REGULATION OF THE DEGRADATION OF ^{125}I-LABELED GLUTAMINE SYNTHETASE INTRODUCED INTO CULTURED HEPATOMA CELLS BY ERYTHROCYTE GHOST–MEDIATED INJECTION

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1. Introduction

The glutamine synthetase (L-glutamate:ammonia ligase (ADP) EC 6.3.1.2) activity of many mammalian cells in culture decreases with increasing glutamine concentrations in the medium [1–4]. Radioimmunoprecipitation experiments with hepatoma tissue culture (HTC) cells have shown that glutamine decreases glutamine synthetase activity by specifically accelerating the degradation of the enzyme [5]. Although a considerable amount of information has been obtained about the properties of the glutamine synthetase degradation system [4–8], details of the pathway of degradation of the enzyme are still unknown. An obstacle to the elucidation of this pathway has been the failure to obtain glutamine-modulated breakdown of glutamine-synthetase in cell-free systems (unpublished). An alternative approach is to introduce labeled enzyme into unlabeled cells by an injection procedure and to study its degradation in vivo. Here, we have microinjected ^{125}I-labeled rat liver glutamine synthetase into HTC cells by erythrocyte ghost-mediated injection [9], and could demonstrate that glutamine specifically accelerates its degradation. Pepstatin, a peptide protease inhibitor impermeable to the cells, was coinjected with ^{125}I-glutamine synthetase and its effects on the degradation of the enzyme were investigated.

2. Materials and methods

2.1. *Cells and media*

Growth of HTC cells, clone GM22 and media were as in [6]. Tricine-buffered saline contained 160 mM NaCl and 20 mM Tricine–NaOH (pH 7.4).

2.2. *Iodination of proteins*

Glutamine synthetase purified as in [10] was iodinated according to [11]. The reaction time was 5 s at 0–4°C. Free ^{125}I was removed by centrifugation through Sephadex G-25 [12]. The preparations contained 1–2% of trichloroacetic acid-soluble ^{125}I. Bovine serum albumin (BSA) was iodinated by the same procedure.

2.3. *Loading of human erythrocyte ghosts*

Erythrocyte ghosts were loaded as in [9]. Briefly, ^{125}I-labeled proteins (0.5 mg/ml) and cytochrome *c* (0.5 mg/ml) were added to 30% (v/v) washed erythrocytes in 160 mM NaCl and 20 mM Tricine–NaOH (pH 7.4). The mixture was dialyzed against 500 vol. 40 mM KCl plus 10 mM Tricine–NaOH (pH 7.4) at 4°C for 90 min. The ghosts were resealed and washed 3 times with Tricine-buffered saline. The washed ghost suspension was found to be virtually free of unbound ^{125}I (<0.3% of the total counts).

2.4. *Fusion of loaded ghosts with HTC cells*

The procedure was essentially as in [9]. The fusion system contained 1×10^7 loaded erythrocyte ghosts/ml, 2×10^6 HTC cells/ml, 0.2 mM $La_2(NO_3)_3$, and 400 hemagglutinating units/ml of Sendai virus in Tricine-buffered saline.

2.5. *Release of trichloroacetic acid-soluble radioactivity from cells*

HTC cells which had been fused with loaded erythrocyte ghosts (5×10^5 cells/ml) were incubated with the appropriate additions at 37°C with gentle shaking. At intervals 1 ml samples were removed, the cells were sedimented, and trichloroacetic acid was added to the

supernatant to a final concentration of 10% (w/v). The trichloroacetic acid-souble counts in the medium were determined in a gamma counter. The cells were washed with phosphate-buffered saline and incubated with 0.83% NH_4Cl at 37°C for 15 min to lyse adsorbed ghosts, washed with phosphate-buffered saline and treated with 10% (w/v) trichloroacetic acid. Radioactivity in the pellets gave the trichloroacetic acid-insoluble counts in cells. Results are expressed as percent of injected counts in trichloroacetic acid-soluble material [(trichloroacetic acid-soluble counts in medium/trichloroacetic acid-insoluble counts in cells at zero time) × 100].

2.6. *SDS–polyacrylamide gel electrophoresis*

Cells were sedimented, washed and treated with 0.83% NH_4Cl as described. Cell pellets containing ~2 × 10^5 cells, were examined by electrophoresis on a 10% SDS–polyacrylamide slab gel followed by autoradiography [13].

2.7. *Materials*

Pepstatin [14] was the generous gift of Dr H. Umezawa, Microbial Chemistry Research Foundation, Tokyo. $Na^{125}I$ was purchased from the Radiochemical Centre, Amersham. Cycloheximide, L-methionine sulfone, L-glutamine and BSA were obtained from Sigma.

3. Results

Glutamine synthetase from rat liver labeled with ^{125}I was trapped in human erythrocyte ghosts and the ghosts' contents were injected into HTC cells using Sendai virus-mediated fusion [9]. The degradation of the labeled enzyme was followed by measuring the release of trichloroacetic acid-soluble counts into the medium. Fig.1 shows the time course of glutamine synthetase degradation in the injected cells. The degradation is markedly accelerated by glutamine, while methionine sulfone, a glutamine antagonist in this system [8], inhibits the degradation of the enzyme (fig.1). The inhibition by methionine sulfone of the glutamine-dependent degradation (fig.1) is apparently due to its competition with endogenous glutamine [8,15]. Therefore, in subsequent experiments degradation in the presence of methionine sulfone is taken as a measure of the basal, glutamine-independent degradation of glutamine synthetase. It should be noted that the glutamine-dependent degradation of injected

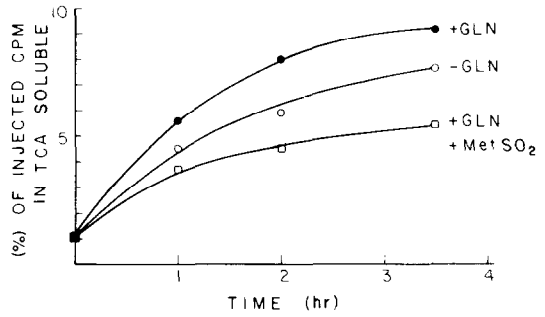

Fig.1. Effect of glutamine and methionine sulfone on the degradation of ^{125}I-glutamine synthetase. Cells were fused with erythrocyte ghosts loaded with ^{125}I-glutamine synthetase. During fusion, 8.5% of the ghosts' contents were transferred to the cells resulting in 17 670 cpm/10^6 cells. The cells were suspended in growth medium without glutamine and incubated with the following additions: none (○); 5 mM glutamine (GLN) (●); 5 mM glutamine + 5 mM methionine sulfone ($MetSO_2$) (□).

^{125}I-glutamine synthetase was only obtained with enzyme preparations which had been iodinated under mild conditions, using short iodination times (<5 s, section 2).

Preincubation of cells with cycloheximide (0.4 mM) partially inhibits the glutamine-dependent breakdown of ^{125}I-glutamine synthetase (fig.2). On the other hand, cycloheximide has no effect on the basal degradation rate of glutamine synthetase determined in the presence of methionine sulfone.

Fig.3 shows the effect of the peptide protease inhibitor pepstatin [14] on the degradation of ^{125}I-glutamine synthetase. Since pepstatin is practically impermeable to cells [16], it was necessary to inject the inhibitor together with the ^{125}I-glutamine synthetase. Under these conditions, pepstatin consistently inhibits the glutamine-dependent breakdown of glutamine synthetase. The effect of pepstatin on the basal degradation varied from experiment to experiment, being very marked in some (fig.3) and practically zero in others (not shown). These results might reflect the variability in the amount of pepstatin which is introduced into the cells during the fusion procedure. Indeed, a correlation was found between the extent of inhibition of the basal degradation of glutamine synthetase and the efficiency of the microinjection.

To test whether the factors affecting the degradation rate of glutamine synthetase are specific for this protein, we have also studied the degradation of ^{125}I-BSA injected into HTC cells. The average degradation

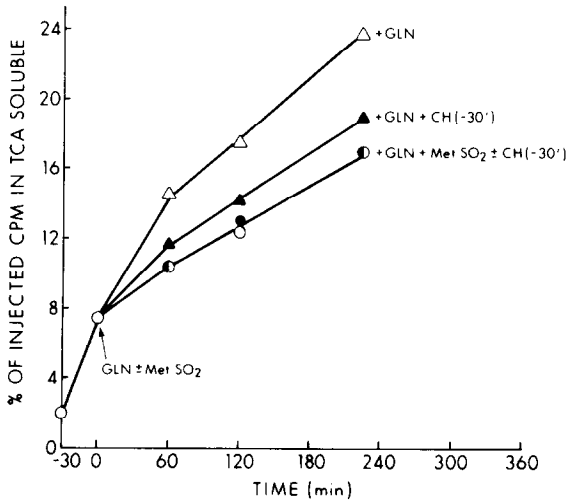

Fig.2. Effect of cycloheximide on the glutamine-dependent degradation of ^{125}I-glutamine synthetase. Cells were fused with human erythrocyte ghosts, and 5% of the ghosts' contents were transferred to the cells resulting in 2450 cpm/10^6 cells. Cells were suspended in growth medium without glutamine, and preincubated for 30 min with (▲,●) or without (△,○) 0.4 mM cycloheximide (CH). At zero time 5 mM glutamine (GLN) with (○,●) or without (△,▲) 5 mM methionine sulfone ($MetSO_2$) was added as indicated, (◐) coinciding with (●,○).

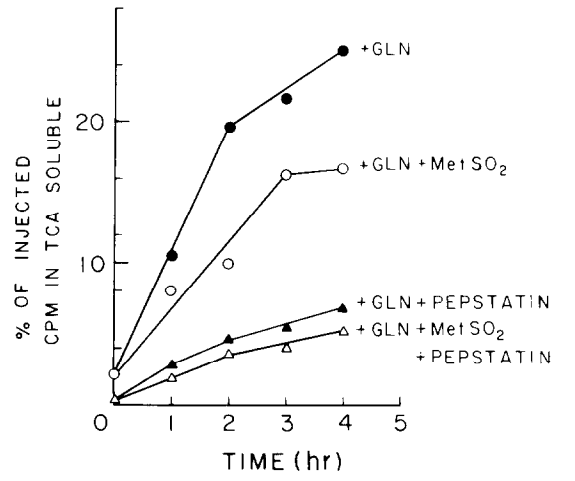

Fig.3. The effect of pepstatin on the degradation of ^{125}I-glutamine synthetase. ^{125}I-Glutamine synthetase was trapped in human erythrocyte ghosts as in section 2, in the presence or in the absence of 1 mg pepstatin/ml. Cells were fused with erythrocyte ghosts loaded in the presence (△,▲) or absence (○,●) of pepstatin. Samples of each batch of cells were incubated either with 5 mM glutamine (GLN) (●,▲) or with 5 mM glutamine + 5 mM methionine sulfone ($MetSO_2$) (○,△).

rate of ^{125}I-BSA is faster than that of ^{125}I-glutamine synthetase and is not affected by substances which modulate glutamine synthetase breakdown, namely glutamine, methionine sulfone, cycloheximide and pepstatin (not shown).

To analyze the degradation products of the injected ^{125}I-glutamine synthetase, extracts from injected cells were subjected to SDS–polyacrylamide gel electrophoresis. Extracts from cells that had been incubated under various conditions, namely with or without glutamine, cycloheximide or pepstatin, revealed no new major radioactive bands (not shown). Thus, there is no evidence for the accumulation of intermediates of glutamine synthetase breakdown using the present technique.

4. Discussion

This investigation shows that the degradation of an injected protein can be subjected to regulation resembling that of the corresponding endogenous protein. Degradation of injected proteins has been investigated [17–19] but regulation of the process by the injected cell had not been demonstrated. This study shows that the degradation of injected ^{125}I-glutamine synthetase, like that of the endogenous enzyme [5,7, 8,15], is accelerated by glutamine and this increased degradation is antagonized by methionine sulfone and inhibited by cycloheximide (fig.1,2). These observations suggest that the glutamine-dependent degradation of injected ^{125}I-glutamine synthetase follows the same pathway as that of the endogenous enzyme.

While the glutamine-dependent degradation of injected ^{125}I-glutamine synthetase was consistently observed, the extent of degradation reached only 10–25% of the injected counts after a 4 h incubation. On the other hand, the extent of degradation of injected ^{125}I-BSA was usually ~40% of the injected counts during a similar incubation period. A possible explanation of these results is suggested by our findings on the subcellular distribution of the injected proteins. When injected cells were fractionated as in [9], 70–90% of the radioactivity of ^{125}I-glutamine synthetase was found in the particulate fraction, whereas the injected ^{125}I-BSA was almost entirely recovered in the soluble (cytoplasmic) fraction. Thus, the extent of ^{125}I-glutamine synthetase degradation might be limited by the amount of enzyme which is free to interact with the degradative system.

The inhibition of the degradation of ^{125}I-glutamine synthetase by pepstatin is particularly interesting. Pepstatin is a peptide protease inhibitor which strongly inhibits pepsin and the lysosomal protease cathepsin D [14]. It is therefore likely that the lysosomal system is involved in the degradation of glutamine synthetase. Inhibition of protein degradation has been reported by pepstatin [16,21] and by other peptide inhibitors of lysosomal proteases [20,21]. Unlike glutamine synthetase, ^{125}I-BSA injected into hepatoma cells is degraded through a pepstatin-insensitive pathway, suggesting that the 2 proteins are degraded by different sets of proteolytic enzymes.

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References

[1] De Mars, R. (1958) Biochim. Biophys. Acta 27, 435–436.
[2] Paul, J. and Fortrell, P. F. (1963) Biochim. Biophys. Acta 67, 334–336.
[3] Kulka, R. G., Tomkins, G. M. and Crook, R. B. (1972) J. Cell Biol. 54, 175–179.
[4] Tiemeier, D. C. and Milman, G. (1972) J. Biol. Chem. 247, 5722–5727.
[5] Arad, G., Freikopf, A. and Kulka, R. G. (1976) Cell 8, 95–101.
[6] Kulka, R. G. and Cohen, H. (1973) J. Biol. Chem. 248, 6738–6743.
[7] Freikopf, A. and Kulka, R. G. (1976) Biochem. Biophys. Res. Commun. 72, 1195–1200.
[8] Freikopf, A. and Kulka, R. G. (1975) Eur. J. Biochem. 56, 484–492.
[9] Wasserman, M., Zakai, N., Loyter, A. and Kulka, R. G. (1976) Cell 7, 551–556.
[10] Tate, S. S., Leu, F. Y. and Meister, A. (1972) J. Biol. Chem. 247, 5312–5321.
[11] Fang, V. S., Cho, A. W. and Meltzer, H. Y. (1975) Biochem. Biophys. Res. Commun. 65, 413–419.
[12] Penefsky, H. S. (1977) J. Biol. Chem. 252, 2891–2899.
[13] Laemmli, U. K. (1970) Nature 227, 680–685.
[14] Umezawa, H. (1972) Enzyme Inhibitors of Microbial Origin, University of Tokyo Press.
[15] Arad, G. and Kulka, R. G. (1978) Biochim. Biophys. Acta 544, 153–162.
[16] Dean, R. T. (1975) Nature 257, 414–416.
[17] Yamaizumi, M., Uchida, T., Mekada, E. and Okada, Y. (1979) Cell 18, 1009–1014.
[18] Zavortnik, M., Thatcher, T. and Rechsteiner, M. (1979) J. Cell. Physiol. 100, 175–186.
[19] Wasserman, M., Kulka, R. G. and Loyter, A. (1977) FEBS Lett. 83, 48–52.
[20] Neff, N. T., De Martino, G. N. and Goldberg, A. L. (1979) J. Cell. Physiol. 101, 439–458.
[21] Hopgood, M. F., Clark, M. G. and Ballard, F. J. (1977) Biochem. J. 164, 399–407.