CHEMICAL EVIDENCE FOR ASSOCIATED TRF WITH SUBCELLULAR FRACTIONS AFTER INCUBATION OF INTACT RAT PROLACTIN CELLS (GH3) WITH ^{3}H-LABELLED TRF

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1. Introduction

The GH3 cell line is a rat pituitary strain [1] which secretes both prolactin and growth hormone [2, 3] and is responsive to the synthetic Thyrotropin Releasing Factor (TRF) (Pyro–Glu–His–Pro–NH_2). TRF increases the prolactin production [4, 5] and modifies both the morphological aspect and the ultrastructural organization of the cells [5].

Previous analysis of the interaction of the ^{3}H-labelled TRF [6] with the GH3 cells [7, 8] allowed establishing several characteristics of the HG3 cells binding sites for TRF. The binding is time dependent, increasing linearly in the first 15 min and thereafter reaching a plateau. This kinetic is closely similar to that of the TRF-induced increase of prolactin release. The binding is highly specific of GH3 cells and of TRF: i) Two other cell lines (3T3 fibroblasts and C6 glial cells) showed a negligible affinity for ^{3}H-labelled TRF; ii) several peptides (Pyro–Glu–His, oxytocin and the synthetic decapeptide LH–RH) did not compete with ^{3}H-labelled TRF for binding to GH3 cells. The number of bound molecules per cell is dose dependent. It increases steeply from 1.35 to 155 nM. Over this concentration and up to 1080 nM, there is no true saturation but a constant low increase. As calculated from Scatchard plot at least two components account for this phenomenon. One with a strong affinity corresponds to the range of biologically active doses (affinity constant: $K_A : 8.5 \cdot 10^{-7}$). The other component with a low affinity is observed for high unphysiological doses ($K_A : 13.3 \cdot 10^{-8}$). The presence of dual types of binding on pituitary thyrotropic cells which recognize TRF has been also recently shown [9].

Moreover, autoradiographic study of GH3 cells previously incubated for 30 min with ^{3}H-labelled TRF revealed the presence of radioactive material within the cells but no concentration on the plasma membrane [7, 8]. A similar localization has been found by dry mount autoradiography in pituitary cells of rat sacrified 1 hr after the injection of ^{3}H-labelled TRF [10]. Such findings asked for an analysis of the nature of the intracellular radioactive material. For this purpose, GH3 cells preincubated with ^{3}H-labelled TRF have been fractionated. Two subfractions, identified by electron microscopy, have been obtained and their radioactive material isolated and analyzed. The results have been compared to those obtained from GH3 homogenate or subcellular fractions incubated with ^{3}H-labelled TRF.

2. Material and methods

2.1. Incubation procedure

Before the experiments, GH3 cells were grown for a week in Roux flasks in HAM F10 medium supple-

mented with 15% horse serum and 2.5% foetal calf serum. The cells were scrapped, collected, washed and then incubated for 20–30 min (10^7 cells/ml–$1–2 \cdot 10^8$ cells/exp.) at 37°C in constant agitation (Gilson omnibain) in regular culture medium supplemented with ^{3}H-labelled TRF (from 200 to 270 nM, 50 ± 5 Ci/mM). As control experiments, GH3 cells and 3T3 fibroblasts have been similarly incubated with ^{3}H-labelled TRF, but after being homogeneized or fractionated as described below.

2.2. Homogenization and fractionation procedures

Following incubation with ^{3}H-labelled TRF, the GH3 cells were washed and centrifuged 6 times in ice cold Earle solution (800 *g*, 3 min, 4°C) (no significant radioactivity was detected in the third washing). Unincubated cells were only washed and centrifuged twice. The homogenization was performed in a manual glass grinder in 5 mM Tris–HCl buffer, 5 mM $MgCl_2$ at pH 7.4.

For subsequent fractionation, this homogenate was supplemented with sucrose (0.25 M) and thereafter centrifuged (1200 *g*, 2–3 hr, 4°C) on a 0.33 M sucrose layer. The resulting pellet which comprises the nuclear fraction was washed 3 times in Earle solution and collected by centrifugation (1200 *g*, 15 min, 4°C) (fraction I). The supernatant was collected and recentrifuged (90000 *g*, 2 hr, 4°C, Rotor Beckman SW 25-1) after lowering the sucrose molarity from 0.25 to 0.1 N. This allowed to recover a pellet which contained ribosomes and other cell organelles as well as the plasma membranes. It is washed and centrifuged in ice-cold Earle solution in the same condition of centrifugation (fraction II).

For control experiments, the GH3 subcellular fractions after their incubation with ^{3}H-labelled TRF were washed and collected as described for their isolation (see above). Homogenates of GH3 and 3T3 cells were either washed 3 times (and collected by centrifugation at 45000 *g*, 30 min, 4°C) or 2 times (and collected by centrifugation at 90000 *g*, 90 min, 4°C).

2.3. Chemical analysis of the radioactive material

Fractions I and II as well as intact cells preincubated with ^{3}H-labelled TRF were submitted to ultrasonic desintegration (10 sec, 45 W, 20 kHz at 4°C in a Sonimasse apparatus T50) in 0.75 ml of distilled water. Approximately 2 μM of unlabelled TRF as carrier were added to those fractions as well as to the cytosol. Thereafter, methanol was added (v/v), the total suspension centrifuged (1500 *g*, 3 min) and filtered on fiber-glass Millipore filters. Each filtrate was lyophilized and rediluted in 10 μl of distilled water. Four extracts were thus obtained, corresponding respectively to the intact cells and to fractions I and II. Thin layer electrophoresis (Selecta G 14.40, 310 V, 12 mA, 210 min, 4°C, E: 15 V/cm in pyridine acetate buffer 0.17 M, pH 4.7) were simultaneously carried out with aliquots of 4 types of extracts as well as with Des–NH_2–TRF, Pyro–Glu–His–OH and histidine. The total radioactivity deposited for one electrophoresis varied from 3000 to 5000 cpm. Peptides and amino acids were localized with Pauly's reagent. The distribution of radioactivity along the thin layer was scanned by scraping off and counting the gel every centimeter.

2.4. Radioactivity determination

The radioactivity was estimated with an Intertechnique SL 30 scintillation spectrometer. Two different mixtures were used as scintillation media: one for cells, cell homogenates, subcellular fractions and media, previously dissolved in 0.2 to 0.5 ml of Soluene Packard, (Toluene: 1 litre, POPOP: 0.236 g, PPO: 5 g and Triton-X-100: 352 ml) and the other for thin layers (for one liter: naphthalene: 80 g, POPOP: 0.5 g, PPO: 4 g, Xylene: 5 vol, Dioxane: 5 vol, Ethanol: 3 vol).

3. Results

After incubation and washings the cells retained about 1% of the medium radioactivity. The repartition of the radioactivity within the subcellular fractions in relation with their protein content is reported on table 1. Mean results obtained from the four experiments showed that 17.5 ± 1.8% of the total cell radioactivity was bound to the fraction I (nuclear pellet) and 18.7 ± 6.8% was bound to the fraction II (ribosomes, organelles and plasma membranes). The cytosol contained 63.5 ± 6% of the total radioactivity of the homogenate. The radioactivity recovered in extracts I and II represents 85–90% of the initial radioactivity of fractions I and II. The electrophoresis revealed that in frac-

Table 1

Repartition of the radioactivity within subcellular fractions of GH3 cells previously incubated for 30 min at 37°C with 200 nM ^{3}H-labelled TRF (50 ± 5 Ci/mM).

Expt.	Nature of the fractions	Total dpm	Bound ^{3}H-labelled TRF fM/fraction	Total proteins mg	dpm/mg prot.	^{3}H-labelled TRF fM/mg prot.	Distribution of radioactivity % per cell**
A	Intact cells	1540000	13880	23.6	65300	588	
	Nuclear	72400	650	2.56	28300	255	17
	Organites	56100	510	0.34	165000	1486	34
	Cytosol	81100	730	0.40	203000	1829	49
B	Intact cells	1896000	17080	23.9	79300	715	
	Nuclear	64200	580	2.78	23100	208	13
	Organites*	42200	380				26
	Cytosol*	51100	460				61
C	Intact cells	1605000	14460	22.3	72000	649	
	Nuclear	112900	1020	2.25	50200	452	22
	Organites	20000	180	0.19	105300	949	10
	Cytosol*	142000	1280				68
D	Intact cells*	2339000	21070				
	Nuclear*	343700	3100				18
	Organites*	468000	4220				33
	Cytosol*	691100	6230				49

* Material which has not been assayed for protein content, because it has been used either for electron microscopic control or for biochemical identification of radioactivity.

** To account for variable yield of fractionation, the % distribution of radioactivity has been calculated on the basis of the number of nuclei finally recovered.

tions I and II as well as in the total homogenate 90–95% of the radioactivity was associated with the TRF carrier spot. No significant radioactivity was detected at the level of the other peptide or amino acid spots (Des–NH_2–TRF, Pyro–Glu–His–OH and histidine).

Results of the experiments in which GH3 cells and 3T3 were homogenized before incubation with ^{3}H-labelled TRF are reported in table 2. The GH3 cells homogenate pellet displays a higher affinity (about 10 times more) for TRF than 3T3 cells homogenate.

Table 2

Radioactivity associated to GH3 cell homogenate or subcellular fractions after their incubation for 30 min at 37°C with 200 nM ^{3}H-labelled TRF (50 ± 5 Ci/mM). 3T3 fibroblast homogenate was used as control.

	Nature of fractions	Total dpm	^{3}H-labelled TRF fM fraction	Total proteins mg	dpm/mg prot.	^{3}H-labelled TRF fM/mg prot.	dpm/10^6 cell
GH3	Intact cells	816000	7350	11.99	68000	613	13600
	Homogenate pellet	40300	360	2.88	14000	126	
GH3	Intact cells	663300	5970	16.39	40500	365	10400
	Nuclear	36200	330	1.17	31000	279	
	Organites	25490	230	1.25	20400	184	
3T3	Intact cells	112000	1010	20.300	5500	49	700
	Homogenate pellet	12500	110	5.900	2100	19	

Similarly the 'nuclei + organites' radioactivity is also higher than that of the 3T3 cells homogenate. In addition the radioactivity of the organites fraction is lowered when the fractionation is performed before the binding. By contrast, the affinity of the nuclear pellet does not seem to be modified whether the fractionation was performed before or after the binding.

4. Discussion

These results demonstrate the existence of chemically unmodified TRF within the GH3 cell at the level of nucleus cytoplasmic organelles and cytosol, after binding of tritiated TRF by intact cells. As it is generally admitted that peptide hormone receptors are localized on plasma membrane, it could be objected that the present results might correspond to a displacement of TRF during the homogenization and fractionation procedures. This possibility however may be ruled out since:

1) as previously shown, cells incubated with ^{3}H-labelled TRF and extensively washed at 4°C retain at this temperature their radioactivity for at least 6 hr [8]. By its stability the binding of ^{3}H-labelled TRF by GH3 cells strongly contrasts with that previously observed for pituitary plasma membrane preparations which is rapidly dissociated at 4°C [11].

2) Previous autoradiographic studies of whole cells submitted to the same treatment showed the presence of radioactive material on the nucleus and cytoplasm without concentration on the plasma membrane. Since 95% of the radioactivity of the whole homogenate as well as of that of fractions I and II consist of TRF, it may be concluded that TRF enters the cells without being chemically modified.

Besides, the fact that GH3 cells homogenate are also able to bind ^{3}H-labelled TRF to higher level than 3T3 fibroblast homogenate suggests that the binding observed for GH3 subfractions corresponds to a specific rather than unspecific phenomenon. The decreased affinity of the homogenate pellet and the 'organite' fractions when isolated before the binding, might be related to an alteration of the binding mechanism by the homogenization. In connection with this point, the nuclei which remain intact after the fractionation retain their affinity for ^{3}H-labelled TRF (tables 1 and 2).

Different results have been reported from similar biochemical analyses of rat pituitary gland taken 1 hr after a TRF intravenous injection [12]. In this study only 60% of the tissue radioactivity originated from TRF and/or Glu–His–Pro–OH. The discrepancy with our results might be due to the different experimental conditions. As far as the intracellular binding of TRF is concerned, Poirier et al. [13] studying the binding of ^{3}H-labelled TRF by several subfractions of bovine pituitary mentioned that, besides the 'pure plasma membrane' fraction, the nucleus and the 'total microsomes' are also able to bind TRF to a lower but unnegligible level. In their opinion this binding would however represent a minor component.

Very recently, Grant et al. [9] reported that no significant intracellular uptake of ^{3}H-labelled TRF has been detected during the first hour of incubation at 37°C of mice tumor pituitary thyrotropic cells (<10.0 pg/10^6 cells). They did not precise their experimental conditions on that point.

In contrasts, our results support the hypothesis that in addition to its possible interaction with a plasma membrane receptor [11, 14] TRF enters the cell and is then tightly bound to some intracellular sites or molecules. The eventual physiological significance of these intracellular events will be examined further.

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References

[1] Yasumura, Y., Tashjian, Jr., A.H. and Sato, G. (1966) Sciences 154, 1186.
[2] Tashjian, Jr., A.H., Yasumura, Y., Levine, L., Sato, G. and Parker, M.L. (1968) Endocrinology 82, 342.
[3] Tashjian, Jr., A.H., Bancroft, F.C. and Levine, L. (1970) J. Cell. Biol. 47, 61.
[4] Tashjian, Jr., A.H., Barowsky, N.J. and Jensen, D.K. (1971) Biochem. Biophys. Res. Commun. 43, 516.
[5] Gourdji, D., Tixier-Vidal, A. and Kerdelhue, B. (1972) Compt. Rend. 274D, 437.

[6] Pradelles, Ph., Morgat, J.L., Fromageot, P., Oliver, C., Jacquet, P., Gourdji, D. and Tixier-Vidal, A. (1972) FEBS Letters 22, 19.
[7] Gourdji, D., Tixier-Vidal, A., Morin, A., Morgat, J.L., Pradelles, Ph. and Fromageot, P. (1972) Lille Medical 17, 1402 abst.
[8] Gourdji, D., Tixier-Vidal, A., Morin, A., Pradelles, Ph., Morgat, J.L., Fromageot, P. and Kerdelhue, B. Exptl. Cell Res., in press.
[9] Grant, G., Vale, W. and Guillemin, R. (1973) Endocrinology 92, 1629.
[10] Stumpf, W.E. and Sar, M. (1973) Fed. Proc. (abst.), in press.
[11] Grant, G., Vale, W. and Guillemin, R. (1971) Biochem. Biophys. Res. Commun. 46, 28.
[12] Redding, T.W. and Schally, A.V. (1971) Endocrinology 89, 1075.
[13] Poirier, G., Labbrie, F., Barden, N. and Lemaire, S. (1972) FEBS Letters 20, 283.
[14] Labbrie, F., Barden, N., Poirier, G. and De Lean, A. (1972) Proc. Natl. Acad. Sci. U.S. 69, 283.