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PARTIAL CHARACTERIZATION AND PROPOSED MODE OF ACTION OF INHIBITORY HeLa CELL SURFACE POLYPEPTIDES

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Summary

Polypeptides removed from the HeLa cell surface by mild pronase treatment rapidly inhibit protein synthesis when added to HeLa cells or cell-free translation systems derived from HeLa cells. The inhibitory activity is heat stable. Protein and carbohydrate components of these polypeptides are required for inhibition of protein synthesis in vivo and in vitro. Two peaks of activity can be recovered from polyacrylamide gels, corresponding to polypeptides with molecular weights of approximately 29000 and 41000. Inhibition of protein synthesis in cell-free translation systems appears to be primarily an effect on elongation of polypeptide chains, whereas in the intact cell the primary target may be polypeptide chain initiation.

Introduction

Cell surface components may play an important role in regulating biosynthetic activities within the cell and in influencing interactions between cells. An earlier report from this laboratory described the inhibitory activity of cell surface polypeptides removed from HeLa cells by mild proteolysis [1]. The crude cell surface polypeptide preparation inhibits protein synthesis, thymidine incorporation and cell division but does not effect cell viability [2]. In this report, we present a preliminary characterization of the active HeLa cell polypeptides that are removed from the cell surface by mild pronase treatment and investigate their mode of inhibition of protein synthesis in vitro and in vivo.

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Abbreviations: HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; SDS, sodium dodecyl sulfate.

Materials and Methods

Assays for inhibition of protein synthesis. Crude cell surface polypeptides were prepared from HeLa S_3 cells as previously described [2]. Labeled polypeptides were prepared by the same method after treating the HeLa cells for 4 h with 2.5 μCi/ml [^{35}S]L-methionine (Amersham Searle, 5.4 Ci/mmol) in minimal essential medium containing 1/20 the normal concentration of methionine. The protein concentration of the polypeptide preparations was determined microfluorometrically [3]. Approximately 60 mg HeLa cell surface peptide is removed by treatment of $2.5 \cdot 10^{10}$ cells. This corresponds to about $2.4 \cdot 10^{-3}$ ng protein removed per cell. Inhibition assays, either in HeLa cells or HeLa cell-free translation systems were carried out as described by Fisher and Koch [2]. After incubation for up to 60 min at 37°C for the in vivo assay or 25°C for the in vitro assay, aliquots of the assay mixtures were spotted onto filter paper discs and trichloroacetic acid precipitable counts were determined by the method of Mans and Novelli [5].

Periodate oxidation, borohydride reduction. HeLa cell surface polypeptides were dialyzed overnight against 100 mM sodium acetate, pH 5.3, containing 10 mM EDTA. A 500 μl aliquot of the sample was treated with 20 μl freshly made 16.9 mM potassium periodate and incubated at 4°C in the dark for 2 h. A drop of glycerol was added to stop the reaction. Half of the sample was dialyzed against 20 mM HEPES buffer, pH 7.1, containing 120 mM KCl, 5 mM $MgCl_2$. The other aliquot was dialyzed for 2.5 h against 100 mM sodium phosphate buffer, pH 8. The latter sample was treated with approximately 0.6 mM sodium borohydride for 3 h in the dark at room temperature. For each 100 μl, 2 μl glacial acetic acid was added. After vigorous shaking, the sample was placed at 4°C for 30 min. The final oxidized and reduced product was dialyzed against HEPES buffer before assaying it for inhibitory activity.

Acid and alkaline hydrolysis. One aliquot of the cell surface peptide was boiled in 1 M HCl for 1 h; another was treated with 0.3 N KOH at 37°C overnight. The samples were neutralized and dialyzed against HEPES buffer before assaying for inhibition.

Cylindrical SDS polyacrylamide gels. 90 mm 12.5% SDS polyacrylamide gels with 10 mm 5% stacking gels were prepared as described by Laemmli and Favre [6]. Samples containing 200—500 μg unlabeled protein or approximately 15000 counts of [^{35}S]methionine-labeled material were layered on the gels. An immune precipitate of MPC-11 (mouse plasmacytoma) cell immunoglobulin H and L chains was used as a molecular weight marker. The H and L chain preparation was the gift of Dr. D.L. Nuss (Division of Laboratories and Research, New York State Department of Health, Albany). The gels were run at 2 mA/gel until the samples had migrated through the stacker at which time the current was increased to 3 mA/gel. The gels were crushed into 2 mm aliquots (unlabeled polypeptides for inhibition assay) or 1 mm aliquots (labeled polypeptides for counting).

Gel fractions were prepared for the inhibition assay by incubating the crushed gel in electrophoresis buffer overnight at 4°C. The soluble supernatant was removed and the SDS in the buffer was precipitated with 3 M KCl. After filtering the samples through 0.22 μm Swinnex filters (Millipore Corp.,

Bedford, Mass.) to remove small particles of acrylamide, the samples were dialyzed overnight against HEPES buffer. The inhibition assays were performed as described in either intact cells or in the cell-free translation system.

Polysome profiles. Polysomes were prepared by the method of Saborio et al. [7] from HeLa cells treated with HEPES buffer or with 3 mg/ml HeLa cell surface polypeptide at 37°C for 15 min or from the HeLa cell-free translation system treated with HEPES buffer or 125 μg/ml polypeptide at 25°C for 20 min. The absorbance of the gradients was monitored at 260 nm with a Gilford spectrophotometer (Gilford Instuments, Oberlin, Ohio).

Results

Inhibitory polypeptides from cell surfaces. HeLa cell surface polypeptides, when added to cells or to cell-free translation systems, decrease the rate of protein synthesis but not the rate of uptake of [^{35}S]methionine within 10—20 min depending on the concentration of polypeptide used [2]. Protein synthesis is completely inhibited after 20 min treatment of HeLa cells with 1.5 mg/ml or 750 μg/ml surface polypeptides (Table I). Treatment of cells with as little as 150 μg/ml for 60 min results in nearly 30% inhibition of protein synthesis. 100 μg/ml HeLa cell surface polypeptide added to the cell-free translation system immediately and completely shuts off protein synthesis. Con-

TABLE I

INHIBITION OF PROTEIN SYNTHESIS BY HeLa CELL SURFACE POLYPEPTIDES

In vivo assay: HeLa cells were treated with the indicated concentrations of HeLa cell surface polypeptide for 20 or 60 min as described [2]. In vitro assay: HeLa S_{10} cell-free translation system was treated with the indicated concentrations of peptide for 20 or 60 min as described. Inhibition of protein synthesis in both assays was determined by incorporation in treated and control samples of [^{35}S]methionine into trichloroacetic acid precipitable material as described in Materials and Methods.

In vivo assay		
Time of assay (min)	Concn. of polypeptides (mg/ml)	Percent inhibition of protein synthesis
20	1.5	72.0
60	1.5	87.9
20	0.75	52.0
60	0.75	79.3
20	0.15	20.0
60	0.15	29.3
In vitro assay		
Time of assay (min)	Concn. of polypeptides (μg/ml)	Percent inhibition of protein synthesis
20	100	100
60	100	100
20	25	86.6
60	25	83.6
20	6.25	48.8
60	6.25	43.3

TABLE II

NATURE OF THE INHIBITORY ACTION OF HeLa CELL SURFACE POLYPEPTIDES

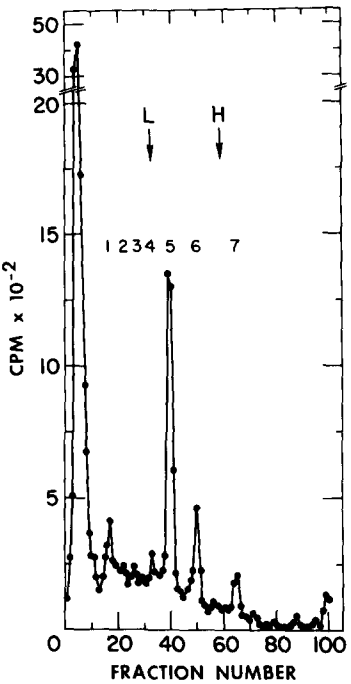
Crude HeLa cell surface polypeptides were treated with the agents above as described in the text and Materials and Methods. The treated samples, along with untreated controls, were assayed in HeLa S_3 cells as described. Incorporation of [^{35}S]methionine into trichloroacetic acid precipitable material after 60 min at 37°C was compared.

Treatment	Effect on inhibitory activity
Pronase (10 μg/ml, 37°C, 15 min)	Decreased by 25%
Alkaline hydrolysis	No effect
Acid hydrolysis	No effect
Periodate	Decreased to 10%
Periodate and borohydride	Restored to 80%
Heat (100°C, 2 min)	Decreased by 40%

centrations as low as 6.25 μg/ml inhibit protein synthesis in cell-free systems by nearly 50%.

Chemical nature of HeLa cell surface polypeptides. The crude polypeptide preparation was treated with a number of agents to determine which chemical components were required for activity. A summary of these results is shown in Table II. Treating crude HeLa surface polypeptide with 10 μg/ml pronase at 37°C for 15 min reduces inhibitory activity approximately 25%. Higher concentrations of pronase were not used because they interfere with the assay systems used. Acid and alkaline hydrolyses were carried out as described in Materials and Methods to determine whether nucleic acid, which may have contaminated the crude preparation, was necessary for inhibitory activity. Boiling the preparation in HCl reduces its inhibitory activity no more than does boiling it in buffer for the same amount of time. Neither the untreated control nor the alkaline-treated sample are reduced in inhibitory activity after overnight incubation at 37°C. Treatment with potassium periodate, as described in Materials and Methods, destroys inhibitory activity, indicating that carbohydrate is important for active HeLa polypeptide. The polypeptide may be labeled in vivo with [^{3}H]glucosamine and in vitro with boro[^{3}H]hydride which also indicates the presence of carbohydrate in the preparation (data not shown). If an aliquot of the same sample is reduced with sodium borohydride after periodate oxidation, most of the inhibitory activity is restored. Crude HeLa cell surface polypeptide is fairly heat stable. Approximately 60% of the inhibitory activity remains after 2 min at 100°C.

Identification of active HeLa cell surface polypeptides by polyacrylamide gel electrophoresis. Samples of the polypeptide preparation either unlabeled or labeled with [^{35}S]methionine were subjected to electrophoresis as described in Materials and Methods. The profile of [^{35}S]methionine labeled material, shown in Fig. 1, contains about 7 major polypeptide bands. Gels containing the unlabeled preparation were crushed and assayed for inhibitory activity in a HeLa S_{10} cell-free translation system as described [2]. The active fractions recovered from gels also inhibit protein synthesis when added to intact HeLa cells. As can be seen in Fig. 2, in addition to a small peak of activity running coincident with the dye front (fraction 5), two peaks of activity are recovered from the gel. Although the relationship between $\log_{10}$ molecular weight and relative migra-



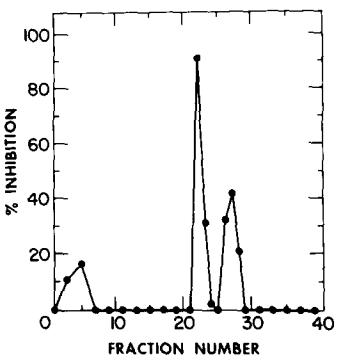


Fig. 1. Labeled polypeptides removed from HeLa cell surface by mild pronase treatment. [^{35}S]Methionine-labeled HeLa cell surface polypeptides were prepared and subjected to electrophoresis on 12.5% cylindrical SDS polyacrylamide gels as described in Materials and Methods. The Bromphenol Blue dye marker is found in fraction 5. The 5% stacking gel begins at fraction 91. Migration positions of myeloma cell immunoglobulin H and L chains (M_r 55000 and 22000, respectively) are marked with arrows.

Fig. 2. Recovery of active HeLa cell surface polypeptide fractions from gels. Unlabeled HeLa polypeptide was subjected to electrophoresis and the gels were crushed into 2 mm fractions as described in Materials and Methods. The fractions were assayed for inhibition of protein synthesis in the HeLa cell-free translation system.

tion of various polypeptides is not strictly linear in this gel and buffer system, an approximation of the relative molecular weights was obtained by comparison with migration of myeloma cell immunoglobulin H and L chains. R_f values of the inhibitory peaks are 0.42 and 0.53 in this gel system. The activity peaks correspond to [^{35}S]methionine labeled polypeptides 5 and 6, having approximate molecular weights of 29000 and 41000, respectively.

Mode of inhibition of protein synthesis in vitro and in vivo. In order to determine whether the inhibitory effect of HeLa cell surface polypeptides in the cell-free translation system and in cells is due primarily to an effect on initiation or elongation of polypeptide chains, polysome profiles were compared in treated and control systems. The HeLa S_{10} translation system reaction was completed as described in Materials and Methods except that [^{35}S]methionine was replaced by unlabeled L-methionine in the reaction mix. An A_{260} scan of the polysome gradients is seen in Fig. 3. After treatment, there is an increase in the region of large polysomes with a concomitant decrease in monosomes and small polysomes. This indicates that in vitro HeLa cell surface polypeptide primarily inhibits elongation of polypeptide chains. Another indication that

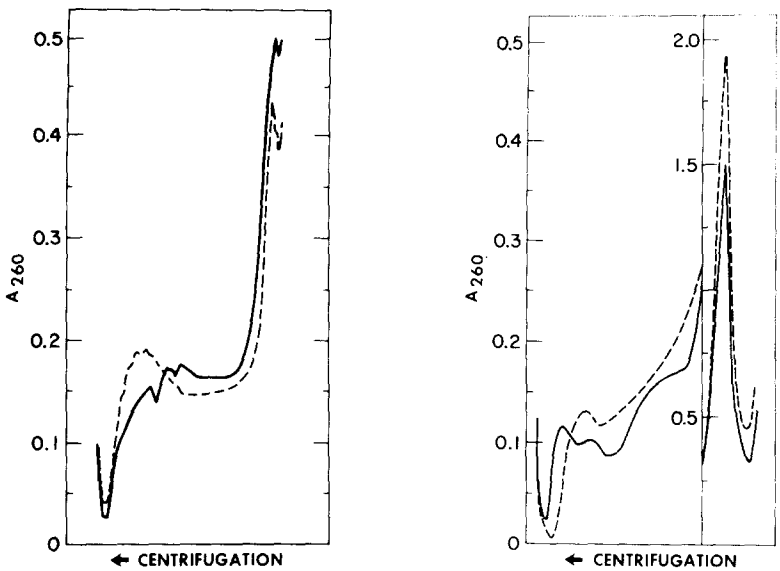


Fig. 3. Effect of HeLa cell surface polypeptides on HeLa S_{10} polysome profiles. Polysome profiles were obtained from untreated and treated HeLa S_{10} as described in Materials and Methods. The cell-free translation system was incubated with HEPES buffer or with 125 μg/ml HeLa peptides at 25°C for 20 min, and then immediately chilled. - - - - - -, Polysome profile of treated S_{10}; ———, polysome profile of control S_{10}.

Fig. 4. Effect of HeLa cell surface polypeptides on HeLa cell polysome profiles. Polysome profiles were obtained from control HeLa cells or cells treated with 3 mg/ml HeLa polypeptide at 37°C for 15 min. The polysome isolation procedure is detailed in Materials and Methods. - - - - - -, Polysome profile of treated HeLa cells; ———, polysome profile of control HeLa cells.

elongation of polypeptide chains is being affected can be found in Table I. The percent inhibition of the cell-free translation system does not increase with time after treatment; it is nearly the same relative to the control through 60 min. This is characteristic of a system where initiation of polypeptide chains is not altered.

Extracts were prepared after treatment of HeLa cells with the crude polypeptide. The polysome profile is shown in Fig. 4. The decrease in the number of larger polysomes is much smaller than would be expected for the amount of inhibition observed under these conditions. Treatment with 3 mg/ml HeLa cell surface polypeptide completely shuts off protein synthesis within 10 min and should, therefore, result in the complete breakdown of polysomes and a large increase in the monosome peak. The fact that the differences between treated and control cell polysome profiles are so slight may indicate that although initiation of polypeptide chains is primarily affected, elongation may also be inhibited. The results summarized in Table I show that compared to the control, the percent inhibition increases with time after treatment of HeLa cells. While this does not constitute definitive proof that polypeptide chain initiation is inhibited, such a pattern of inhibition is characteristic of initiation inhibition.

Discussion

We have described the isolation of heat stabile glycopolypeptides that inhibit protein synthesis and that are removed from cell surfaces specifically by protease treatment [2]. Two active components can be isolated from crude HeLa cell surface polypeptides which both inhibit protein synthesis in vivo and in vitro. The active components correspond to polypeptides with approximate molecular weights of 29000 and 41000.

Proteins found at the cell surface may be important in regulating many types of cell to cell interactions. It has been suggested that the presence or absence of such proteins may be responsible for differences observed in the behavior of contact inhibited and transformed cells [8—10]. The elevation of glycosidase and proteolytic activity occurring in transformed cells may alter cell surface glycopeptides [11—13]. These alterations become extremely important if the molecules involved take part in interactions such as cell-cell adhesion, cell-surface adhesion, contact inhibition, metastasis or interaction with extracellular macromolecules.

Within a cell population, two possible modes of action for regulation of protein synthesis by glycopolypeptides can be envisioned. The polypeptides on a cell's surface could impose a restraint on protein synthesis in that cell. Alternatively, polypeptides lost from the cell surface as a result of proteolysis may exert an inhibitory effect on all of the cells in the immediate environment. Considering the approximate yield of surface polypeptide from one cell (see Materials and Methods) and the fact that approximately 10% of the crude peptide actually enters the cell [2], it can be calculated that protein synthesis in 1 cell may be inhibited 30% in 60 min by the polypeptides removed from approximately 6 cells.

The elongation of polypeptide chains appears to be the site of inhibition of HeLa surface peptide-treated cell-free translation systems; however, the effect of the surface polypeptides on polysome patterns in the whole cells may be complicated by the fact that two components of the crude preparation inhibit protein synthesis. It is conceivable that one component could affect initiation while the other affects elongation of polypeptide chains. In order to solve this problem satisfactorily, the two purified polypeptides must be obtained in sufficient quantity to study individually their effects on the polysome profiles of treated cells.

A number of cell surface-derived polypeptides and glycopeptides have been described that exert inhibitory or enhancing effects on cell growth and macromolecular synthesis [14—16]. The HeLa cell surface polypeptides described in this report appear to be members of this class of regulatory molecules.

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