REVERSIBLE INHIBITION OF PROTEIN SYNTHESIS IN HELA CELLS BY EXPOSURE TO PROTEOLYTIC ENZYMES

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SUMMARY

Protein synthesis in suspended HeLa S_3 cells is inhibited by more than 50% immediately after addition of 100 µg pronase/ml or 500 µg trypsin/ml. Polyribosome profiles are not altered by exposure of cells to 1 or 2 mg trypsin/ml suggesting that the inhibition affects peptide chain elongation. Protein synthesis resumes after removal of proteases by sedimentation and resuspension of the cells.

INTRODUCTION

Trypsin at concentrations of 100 µg/ml to 2 mg/ml has been used routinely for many years in the cultivation of tissue culture cells, particularly for the preparation of primary cultures and for disaggregation of cells grown in monolayer cultures (1). Surface adhesion of cells is inhibited by trypsin treatment (2) and protein synthesis is required to overcome this inhibition (2). Increased (3, 4) and altered (5) protease activities have been found in transformed cells. It was suggested that a protease-like activity is required by transformed cells for unrestrained growth (6). Mouse fibroblasts in culture are released from contact inhibition or density inhibition by exposure to either trypsin (70 µg/ml) or pronase (5 µg/ml) (6). Similar observations have been made with chick embryo cells in culture (7). The lower sensitivity of transformed cells to density inhibition has been correlated with the excretion of proteolytic enzymes by these cells (6). In the presence of 2 µg trypsin per ml in fluid maintenance medium, primary chick embryo fibroblasts yield higher titers of infectious influenza B virus. The plating efficiency of

influenza B virus is also increased when trypsin is incorporated in the same amount in the agar overlay (8). Recently, proteolytic enzymes have been applied extensively in studies on the composition of the cell membrane (9, 10). The glycoproteins released from normal and transformed cells by proteolytic enzymes differ in size and composition (11, 12). In experiments (13) aimed at reversing the block in protein synthesis in HeLa cells elicited by L-1-tosyl-amido-2-phenyl-ethyl chloromethyl ketone (TPCK), a specific inhibitor of chymotrypsin (14), we observed that exposure of cells to proteolytic enzymes also results in a rapid cessation in the incorporation of radioactive amino acids into protein. This cessation is related to the release of membrane proteins (15).

MATERIALS AND METHODS

HeLa S_3 cells were grown in suspension in Joklik-modified Eagle's minimal essential medium (MEM; GIBCO No. F-13) supplemented with 5% fetal calf serum. Cell density varied between 2×10^5 and 5×10^5 cells/ml. Cells were collected by centrifugation and resuspended at a density of 4×10^6 cells/ml in MEM buffered with 25 mM HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane sulfonic acid; Calbiochem). Proteolytic enzymes (trypsin, 2.5% solution; Microbiological Associates, or pronase, B grade; Calbiochem) and radioactive amino acids ([^{35}S]methionine, specific activity 190 Ci/mmole; Amersham Searle, or [^{3}H]alanine, specific activity 54 Ci/mmole; NEN) were added as indicated in the legends, and incorporation of labeled amino acids into cellular protein was measured by the filter paper disc method (16). Pronase was suspended in 20 mM Tris, pH 7.4, 100 mM NaCl at a concentration of 120 mg/ml. Insoluble material was removed by centrifugation at 12,000 g for 20 min at 4° C. The protein remaining in solution was diluted to 20 mg/ml with the same buffer.

RESULTS

Inhibition of Protein Synthesis in HeLa Cells by Exposure to Proteolytic

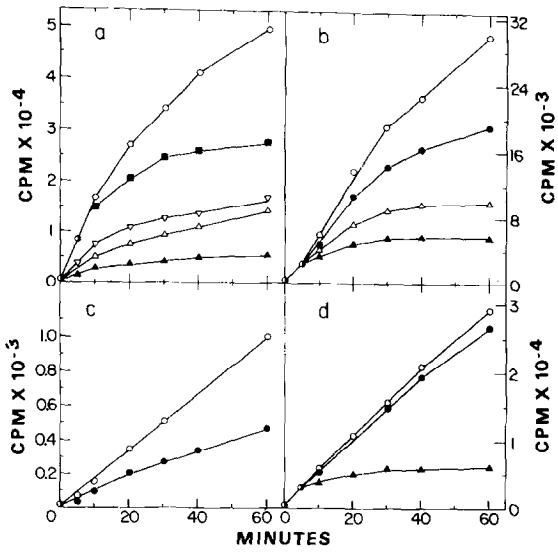

Fig. 1. Effects of Trypsin and Pronase on Amino Acid Incorporation into Cellular Protein.
Aliquots of concentrated HeLa cell suspensions (4 x 10^6 cells/ml) were incubated at 37° C with [^{35}S]methionine (10 µCi/ml) in the absence (control) or presence of various concentrations of trypsin or pronase. Protein synthesis was determined as described (17).
Panel a shows data obtained by incubation of cells (4 x 10^6/ml) in MEM with various concentrations of trypsin/ml:
o (control, 0 µg/ml); ■ 100 µg/ml; ▽ 500 µg/ml; Δ 1 mg/ml; ▲ 2 mg/ml.
Panel b shows data obtained by incubation of cells (4 x 10^6/ml) in MEM with various concentrations of pronase/ml; pronase was added to the cells 5 min after [^{35}S]methionine for final concentrations:
o (control, 0 µg/ml); • 20 µg/ml; Δ 100 µg/ml; ▲ 500 µg/ml.
Panel c shows the effect of 100 µg trypsin/ml on cells (4 x 10^5/ml) incubated in MEM supplemented with 5% fetal calf serum.
o (control, 0 µg/ml); • (100 µg/ml).
Panel d shows HeLa cell cultures with 4 x 10^6 cells/ml incubated with [^{35}S] methionine for 5 min at 37° C. One culture served as control (o), the other two received pronase at 5 min up to 500 µg/ml (▲) or pronase which had previously been exposed to 100° C for 2 min to 500 µg/ml (•).

Enzymes. The effect of varying concentrations of pronase and trypsin on the incorporation of radioactive amino acids into HeLa cell proteins is presented in Fig. 1. Upon addition of 20 µg of pronase/ml, the rate of protein synthesis declines to 40% after 20 min (Fig. 1b). With 100 or 500 µg pronase/ml, protein synthesis is inhibited immediately and ceases after 40 and 30 min, respectively. To obtain a corresponding degree of protein synthesis inhibition, 4-5 fold higher concentrations of trypsin are required (Fig. 1a). The presence of serum does not prevent this in-

hibitory effect of trypsin (Fig. 1c). Addition of heat-inactivated pronase (Fig. 1d) or trypsin (data not shown) results in no significant effect on protein synthesis, suggesting that proteolytic activity is required for the inhibition of protein synthesis.

The rapid inhibition of protein synthesis caused by exposure to proteolytic enzymes could be accompanied by either breakdown or stabilization of polyribosomes. Fig. 2 shows that the polyribosome profile is not altered significantly by a 10-minute exposure of the cells to 1 or 2 mg of trypsin/ml. Based on the assumption and general observation that inhibitors of elongation stabilize polyribosomes, this result suggests that the inhibition affects peptide chain elongation.

Restoration of Protein Synthesis After Removal of Proteolytic Enzymes By Centrifugation. To determine whether the inhibition of protein synthesis

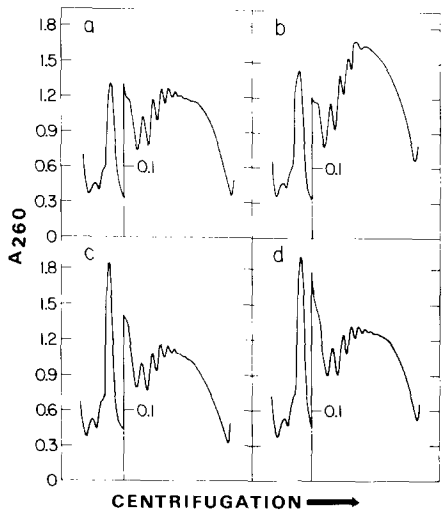

Fig. 2. Unaltered Polyribosome Profiles after Incubation with Trypsin. The preparation of cytoplasmic extracts from HeLa cells and the analysis of polyribosome profiles by centrifugation in 15-45% linear sucrose gradients has been described previously (17). Trypsin was added to HeLa cell cultures at a density of 4 x 10^6 cells per ml at 0° C to 1 mg/ml and 2 mg/ml. From the latter culture, one portion was lysed immediately (panel a), other portions were incubated at 37° C before lysis for 4 min (panel b) or 10 min (panel d). The culture which had received 1 mg trypsin/ml was also incubated for 10 min at 37° C before lysis (panel c).
Note that the scale was six-fold amplified at the beginning of the polyribosome profile.

caused by proteolytic enzymes is reversible, the incorporation of radioactive amino acids into proteins was examined after removal of proteolytic enzymes by centrifugation and resuspension of cells in fresh medium. As shown in Fig. 3, the rate of incorporation of [^{35}S]methionine in cells pretreated for 30 min at 37° C with 2 mg trypsin/ml was 90% of the control in the absence (Fig. 3a) and presence (Fig. 3b) of serum. The recovery of

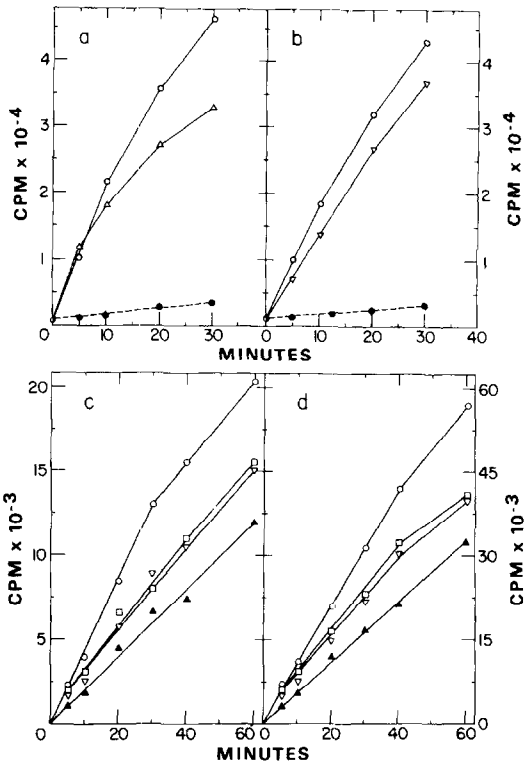

Fig. 3. Reversibility of the Inhibition of Protein Synthesis Induced by Trypsin or Pronase.
HeLa cell suspensions (4 x 10^6 cells/ml) were incubated for 30 min at 37° C in the absence and presence of 2 mg trypsin/ml. The cells were sedimented and resuspended in the original medium containing trypsin (●) or in an equal volume of fresh MEM: control, cells minus trypsin (o), cells previously incubated with 2 mg trypsin/ml and resuspended in fresh medium (Δ). All cultures received then [^{35}S]methionine (10 µCi/ml) and the incubation was continued at 37° C (panel a). The experiment was repeated as described above, but all cultures received 3% fetal calf serum after resuspension and with the addition of [^{35}S]methionine (panel b).
In another experiment, cells at 4 x 10^6/ml were incubated for 30 min at 37° C with various concentrations of pronase. After centrifugation and resuspension in MEM, the cells were incubated with [^{3}H]alanine (40 µCi/ml) (panel c) and [^{35}S]methionine (10 µCi/ml) (panel d):
o (control, 0 µg/ml); ▫ 20 µg/ml; ▽ 100 µg/ml; ▲ 500 µg/ml.

protein synthesis in HeLa cells from inhibition by pronase is shown in Fig. 3c and d. The incorporation of [^{35}S]methionine (Fig. 3d) and [^{3}H] alanine (Fig. 3c) was 70% of the control after pre-exposure to 20 or 100 µg pronase/ml and 55-60% after pre-exposure to 500 µg pronase/ml. The rapid recovery in protein synthesis after removal of proteases indicates that the viability of the cells is not severely affected. The number of cells which are stained with trypan blue does not increase by exposure to proteases. Furthermore, cells exposed to 100 µg pronase/ml or 500 µg trypsin/ml for 15 min at 37° C divide at the same rate as untreated cells upon return to normal growth medium.

DISCUSSION

Protein synthesis in HeLa cells is inhibited by a number of agents, including TPCK (13), DMSO (17), ethanol (18), hypertonicity (19) or cytochalasin B (20). All these agents in comparable concentrations produce little or no effect on *in vitro* protein synthesis in cell-free extracts prepared from HeLa cells or L cells (manuscript in preparation). These observations support the conclusion that protein synthesis *in vivo* can be regulated by membrane-mediated events (13, 21). Proteolytic enzymes have been used to release cells from density inhibition (6, 7) and to enhance influenza B virus replication (8). We were, therefore, surprised to find that incubation of HeLa cells at 37° C in the presence of pronase (100 µg/ml) or trypsin (500 µg/ml) results in a rapid inhibition of protein synthesis. Proteolytic enzymes exert their primary action on the cell membrane, suggesting that the inhibition of protein synthesis here is also membrane-mediated. The inhibition of protein synthesis by proteolytic enzymes is not accompanied by a breakdown of polyribosomes, suggesting that protein synthesis ceases by inhibition of peptide chain elongation. Incubation of HeLa cells for 30 min in the presence of 500 µg pronase/ml or 2 mg trypsin/ml and subsequent removal of the proteolytic enzymes by centrifugation and resuspension of cells in fresh medium results in a partial

(60-90%) but rapid recovery of incorporation of labeled amino acids into proteins. Heated enzyme solutions are ineffective with regard to HeLa cell protein synthesis. However, heated supernatants obtained by centrifugation of cells exposed to pronase, when added to other cells which had been pre-incubated with labeled amino acids for 5 min at 37° C, cause immediate inhibition of protein synthesis (15). Being that this inhibition is immediate and does not increase with time, it cannot be due solely to a reduced uptake of amino acids. The protease-released glycopeptides can be separated from proteolytic enzymes by ethanol precipitation and differential solubility in buffers with low ionic strength. Addition of the non-dialyzable glycopeptides (10 to 100 µg/ml) to suspended HeLa cells results in a rapid inhibition of protein synthesis (15). We conclude from these results that protease-released membrane components are responsible for the inhibition of protein synthesis reported here. The membrane components, when still cell-attached, might act likewise in cell-cell interactions and cause cessation of growth in cultures with high cell densities.

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