Degradation of short-lived proteins is decreased by centrifugation

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We have examined the effects of enucleation and of inhibitors of mRNA synthesis (actinomycin D and cordycepin) on protein turnover of HeLa cells. Enucleation markedly inhibited the rate of protein degradation for short-lived proteins. However, cells centrifuged in the absence of cytochalasin B at the speed required to obtain cytoplasts showed protein degradation rates identical to those of cytoplasts, while inhibitors of mRNA synthesis did not affect the process. Although enucleation may affect degradation of specific proteins, these results suggest that centrifugation is largely responsible for the inhibition of protein degradation in cytoplasts.

Centrifugation *Cytochalasin B* *Enucleation* *HeLa cell* *mRNA* *Proteolysis*

1. INTRODUCTION

Proteins of mammalian cells have different half-lives [1]. Thus, whatever the mechanism(s) of turnover, selective factors should be part of intracellular protein degradation [2]. An important question is whether the nucleus partakes in this process. This has been tested using cytochalasin B to cause enucleation of cells.

Enucleation completely inhibited degradation of proteins in FU5-5 rat hepatoma tissue culture cells [3]. However, it was claimed that enucleation does not strongly affect protein degradation in general, but may affect the degradation of some specific proteins in HTC cultured rat hepatoma cells [4,5]. To clarify this apparent disagreement, we have examined the effects of enucleation and of inhibitors of mRNA synthesis on protein turnover of HeLa cells and found a marked inhibition of short-lived protein degradation after enucleation. However, centrifugation in the absence of cytochalasin B, at the speed required for enucleation, produced identical inhibition rates of short-lived protein degradation. However, inhibition of mRNA synthesis did not affect the process. Although enucleation may affect degradation of specific proteins, our findings strongly suggest that centrifugation rather than enucleation is largely responsible for the inhibition of protein turnover in cytoplasts.

2. MATERIALS AND METHODS

2.1. *Materials*

Actinomycin D, cordycepin and cytochalasin B were from Sigma Chemical Co. L-[4,5-^{3}H]Leucine (65 Ci/mmol) was from Amersham Radiochemical Centre.

2.2. *Cell culture*

HeLa cells were cultured in monolayer in 25 cm^2 plastic flasks as in [6]. Cell viability was checked by trypan blue exclusion.

2.3. *Enucleation of cells*

Cells were enucleated by a modification of the method in [7]. Briefly, the flasks containing exponentially growing cells were filled (~60 ml) with Eagle's minimal essential medium, 10% foetal bovine serum, 1 mM L-leucine and 10 μg cytochalasin B/ml. Centrifuge (Sorvall RC-5), rotor

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(GSA), polycarbonate bottles (Sorvall, ref. 03526) containing 110 ml of water and medium, were all pre-warmed at 37°C. Culture flasks were inserted into the bottles, cap upward and with the cells closest to the rotor axis, and centrifuged at 8500 rev./min, 30 min, and adjusting the temperature control setting of the centrifuge to maintain 37°C in the flasks (conditions for optimum enucleation, as determined from a previous study of variations in time and speed of centrifugation). Percentage of enucleation was 70% as estimated by Giemsa staining. Carioplasts formed a pellet in one of the corners of the flasks and were easily washed out. Controls were performed without centrifugation and/or without cytochalasin B.

2.4. *Intracellular protein degradation*

Cells were incubated in medium with 5 μCi [^{3}H]leucine/ml for 15 min and washed 3 times with medium containing 1 mM cold L-leucine (short-lived proteins). To label long-lived proteins, cells were incubated with 2 μCi [^{3}H]leucine/ml for 24 h, washed 3 times with medium containing 1 mM leucine and incubated in the same medium for 15 h. The labeled cells were used immediately for enucleation or controls. Then the cells were washed 3 times with medium plus 1 mM leucine, placed in the same medium, and, after 10 min, the degradation of intracellular proteins was measured as in [8]. For the inhibitor experiments this medium was supplemented with 15 μg cordycepin/ml or 1 μg actinomycin D/ml.

2.5. *Electron microscopy*

Electron microscopy of whole HeLa cells, cytoplasts and caryoplasts thereof was carried out using Vestopal W as embedding medium, following standard methods [9].

3. RESULTS AND DISCUSSION

Fig. 1 shows the electron microscopic appearance of HeLa cells, cytoplasts and caryoplasts thereof. Turnover of both short- and long-lived proteins was compared in whole cells and cytoplasts. Degradation of short-lived proteins was ~30–50% inhibited. The degradation of long-lived proteins, however, was about the same as in control cells. Similar values for short-lived proteins have been obtained in HTC cells [5]. The turnover of both

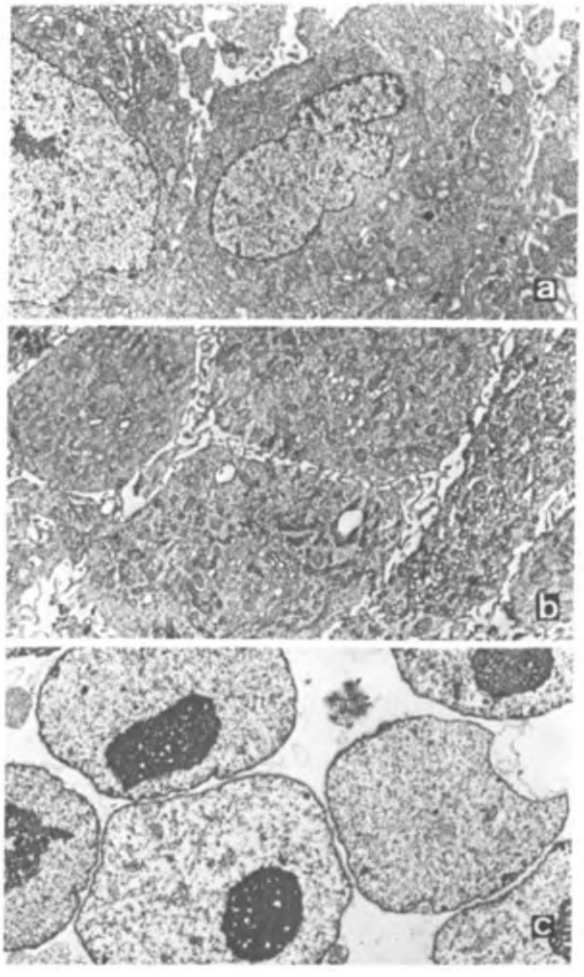

Fig. 1. Electron micrographs of: (a) HeLa cells; (b) cytoplasts; (c) caryplasts thereof; ×5000.

pools of proteins was followed in the presence of actinomycin D and cordycepin used at concentrations known to inhibit mRNA synthesis [10]. The rate was equal in control and in enucleated cells (fig. 2). Therefore, although mRNAs of HeLa cells with a half-life of $\leq$2 h have been described [11], the observed effect cannot be simply attributed to inhibition of the synthesis of an mRNA with a short half-life.

This inhibition could then be due to toxicity of cytochalasin B or to centrifugation rather than to enucleation itself. Fig. 3 shows that cytochalasin B had no inhibitory effect, whereas centrifugation had the same effect as enucleation. We conclude,

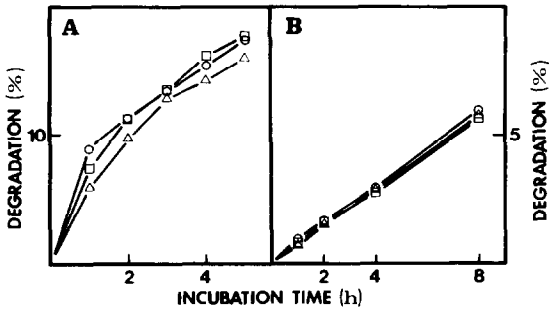

Fig. 2. Effects of actinomycin D and cordycepin on the degradation of short-lived (A) and long-lived (B) proteins. Short-lived proteins were labeled with [^{3}H]leucine for 15 min, and long-lived proteins were labeled for 24 h and chased for 15 h. Labeled cells were incubated with no addition (□—□); with 1 μg actinomycin D/ml (△—△); or with 15 μg cordycepin/ml (○—○). After 10 min protein degradation was measured, at the times indicated, as the release of acid-soluble [^{3}H]leucine from radioactive proteins present at the start of the chase period. Each point is the mean of 3 separate experiments with duplicated samples; SE-values were 4–9%.

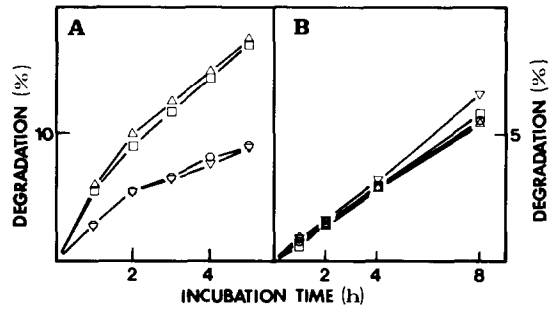

Fig. 3. Degradation of short-lived (A) and long-lived (B) intracellular proteins in HeLa cells. Untreated cells (□—□); cells treated 30 min with 10 μg cytochalasin B/ml (△—△); cells treated with cytochalasin B and centrifuged at 8500 rev./min (11800 × g) for 30 min to obtain cytoplasts (▽—▽), and cells centrifuged for 30 min at the same speed required to obtain cytoplasts (○—○). HeLa cells were labeled as described in fig. 2. Labeled cells were used immediately for treatments and then degradation at the indicated times was measured. Each point is the mean of 5 separate expt with duplicate samples. SE-values were 2–6%.

therefore, that cytochalasin B treatment, followed by its removal by washing the cells, is not toxic, supporting the results of others [5], and that the observed inhibition of short-lived protein degradation in cytoplasts can be explained by a centrifugation effect. Similar results were obtained in Balb/3T3 mouse fibroblasts and its SV-40 transformed derivative SV3T3 and in HTC cells (not shown). Therefore, the finding exemplified here should be important in experiments with cytoplasts, at least in those concerning protein turnover. Most of the methods of enucleation combine treatment with cytochalasin B and centrifugation, either in iso-osmotic Ficoll density gradients, for cells grown in suspension or loosely attached in monolayer [12], or in a substrate placed directly in the centrifugal field, for cells grown in monolayer [7]. Obviously it is possible that enucleation affects degradation of specific proteins in some cell types [3–5], but it is clear that this effect is quantitatively unimportant for HeLa and other cells.

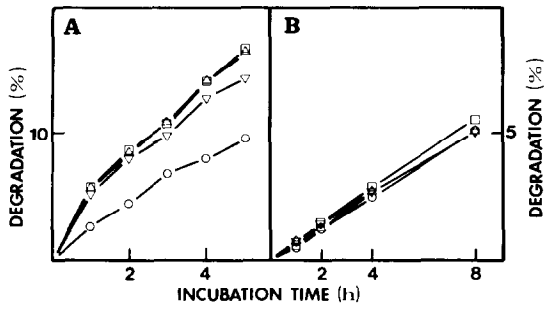

Fig. 4. Effects of different centrifugation speeds on the degradation of short-lived (A) and long-lived (B) proteins. HeLa cells were labeled as in fig. 2 and non-centrifuged (□—□); or centrifuged at 3000 rev./min (1500 × g, △—△); 6000 rev./min (5900 × g, ▽—▽), or 8500 rev./min (○—○), for 30 min and then degradation was measured. Each point is the mean of 3 separate expt with duplicate samples; SE-values were 2–8%.

As for the inhibitory effect observed here, it may be explained by a modification of cellular function. Indeed, we have found no difference in viability or in electron microscopic appearance of centrifuged and non-centrifuged cells. Also, the effect cannot be explained by removal of some cell structure, because the very small pellet that sometimes appeared on centrifugation consisted of some few dead cells (in cytochalasin B treated cells the pellet was bigger and mainly composed of caryoplasts). It is important to point out that the effect of centrifugation is not graded as can be seen in fig. 4. A critical centrifugation force has to be

exceeded to observe inhibition of protein degradation and higher centrifugation forces (up to $20000 \times g$) do not increase the inhibitory effect. Therefore, it would be most interesting to clarify whether unidentified cell component(s) are modified under our conditions. At any rate, these findings clearly show that centrifugation of cells in the absence of cytochalasin B at the same speed used to obtain cytoplasts is a necessary control for a valid interpretation of results with enucleated cells.

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