SYNTHESIS OF FERRITIN IN CULTURED HEPATOMA CELLS

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1. Introduction

Ferritin is the major intracellular site for iron storage [1]. It has many useful properties and advantages, despite the fact that it is not an enzyme, for studying the regulation of protein synthesis and turnover in mammalian tissues [2–4].

In the present report the synthesis of ferritin has been studied in a cell line of a hepatoma tissue culture called 'HTC cells'. These cells were derived from a malignant tumor of rat liver parenchymal cells (Morris hepatoma 7288 C) and adapted to growth in cell culture by Thompson et al. [5]. The outstanding characteristic of HTC cells is their response to the adrenal steroids. The synthesis of ferritin has been studied in suspension cultures of HTC cells as well as in cell free extracts incubated in vitro by measuring the incorporation of [^{3}H]leucine into proteins. The labelled ferritin was isolated by antibody precipitation after a preliminary purification in the presence of carrier rat liver ferritin. Apoferritin was synthesized to the same rate by the whole cells and by the cell free extracts but only a slight increase in ferritin protein concentration following iron treatment was observed. No measurable effect of glucocorticoids was observed on ferritin synthesis. Thus, it becomes interesting to retain ferritin as an internal control protein in the study of hormonal induction of tyrosine aminotransferase (TAT) in HTC cells.

2. Material and methods

2.1. *Preparation of cell extracts*

HTC cells were grown at 37°C in suspension cultures in Swim's 77 medium supplemented with 10% calf serum [6]. For steroid induction, dexamethasone was added to a final concentration of 1 μM. For labelling experiments in whole cells, induced and uninduced HTC cells were exposed for 45 min to L-[^{3}H]leucine (1 mCi/1; specific activity, 40 Ci/mM; CEA, Saclay). Cells were collected and homogenized as previously described [7] to obtain the 125 000 *g* supernatant fractions.

2.2. *Cell free protein synthesis*

'S 20' fractions were obtained containing about 20 mg of protein per ml [7]. Cell free protein synthesis was done in volumes of 10 ml containing about 6 mg of S 20 protein per ml, 1 mM ATP, 0.6 mM GTP, 10 mM phosphoenolpyruvate, 30 μg pyruvate kinase per ml, 4.8 mM $MgCl_2$, 75 mM KCl, 22 mM Tris–HCl (pH 7.2) and 2 mCi of L-[^{3}H]leucine. Incubations were performed at 37°C for 45 min, after which the samples were cooled on ice and centrifuged at 125 000 *g* for 1 hr.

2.3. *Purification of labelled proteins*

The 125 000 *g* supernatant labelled in vivo or in vitro, adjusted to contain 0.05 M phosphate buffer

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(pH 6.5), 2 mM 2-oxoglutarate, 0.1 mM pyridoxal phosphate, 100 μg purified rat liver ferritin per ml [4], was heated at 65°C for 7 min and quickly cooled. The coagulated proteins were discarded by centrifugation (15 min at 20 000 *g*), 5 mg/ml of bovine serum albumin was added and the mixture was dialyzed against 25 mM phosphate buffer. At this step further purification was obtained separately for ferritin and for TAT. To further purify TAT activity, the heated fraction was chromatographed on DEAE cellulose [7] and the 0.3 M KCl eluate was collected. For ferritin purification, the heated 125 000 *g* supernatant was brought to pH 5.5 with 0.5 M acetic acid. The precipitate was removed and ferritin was precipitated from the supernatant with $(NH_4)_2SO_4$ at a final concentration of 2.2 M. Dialysis in the presence of carrier leucine was performed as described by Chu and Fineberg [8] and the solution was brought up to the initial volume.

2.4. *Antibody precipitation*

Immunoprecipitations of ferritin were performed in conical tubes on 100 μl samples for extracts from in vivo labelled proteins and on 1 ml samples for extracts labelled in vitro. The incubation mixtures were brought respectively to a final volume of 1 ml and 2 ml which also contained 25 mM potassium phosphate (pH 7.6), 150 mM KC1, 5 mg of bovine serum albumin and 100 μl of rabbit antisera prepared by Saddi et al. [4] against twice crystallized rat ferritin; carrier ferritin purified from rat liver [4] was added to equivalence (100 μg per assay). The antigen-antibody complex was allowed to precipitate at 37°C for 1 hr, then transferred to 4°C overnight. The precipitates were collected by centrifugation and the supernatant solutions were submitted successively to a second and a third immunoprecipitation by addition of portions of both carrier ferritin and antibody. The complexes were washed three times by suspension in 1 ml of 0.15 M NaC1 and recentrifugation. They were then dissolved in 0.1 ml of 0.5 N NH_4OH, transferred to Whatman filter paper disks, and radioactivity was counted.

Labelled TAT was isolated immunochemically as described earlier [7].

2.5. *Protein, TAT and ferritin titration; counting of radioactivity*

Protein concentration was determined by the method of Lowry et al. [9]; TAT enzymic activity was assayed by the method of Diamondstone [10]. Ferritin titration was carried out using the extinction coefficient at 325 nm as described by Saddi et al. [4]. The acid-precipitable radioactivity was determined by precipitation of aliquots with trichloracetic acid on Whatman paper disks, by the method of Mans and Novelli [11]; the disks were counted in 10 ml of scintillation fluid (4 g Omnifluor per liter of toluene). Values shown represent the average of triplicate determinations.

3. Results

In the first study, purification and immunoprecipitation of ferritin were performed on the 125 000 *g* supernatant containing the released protein from cells labelled in vivo. The results in table 1 show the radioactivity recovered in three successive immunoprecipitations. It can be seen that the number of counts recovered in the second immunoprecipitate is low and very close to that found in the third. Therefore, substraction of the radioactivity recovered in the second precipitate from that in the first gives a valid estimate of the incorporation into a specific immunoprecipitate. Although the radioactivity in the second and third precipitate is higher for the crude heated fraction as compared to the values obtained with the purified supernatant, our studies show that there exists a good correlation between ferritin estimation on crude or further purified fractions; therefore it seems sufficient for our comparative study to work on crude heated extracts where the TAT enzymic activity (stimulated to a 5–15-fold increase by dexamethasone) is not discarded. The results in table 1 show that the isolated ferritin from dexamethasone induced and uninduced cells is very similar. For 10 experiments, the range is 0.22–0.32% of the total released radioactivity incorporated into ferritin. The addition of iron to the cell culture led to irregular effects; in no case was ferritin synthesis more than 20–30% over the control.

A second study was done on 'S 20' cell free extracts from both uninduced and dexamethasone induced HTC cells. These cell-free extracts incorporate, per mg of protein in the mixture, 3 to 4 × 10^5 cpm of labelled [^{3}H]leucine during a 45 min incubation at 37°C, whereas the samples kept on ice give a blank value of

Table 1
Radioactive amino acid incorporation into ferritin by HTC cells induced or uninduced by dexamethasone

Stage of purification	Steroid induction	TAT enzymic activity mU/mg protein	Total cpm recovered in the immunoprecipitates 1st	2nd	3rd	cpm in ferritin 1st minus 2nd precipitate	% total released radioactivity in ferritin
Heated 125 000 *g*	+	146	13 750	875	500	12 885	0.32
supernatant	–	18	12 090	900	700	11 190	0.28
Purified	+	0	10 600	190	135	10 400	0.31*
supernatant	–	0	9 800	180	120	9 714	0.27*

The total released radioactivity in 100 μl of the 125 000 *g* supernatant was respectively 4×10^6 cpm and 3.95×10^6 cpm for the steroid induced and uninduced cells. The immunoprecipitations represent the mean of 3 separate incubations of 100 μl samples.
* These values have been corrected to the initially added amount of carrier ferritin using the absorbance at 325 nm of the clarified precipitate at the end of the purification procedure; this correction accounts for the losses of material during purification [4].

less than 2%. After incubation, 45–55% of the acid-precipitable counts remain in the 125 000 *g* supernatant layer. To investigate whether this extract would incorporate labelled aminoacids specifically into the polypeptide chains of ferritin, we resorted to immunochemical isolation after partial purification. The results in table 2 show that 0.24–0.30% of the total released radioactivity is found in the ferritin immunoprecipitates. The radioactivity is the same when the 'S 20' is derived from uninduced or steroid induced cells, whereas the radioactivity in immunoprecipitated TAT is about 8 times greater when the 'S 20' is derived from induced rather from uninduced cells.

4. Discussion

These experiments suggest that HTC cells are able to synthesize a ferritin-like substance on the basis of labelled counts immunoprecipitated by antibody. The rate of synthesis (0.22–0.32%) is very close to the one found in rat liver. It is higher than that found in several Morris hepatoma tumors [12] and in He La cells where ferritin reaches only one-thousandth of the rate of general protein synthesis [8]. Our experiments show that iron induces only a slight increase of ferritin synthesis in HTC cells, whereas the iron-induced increase of leucine uptake in ferritin was found to be

Table 2
Synthesis of ferritin in HTC cell free extracts

	Steroid induction	Radioactivity in released polypeptides (1 ml) $\times 10^{-3}$	cpm recovered in the immunoprecipitates 1st	2nd	cpm in ferritin 1st minus 2nd	% total radioactivity released in ferritin	TAT
Exp. 1	+	1153	3312	247	3065	0.26	0.14
	–	1012	3269	232	3037	0.30	0.016
Exp. 2	+	720	2224	200	2024	0.28	0.18
	–	905	2650	235	2415	0.24	0.023

Ferritin immunoprecipitations were performed on 1 ml samples of the 125 000 *g* supernatant containing the released polypeptides, after the heating step; the values represent the mean of 3 separate incubations.

60% in He La cells which originated from a human uterine cervical carcinoma. In Morris hepatomas grown as solid intraperitoneal or intramuscular tumors with growth rates ranging from 3 to 12 days doubling time, Linder et al. [12] have found that, with increasing rate of growth, the hepatomas showed a progressive reduction of iron inducible ferritin synthesis. Our results are in agreement with this relationship: HTC cells have a doubling time of only 24 hr and present the lowest iron induction of ferritin synthesis up to now found in hepatoma cells.

The present results show that cell free preparations of HTC cells can translate endogenous ferritin messenger RNA and release complete polypeptide chains identical by their antigenicity to those synthesized in whole cells. Furthermore the percentage of ferritin in total proteins labelled in vivo or in vitro is identical. Ferritin synthesis is not affected by dexamethasone which stimulates TAT synthesis. Therefore it appears that ferritin synthesis, by simultaneous labelling, provides an internal control for the study of translational regulation of TAT synthesis in HTC cells. This is a very interesting point, for HTC are no longer able to synthesize albumin [13] which has generally been used as an internal control in cell free protein synthesis from liver extracts [14].

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