INTRACELLULAR SITE OF PROLACTIN SYNTHESIS IN RAT PITUITARY CELLS IN CULTURE

Debajit K. Biswas and Armen H. Tashjian, Jr.

Laboratory of Pharmacology, Harvard School of Dental Medicine

and

Department of Pharmacology, Harvard Medical School, Boston, Mass. 02115

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SUMMARY

Free and membrane-bound polyribosomes were isolated from control and thyrotropin releasing hormone-treated GH_3 cells. The two polysome fractions were used to direct {^{3}H}leucine incorporation into prolactin in both heterologous and homologous cell-free protein-synthesizing systems. Prolactin was measured by immunoprecipitation and SDS-disc gel electrophoresis of the reaction products. Only membrane-bound polysomes directed incorporation of {^{3}H}leucine into labeled prolactin. In additon, intact cells were pulse-labeled with {^{3}H}leucine, free and membrane-bound polysomes were isolated, and newly synthesized prolactin associated with each polysome fraction was measured. In control cells, {^{3}H}prolactin represented about 0.4 and 4.2% of total acid-insoluble radioactivity in free and membrane-bound polysomes, respectively; whereas, in thyrotropin releasing hormone-treated cells, these values were about 1 and 20%, respectively. Added {^{3}H}prolactin did not associate nonspecifically with membrane-bound polysomes. We conclude that prolactin is synthesized predominantly on membrane-bound polysomes in GH_3 cells.

INTRODUCTION

The GH_3 strain of rat pituitary tumor cells produces two protein hormones, prolactin (PRL) and growth hormone (GH) (1,2). These cells respond to a variety of physiological stimuli (3,4). Thyrotropin releasing hormone (TRH) stimulates markedly (2- to 5-fold) the synthesis of prolactin in GH_3 cells (3). Dibutyryl cyclic AMP (db-cAMP) also stimulates prolactin production by these cells (4).

It is presumed that the protein hormones of the anterior pituitary gland are synthesized on membrane-bound polyribosomes, like many other secretory proteins (5,6), but there is no direct evidence for this hypothesis in the

case of PRL. In this report, evidence is presented that leads us to conclude that, in hormone-producing cells in culture, PRL is synthesized on membrane-bound polysomes. In a substrain of GH cells, Bancroft has reported that newly synthesized GH is membrane-associated (7).

METHODS AND MATERIALS

Isolation of polysomes from GH_3 cells. Cells were grown in suspension culture to a density of approximately 1 x 10^6/ml. All subsequent operations were performed at 2-4°C. Cells (200-500 ml) were harvested, washed 3 times with TBS buffer (20 mM Tris-HCl, pH 7.5; 146 mM NaCl; 11 mM sucrose), and resuspended in twice the packed cell volume of hypotonic buffer (10 mM Tris-HCl, pH 7.5; 1.5 mM $Mg(CH_3COO)_2$; 6 mM 2-mercaptoethanol; 15 mM KCl) for 10 min. The suspension was then homogenized by 10 strokes in a Dounce homogenizer with a loose-fitting pestle. HEPES buffer (200 mM HEPES, pH 7.5; 850 mM KCl; 30 mM $Mg(CH_3COO)_2$; 60 mM 2-mercaptoethanol) was added (one tenth the volume of the homogenate) to the suspension, and the mixture centrifuged at 500 x g. The supernatant solution was saved and the pellet resuspended in 2 volumes of hypotonic buffer, homogenized and recentrifuged. The two supernatants were pooled and centrifuged at 21,000 x g for 10 min. Polyribosomes in the 21,000 g supernatant (designated "free polysomes") were isolated by sedimentation at 105,000 x g for 5 hr through 50% sucrose. The pellet was dissolved in polysome-buffer (20 mM Tris-HCl, pH 7.5; 3.5 mM $Mg(CH_3COO)_2$; 100 mM KCl; 6 mM 2-mercaptoethanol).

The pellet from the 21,000 x g centrifugation (containing membrane-bound polyribosomes) was suspended in polysome-buffer, and the nonionic detergent, Nonidet P-40 (Shell Oil Co.), was added to a final concentration of 0.5%. The mixture was centrifuged at 21,000 x g for 10 min and membrane-bound polyribosomes released into the supernatant were concentrated by centrifugation at 105,000 x g for 5 hr. The pellet was resuspended in polysome buffer and this fraction was designated "membrane-bound polysomes." Ribosomal content of these preparations was estimated spectrophotometrically by assuming that preparations having an A_{260}/A_{280} ratio of 1.8 contained 1.0 mg of ribosomes when the A_{260} was 11.0 (8).

Measurement of cell-free prolactin synthesis. Incorporation of {^{3}H}leucine into PRL directed by free or membrane-bound polysomes was measured in both Krebs-II ascites cell-free extracts (9) and in a cell-free system prepared from GH_3 cells. The incubation mixtures, in a final volume of 50 µl, contained the following: 15 µl of either Krebs-II extract or a pH 5 enzyme preparation from GH_3 cells (10), 1.5 mM ATP, 0.5 mM GTP, 10 mM creatine phosphate, 20 µg/ml creatine kinase, 20 mM Tris-HCl, pH 7.4, 100 mM KCl, 3.5 mM $Mg(CH_3COO)_2$, 40 µM each of 19 nonradioactive amino acids, 4 µM {^{3}H}leucine (5 Ci/mmole), and 40-60 µg of either free or membrane-bound polysomes. Incorporation of {^{3}H}leucine into hot trichloroacetic acid insoluble material was linear for incubations of up to 60 min duration at 37°C. Background radioactivity was estimated by TCA precipitation of zero-time samples; such precipitates contained 10-20% of the counts incorporated after 60 min. {^{3}H}PRL was separated from other radioactive proteins by immunoprecipitation of the reaction mixture with PRL-specific antibodies, dissociation of the {^{3}H}PRL-antiPRL complex in SDS buffer and disc gel electrophoresis as described by Dannies and Tashjian (11).

Measurement of prolactin synthesis in intact cells. Control and TRH-treated (3 days, 28 nM) GH_3 cells (2 x 10^8 cells) were harvested, washed and resuspended in 20 ml of pre-equilibrated leucine-free Eagle's minimal essential medium containing 10% dialysed fetal calf serum. Following incubation for 30 min at 37°C in 5% CO_2 and 95% air, the cells were exposed to {^{3}H}leucine (50 µCi/ml) for 2 or 5 min. The cells were then poured onto frozen TBS buffer, washed and free and membrane-bound polysomes were prepared as described above. Total hot TCA insoluble radioactivity in each of these fractions was determined as was the incorporation into {^{3}H}PRL.

RESULTS

Cell-free incorporation of {^{3}H}leucine into acid insoluble material directed by free and membrane-bound polysomes from control GH_3 cells was approximately the same in 3 experiments (Table 1). Treatment with TRH or

Table 1

INCORPORATION OF {^{3}H}LEUCINE INTO ACID-INSOLUBLE MATERIAL IN CELL-FREE SYSTEMS DIRECTED BY POLYSOMES PREPARED FROM CONTROL AND TRH-TREATED CELLS

Expt. No.	Source of Polysomes	Translation System	{^{3}H}leucine Incorporation (cpm/100 µg ribosomes)	
			Free	Bound
1	control	Krebs	1780	1785
	TRH	Krebs	1744	4272
2	control	Krebs	1200	1220
	TRH + db-cAMP	Krebs	2100	7550
3	control	GH-cell	2600	1900
	TRH	GH-cell	1718	5030

Free and membrane-bound (bound) polysomes were isolated from control or treated cells as described under Methods. Cells were treated with TRH (28nM) or TRH plus db-cAMP (1mM) for 3 days before harvesting. At the end of the 60-min incubation, 2 ml of 10% TCA was added and the suspension heated at 85°C for 20 min. Samples were cooled, the precipitates were collected on GFC filters, washed 3 times with TCA and then with 95% ethanol. Filters were dried and radioactivity determined in Bray's solution at an efficiency 35%. Each value is the mean of duplicate determinations. Zero-time controls with each series of experiments gave values of 150-200 cpm. Values obtained with Krebs extracts alone (in the absence of added polysomes) were 500-600 cpm and with GH-cell pH 5 extracts were 500-700 cpm at the end of the 60-min incubation.

TRH plus db-cAMP did not consistently affect the incorporation directed by free polysomes; however, the incorporation directed by membrane-bound polysomes isolated from treated cells was substantially higher in each experiment (Table 1).

Incorporation of {^{3}H}leucine into PRL directed by free and membrane-bound polysomes in the Krebs cell-free system is shown in Fig. 1. No radioactive peak in the region of PRL was observed when free polysomes from control or TRH-treated cells were used (Fig. 1, bottom). However, a

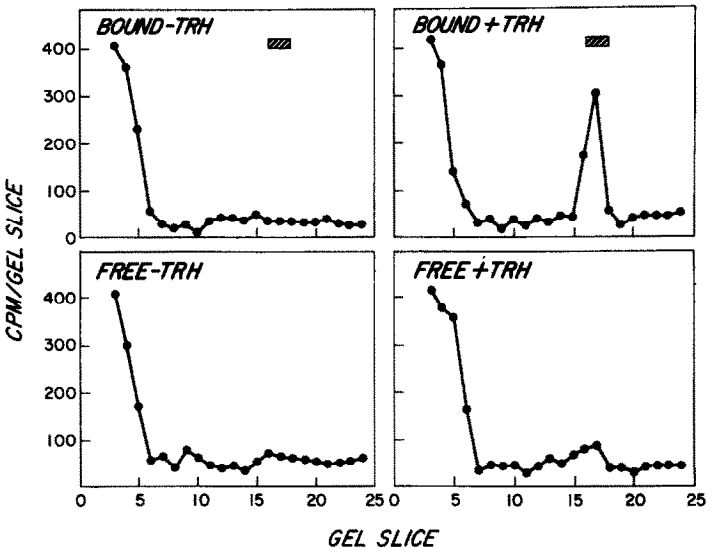

Fig. 1. Identification of {^{3}H}PRL synthesized in the Krebs cell-free system as directed by free or membrane-bound (bound) polysomes isolated from control (-TRH) or TRH-treated (+TRH) cells. Treatment with TRH (28mM) was for 3 days. At the end of the cell-free incubation an aliquot was taken for determination of total, hot acid-precipitable radioactivity and immunoprecipitation. The figure shows the patterns of radioactivity of immunoprecipitates electrophoresed on SDS gel. Each point is the mean of duplicate gels. The dashed bars at the top of the upper panels indicate the position of standard rat PRL which was run in separate gels and identified by staining with amido black.

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distinct radioactive peak, representing about 13% of the total TCA insoluble radioactivity in anequivalent volume of incubation mixture, was seen with membrane-bound polysomes from TRH-treated cells (Fig. 1, top). No peak was observed with membrane-bound polysomes from control cells

which produced only 20% as much PRL as treated cells. The results obtained with polysomes from TRH-treated cells were consistent with the hypothesis that PRL is synthesized predominantly on membrane-bound polysomes. These findings derived from cell-free protein synthesis experiments were extended by studies with intact cells.

Results of two such experiments are presented in Table 2. In the first

Table 2

NEWLY SYNTHESIZED {^{3}H}PROLACTIN ASSOCIATED WITH FREE AND MEMBRANE-BOUND POLYSOMES ISOLATED FROM LABELED INTACT GH_3 CELLS

Expt. No.	Type of Polysome	Treatment	Incubation with {^{3}H}leucine (min)	{^{3}H}leucine Incorporation (cpm x 10^{-3}) Total	Prolactin	% {^{3}H}leucine in PRL
1	Free	Control	5	16	0.069	0.43
	Bound	Control	5	6	0.254	4.2
	Free	TRH	5	15	0.116	0.77
	Bound	TRH	5	7	1.400	19.5
2	Free	TRH	2	12	0.130	1.1
	Free	TRH	5	17	0.250	1.5
	Bound	TRH	2	3.3	0.790	24
	Bound	TRH	5	5.5	1.380	25

Control or TRH-treated (28 nM for 3 days) GH_3 cells were pulse-labeled with {^{3}H}leucine for 2 or 5 min as described under Methods. Free and membrane-bound polysomes were isolated and total acid precipitable radioactivity was determined in duplicate 200 µl aliquots. {^{3}H}PRL was determined in duplicate 200 µl aliquots by immunoprecipitation and disc gel electrophoresis. Mean values are given.

experiment, it is seen that newly synthesized {^{3}H}PRL is associated predominantly with membrane-bound polysomes in both control and TRH-treated cells. In the second experiment, using only TRH-treated cells, the as-

sociation of {^{3}H}PRL with bound-polysomes is again noted after {^{3}H}leucine pulses of both 2 and 5 min. Fig. 2 shows the SDS-disc gel electrophoresis

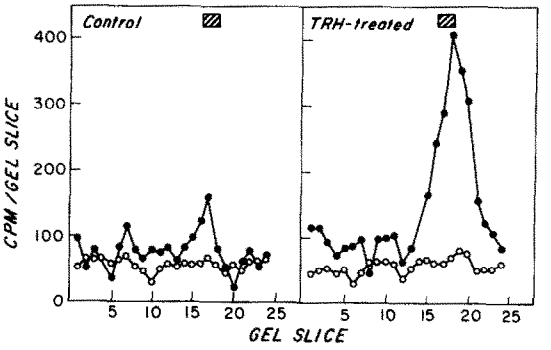

Fig. 2 Identification of {^{3}H}PRL synthesized by intact GH_3 cells. After a 5-min pulse with {^{3}H}leucine, {^{3}H}PRL associated with free (o-o) and membrane-bound (•-•) ribosomes was identified as described in Methods. SDS gel patterns of immunoprecipitates from TRH-treated and control cells are shown. The bars at the top of each gel pattern give the position of the rat PRL standard.

patterns of immunoprecipitates prepared from free and membrane-bound polysomes isolated from both control and TRH-treated cells.

It could be argued that the association of {^{3}H}PRL with membrane-bound polysomes does not represent synthesis of PRL on these structures but merely reflects nonspecific binding of {^{3}H} PRL with membrane-bound polysomes during isolation. In order to test this possibility, 32,000 cpm {^{3}H}PRL (10^4cpm/μg, isolated and purified by Dr. T.F.J. Martin) were incubated with the unlabeled 21,000 x g pellet (containing approximately 1 mg of membrane-bound polysomes) at 37°C for 10 min. The mixture was then treated with Nonidet P-40 and released, membrane-bound polysomes were isolated as described in Methods. Less than 0.1 pmole of {^{3}H}PRL was associated with 100 pmoles of ribosomes (less than 1% of the {^{3}H}PRL added). Thus, we conclude that the {^{3}H}PRL associated with membrane-bound polysomes in both cell-free and intact cell experiments was not due to nonspecific binding of {^{3}H}PRL with these structures.

DISCUSSION

The results of this investigation lead us to conclude that in GH_3 cells PRL is synthesized on membrane-bound polyribosomes. In the related strain of GC cells, Bancroft has shown that 86% of newly synthesized GH is also membrane-associated (7). Because GH_3 cells synthesize both PRL and GH it was important to show that the method used by us to measure newly synthesized PRL was specific for PRL and did not measure GH as well. The antiserum used in the immunoprecipitation experiments did not fix complement with rat GH (2). In addition, ^{125}I-labeled rat GH was incubated with anti-PRL under the conditions of PRL immunoprecipitation, dissociation and identification on disc gel electrophoresis, and no radioactivity was detected in the gel in the position of either GH or PRL.

The conclusion that PRL is synthesized on membrane-bound polysomes was drawn from experiments in both cell-free systems (Fig. 1) and in intact cells (Table 2 and Fig. 2). The results in Table 2 show that, in TRH-treated cells, about 20-25% of the acid insoluble material synthesized on membrane-bound polysomes was PRL whereas only 0.4-1% was PRL in free polysomes. The small amount of {^{3}H}PRL in the free polysome fraction may well represent contamination from membrane-bound polysomes that occurs during the isolation procedure.

It is noted that the peaks of radioactivity in the PRL regions of the gels is broader in experiments in which labeling is performed with intact cells (Fig. 2) as compared to results obtained in cell-free systems (Fig. 1). This finding may represent greater heterogeneity in the size of nascent PRL chains in the intact cell experiments, or possibly the association of tRNAs with nascent chains and a consequent alteration in electrophoretic mobility.

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