ROLE OF THE NUCLEUS IN THE GLUTAMINE-DEPENDENT DEGRADATION OF GLUTAMINE SYNTHETASE IN CULTURED HEPATOMA CELLS

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1. Introduction

Glutamine accelerates the degradation of glutamine synthetase (GS, L-glutamate:ammonia ligase (ADP) EC 6.3.1.2) in cultured rat hepatoma (HTC) cells [1]. The mechanism of this regulatory process, in which the product of an enzymatic reaction accelerates the degradation of the enzyme producing it, is not well understood. Apparently glutamine does not facilitate GS degradation simply by binding to its active site [2]. Inhibitors of protein synthesis prevent the decay of GS activity caused by glutamine [3]. It has therefore been proposed that a labile polypeptide factor is implicated in the process [3]. In this respect the GS system resembles certain other protein degradative systems which require concomitant protein synthesis for activity [4].

The aim of the present investigation was to examine the effects of inhibitors of messenger RNA synthesis and of enucleation on GS degradation. It is shown that inhibitors of RNA synthesis inhibit the glutamine dependent degradation of GS. The inhibition increases progressively with time of incubation and is complete after incubations of $\geqslant$6 h. Enucleation of cells with cytochalasin B immediately inhibits the degradation of GS. These results are discussed in terms of a model proposed earlier for the degradation of glutamine synthetase.

2. Materials and methods

2.1. *Materials*

Actinomycin D and cordycepin (3′-deoxyadenosine) were from Sigma. Cytochalasin B was from Serva and α-amanitin was purchased from Boehringer. Calf serum was obtained from Bio-Lab, Israel. [4-^{3}H]-Leucine was purchased from the Nuclear Research Center, Israel.

2.2. *Growth of cells and induction of GS*

Hepatoma tissue culture cells, subclone GM 22, were grown in suspension, as in [5]. Glutamine synthetase was induced with methionine sulfone, as outlined in [1]. The composition of growth medium and induction medium were the same as in [1].

2.3. *Enucleation of cells*

Cells in suspension were enucleated with the aid of cytochalasin B as in [6]. Staining with Giemsa stain showed that the cytoplasts were essentially free from nucleated cells (<2%).

2.4. *Labeling of long-lived proteins*

Cells were incubated in growth medium with 1 μCi/ml of [^{3}H]leucine for 36 h. They were then washed once with growth medium, containing 1 mM leucine, and incubated in the same medium for a further 10 h before the start of the experiment.

2.5. *Labeling of short-lived proteins*

Cells were incubated in growth medium with 5 μCi/ml of [^{3}H]leucine for 30 min, washed once with growth medium, containing 1 mM leucine, and used immediately for the experiment.

2.6. *Intracellular protein degradation*

Labeled cells or cytoplasts prepared from them were incubated at a density of 5×10^5/ml, at 37°C, in growth medium containing 1 mM leucine, with gentle shaking. Samples of 1.0 ml were removed at

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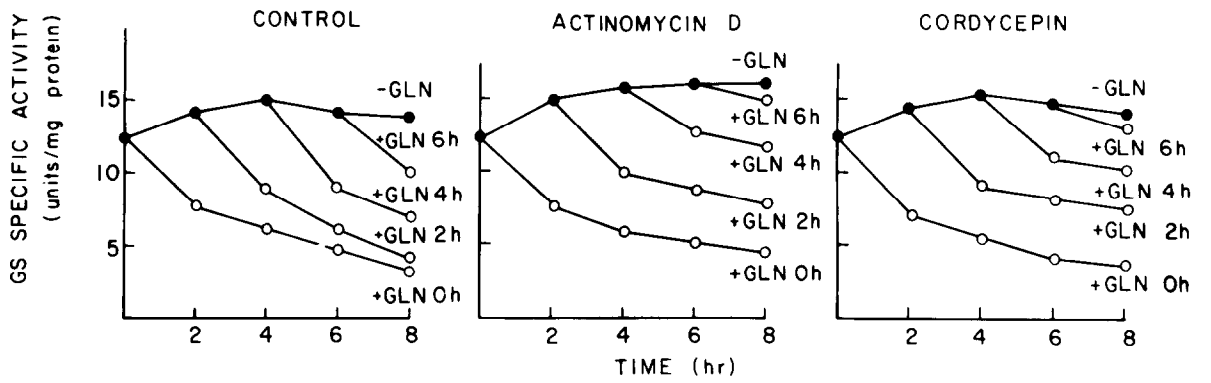


Fig.1. The effect of cordycepin and actinomycin D on the glutamine-dependent inactivation of GS. HTC cells were induced as in section 2. The cells were suspended in fresh induction medium and incubated as in [1]. To one set of 5 vessels 15 μg cordycepin/ml was added. To another set 1 μg actinomycin D/ml was added, and a third set contained no additions. At different times 5 mM glutamine was added to each system (○). One system (–GLN) remained without glutamine (●). Incubation was continued for a further time. Samples were taken and GS activity and amount of protein were estimated.

intervals and the cells were sedimented. To the supernatant trichloroacetic acid was added to 10% final conc. (w/v). After 10 min at 0°C the precipitate was removed by centrifugation, and trichloroacetic acid-soluble counts were measured on 0.5 ml supernatant in a 10 ml toluene–Triton X-100 (2:1) scintillation mixture. The counts in the cell pellet were determined after suspension in 1 ml 1.0 N NaOH. '% Trichloroacetic acid-soluble cpm' are the percentage of the total counts in the sample, which are present in the trichloroacetic acid supernatant.

2.7. *Analytical methods*

GS activity was determined by the glutamotransferase assay as in [5]. Protein was measured as in [7] using bovine plasma albumin as standard.

3. Results

3.1. *Effects of mRNA synthesis inhibitors*

Fig.1 shows the effects of actinomycin D and cordycepin on the glutamine-modulated degradation of GS. 'Induced' cells containing high levels of glutamine synthetase were incubated, in the presence or absence of inhibitor, in medium without glutamine. Glutamine was added at various times after the addition of inhibitor, and the incubation was continued. When glutamine was added at zero time with actinomycin D or cordycepin, GS activity decayed rapidly, at the same rate as in the control. After continued incubation with the inhibitors, the effect of glutamine on GS activity was progressively diminished and was completely abolished after 6 h. Essentially similar results were obtained with α-amanitin (not shown).

3.2. *Effect of enucleation*

Fig.2 shows that in cytoplasts, prepared by density gradient centrifugation of HTC cells in the presence of cytochalasin B, glutamine does not stimulate

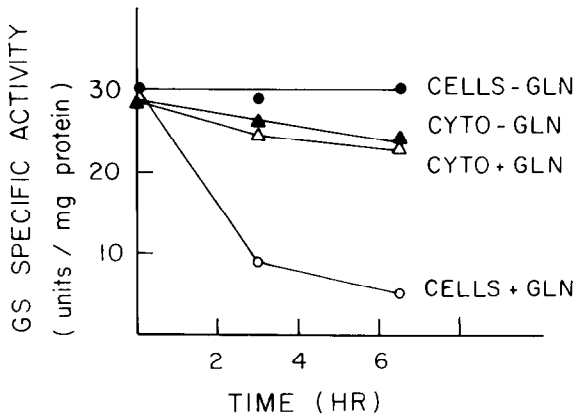


Fig.2. Effect of enucleation on the glutamine-dependent inactivation of GS. Induced HTC cells and cytoplasts prepared from these cells were incubated in 'induction medium'. (●) Cells in absence of glutamine; (▲) cytoplasts in absence of glutamine; (○) cells in the presence of 5 mM glutamine; (△) cytoplasts in the presence of 5 mM glutamine. At different times samples were taken for the estimation of the amount of protein and GS activity.

Table 1
Effect of pretreatment with cytochalasin B on the glutamine-dependent inactivation of GS (GS units/mg protein)

Time (h)	Untreated cells		Cells pretreated with cytochalasin B	
	– Gl	+ Gl	– Gl	+ Gl
0	25	25	30	30
1	34	19	37	21
3	31	10	34	14

Part of the cells were preincubated in the presence of 10 μg/ml cytochalasin B for 2 h. The cells were washed with 'induction medium', suspended in the same medium, and incubated for 0, 1 and 3 h in the absence or presence of 5 mM glutamine. Control cells were treated the same way, omitting the preincubation with the inhibitor. Cells were harvested and GS activity and amount of protein were estimated as in section 2

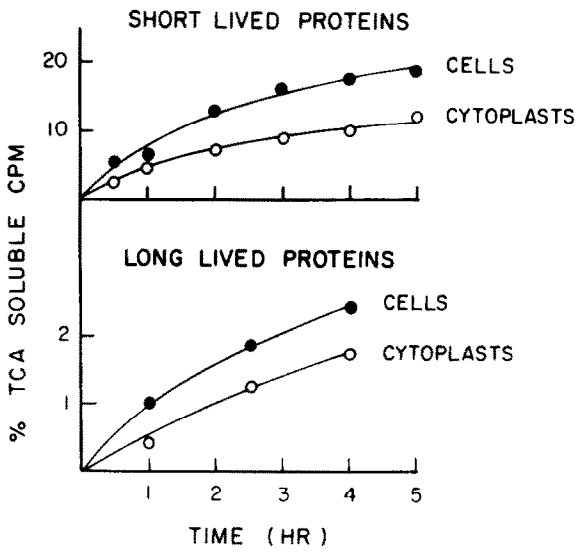


Fig.3. Degradation of intracellular proteins in HTC cells and cytoplasts. Long- and short-lived proteins were labeled with [^{3}H]leucine as in section 2. The labeled cells (•) and the labeled cytoplasts (○) were suspended in 'growth medium'. At different times samples were taken and the release of trichloroacetic acid-soluble radioactivity was determined.

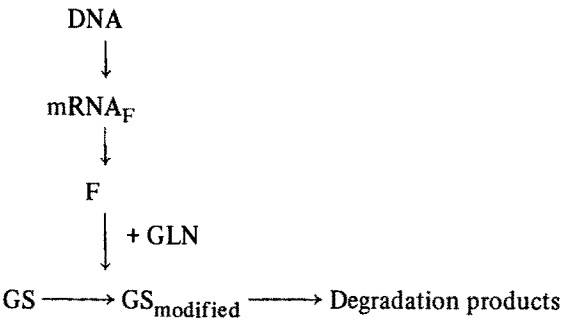
the decay of GS activity. Thus, enucleation, unlike the inhibition of mRNA synthesis, causes an immediate abolition of the effect of glutamine.

The possibility arose that the abolition of the glutamine effect was due to a toxic effect of cytochalasin B, rather than to the enucleation itself. Table 1 shows that the treatment of cells with cytochalasin B for 2 h, followed by its removal by washing the cells, had little or no effect on the glutamine-modulated decay of glutamine synthetase. Since the enucleation procedure involves exposure of the cells to cytochalasin for only 1 h, a toxic effect of the cytochalasin B seems to be ruled out.

Another possibility was that enucleation inhibits cellular protein degradation in general. Therefore the degradation of both 'short-lived' proteins and 'long-lived' proteins [4,8] was compared in cells and in cytoplasts. The turnover of both classes of proteins in cytoplasts was 60–70% of that found in the intact cells (fig.3). This rather small decrease in general protein degradation is not likely to account for the complete and immediate effect of the enucleation on GS degradation.

4. Discussion

In [3] we suggested the following mechanism to explain the effects of protein synthesis inhibitors on the glutamine-stimulated degradation of GS:



According to this scheme, F is a labile polypeptide factor which, in the presence of glutamine, promotes GS modification and degradation. mRNA$_F$ is the specific messenger RNA of the factor F.

As the glutamine-dependent degradation is rapidly inhibited by protein synthesis inhibitors, it was proposed that the factor F has a short half-life (~30 min) in the absence of glutamine [3]. Thus, the maintenance of a pool of F is dependent on its constant synthesis and, therefore, dependent on the maintenance of a pool of its messenger RNA (mRNA$_F$).

The effects of the inhibitors of cytoplasmic mRNA synthesis (fig.1) could readily be explained in terms of this model. The effect of glutamine is abolished

upon treatment for several hours with actinomycin D, cordycepin, and α-amanitin. The fact that similar results were obtained with the 3 inhibitors, each of which is known to prevent the accumulation of cytoplasmic mRNA by a different mechanism [9], suggests that the effects of the inhibitors on the degradation of GS is due to inhibition of the synthesis of a specific mRNA. If the life span of $mRNA_F$ were ~6 h, then inhibition of its synthesis would result in a gradual decay and the eventual abolition of the glutamine effect. The data thus suggest that the factor F involved in glutamine synthetase degradation is more labile than its mRNA.

If the effect of enucleation were simply the removal of the source of cytoplasmic RNA, then the results of enucleation should be the same as those of inhibition of RNA synthesis. Since enucleation causes a complete and immediate cessation of the glutamine-stimulated degradation of GS, it seems that enucleation must have some effect additional to the depletion of $mRNA_F$. Our experiments indicate that the loss of activity after enucleation is not due to non-specific toxicity of cytochalasin B or to a general inhibition of protein degradation. An alternative explanation is that enucleation removes or disrupts some unidentified cellular structure or function. Clearly, enucleation does more than simply stop the flow of RNA from the nucleus to the cytoplasm. Furthermore, the above results indicate that the degradation of GS involves enucleation-sensitive steps not shared with the degradative pathways of most other cellular proteins.

As there is some disagreement in [10,11] with respect to the effects of enucleation on protein turnover, it is of interest that in our experiments the rate of general protein turnover was only slightly reduced in cytoplasts. In [10] enucleation resulted in a complete inhibition of the degradation of the cellular proteins. In [11] however, using two-dimensional electrophoresis, observed that enucleation does not affect the breakdown of many HTC cell proteins, although it does specifically inhibit the degradation of tyrosine aminotransferase and a few other specific proteins. Our results support the conclusions in [11] that enucleation does not strongly affect protein breakdown in general but may affect the degradation of individual proteins.

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