whether the inhibitory effect of estradiol on the stimulation of the binding by ethanol has any correlation with results reported by Rabin's group. A single experiment with male rat liver cell extract showed that testosterone did not inhibit the stimulation of the binding by ethanol. It has been shown that nascent chains on polysomes bound to membranes in vivo are released into a deoxycholate-soluble fraction of membranes when incubated with puromycin (14,15). We have found that nascent chains on polysomes bound to membranes in our system are also released into deoxycholate-soluble fraction of membranes when incubated in the presence of a protein synthesizing system (a manuscript in preparation).

In an extension of our attempts to influence binding capacity of membranes by physiological measures, we have shown that phenobarbital treatment increases, while liver regeneration decreases qualitatively the polysome-binding capacity of the cytoplasmic extract. Relative increase in the quantity of membrane-bound polysomes in phenobarbital treated rat liver cells (16) and relative increase of the quantity of free polysomes in regenerating liver cells (17) observed in vivo may be related to the qualitative change of membranes found in the in vitro binding experiments reported here.

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