IDENTIFICATION OF A GLUCOCORTICOID-SENSITIVE HISTONE PROTEIN FROM MOUSE FIBROBLAST NUCLEI

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SUMMARY: The acid-soluble proteins from mouse fibroblast nuclei show a typical elution profile for histone proteins on Bio-Rex 70 columns. Glucocorticoid treatment of cells growing in vitro decreases labeled amino acid incorporation into one specific histone region, corresponding to a peak of a lysine-rich histone. The single histone affected is present in only small amounts in the cell; it normally represents about 6% of the H_1 protein, and less than 1% of the total histones. As measured by total protein nitrogen of the affected peak, glucocorticoid inhibition of this protein is first apparent between one and two hours after the start of steroid treatment.

INTRODUCTION

Mouse fibroblast cells growing in vitro are among many cell types which respond with metabolic changes after exposure to glucocorticoids. Inhibition of cell growth and decreased synthesis of DNA can be demonstrated readily after about 6 to 12 hours of steroid treatment (1). Even earlier, between 1 and 2 hr, there is a marked decline in the rate of hexose uptake by these cells (2). However, all these effects are thought to be secondary to some initial effect of the steroid after translocation of the cytoplasmic steroid-bound receptor to a nuclear locus.

In the rat uterus system, estrogen treatment has been demonstrated to increase the incorporation of labeled amino acid into a specific protein when soluble proteins of the uterus were separated by starch gel electrophoresis (3). This induced protein (IP) response to estrogen was shown to be blocked when RNA synthesis was completely inhibited (4) and can be prevented by some antiestrogens (5). All attempts by us to isolate a similar induced protein or even to demonstrate a differential incorporation of labeled amino acid into fibroblast

cytosol proteins as a result of glucocorticoid treatment were unsuccessful. That result prompted a shift in focus to the fibroblast nucleus where the effect of glucocorticoid treatment on the rate of synthesis of acid-soluble proteins was examined. We find that there is a single acid-soluble nuclear protein, synthesized under normal growth conditions by the L929 fibroblast, which is affected by glucocorticoids. Cells respond to steroid by an apparently decreased synthesis and a loss of this protein.

MATERIALS AND METHODS

Materials: The protein labeling mixtures of [^{14}C] or [^{3}H]L-amino acids were obtained from New England Nuclear, Boston, Massachusetts. Each mixture consisted of purified L-amino acids combined in the same relative proportions as found in a typical algal protein hydrolysate. Triamcinolone acetonide was purchased from Sigma Chemical Co., St. Louis, Missouri. Bio-Rex 70 was obtained from Bio-Rad Laboratories, Richmond, California. All other chemicals used were reagent grade and were used without further purification.

Cell culture and incubation: Monolayer cultures of mouse fibroblast L929 cells were maintained on Joklik medium and harvested as previously described (1). Cells were resuspended at approximately 10^7 cells/ml in growth medium without serum but with an amino acid level 100 times lower than the maintenance medium and buffered with 25 mM Hepes, pH 7.35. Triamcinolone acetonide (prepared at 100 times the final concentration desired in 10% ethanol) was added to give 10^{-7} M and the corresponding volume of 10% ethanol added to control aliquots. Solutions of the labeled amino acid mixtures were prepared in 250 mM Hepes buffer, pH 7.35 to give 20 µCi/ml for the [^{3}H] and 6 µCi/ml for the [^{14}C]L-amino acids, which were added when desired in the ratio of 1 ml to 100 ml of incubation volume. Incubations were performed at 37°C with constant stirring.

Preparation of acid-soluble proteins: The cell suspension was harvested by centrifugation at 600g for 10 min at 4°C and washed in 10 volumes of a cold balanced salt solution. The washed cells were resuspended in 1.5 volumes of hypotonic solution (0.1 mM EDTA and 10 mM Tris, pH 7.35) for 5 min, then homogenized with 15 strokes in a Dounce-type homogenizer. Hypertonic sucrose solution (1.25 M sucrose, 15 mM $CaCl_2$, 5 mM $MgCl_2$, and 250 mM Tris, pH 8.0) was added to bring the homogenate to 0.25 M sucrose and the suspension was centrifuged at 10,000g for 10 min. The 10,000g pellet was washed with 2 volumes of an isotonic sucrose solution (0.25 M sucrose, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 50 mM Tris, pH 7.5) containing 1% Triton X-100 and then centrifuged at 700g for 10 min. The washed pellet was resuspended in another 2 volumes of isotonic sucrose solution (without Triton X-100) and centrifuged at 700g for 10 min. The nuclear pellet was extracted with 5 volumes of 0.14 M NaCl in 10 mM Tris, pH 7.5 for 30 min at 4°C with stirring and then centrifuged at 7000g for 10 min. The 7000g pellet was resuspended in 2 volumes of 2 M NaCl in 10 mM Tris, pH 7.5 with 50 mM $NaHSO_3$ added as a protease inhibitor. The suspension was extracted in the presence of 0.25 N H_2SO_4 for 60 min at 4°C with constant stirring and then centrifuged at 10,000g for 20 min. The resultant supernatant was retained and the pellet was resuspended in 2 volumes of 2 M NaCl and extracted with 0.25 N H_2SO_4 for 16 hr at 4°C with stirring. The extract was centrifuged at 10,000g for 20 min, the two extracts pooled and the sulphates of the acid-soluble proteins were precipitated by addition of 5 volumes

of cold 100% ETOH and storage for 18 hr at -7° C. The precipitate was collected by centrifugation at 10,000g for 10 min, washed 2 times with 100% ETOH and dissolved in 1 volume of 7% guanidinium chloride in 0.1 M phosphate buffer, pH 6.8 (GuCl-PO_4).

Fractionation of acid-soluble proteins: The combined acid-soluble proteins were fractionated on a column (1.4 x 20 cm) of Bio-Rex 70, according to the method used by Fambrough and Bonner (6). The sample was applied to the column and eluted with a linear gradient of 7 - 15% GuCl-PO_4 (collecting 0.5 ml fractions) until a total volume of 90 ml was collected, then the column was washed with 30% GuCl-PO_4 until another 70 fractions were collected. Protein content in each fraction was determined by turbidity (absorption at 420 nm using a Brinkman PC/600 probe colorimeter) after addition of 2.5 ml of 1.25 M trichloroacetic acid to each sample (7).

Assays for radioactivity and total protein: The precipitate from pooled fractions (every 5 tubes) was collected on glass fiber filters (Gelman type A-E) using a Millipore multiple sampling manifold. Each filter was washed with 5 ml 0.5 M cold trichloracetic acid, then 5 ml cold H_2O, dried, and placed in a scintillation vial with 10 ml of a toluene scintillation solution and counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 3310. Protein nitrogen determinations on pooled samples were performed according to the method of Oyama and Eagle (8).

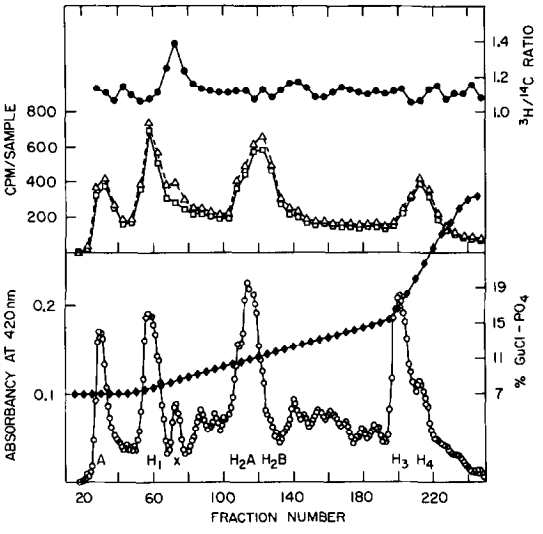

Figure 1: Fractionation of labeled fibroblast acid-soluble nuclear proteins on Bio-Rex 70. Replicate cell suspensions were incubated with either 10^{-7} M triamcinolone acetonide and [^{14}C] L-amino acid mixture or 0.1% ethanol and [^3H] L-amino acid mixture for 4 hr at 37°C with stirring. The cells were harvested at the end of the incubation period and the acid-soluble proteins were isolated as described under Materials and Methods. An equal volume of the acid-soluble proteins dissolved in 7% GuCl-PO_4 from the control and treated samples were mixed together and fractionated as described. Protein concentration as determined by turbidity at 420 nm (o), [^{14}C] radioactivity (□), and [^3H] radioactivity (Δ) were assayed as described under Materials and Methods. The concentration of GuCl-PO_4 (◆) in the effluent was determined by refractive index.

RESULT AND DISCUSSION

The elution of acid-soluble extracts of mouse fibroblast nuclei on a Bio-Rex 70 column yielded a protein pattern (Fig. 1) quite similar in its major profile to that obtained by Fambrough and Bonner with pea bud histones (6) and Barker, using rat uterine extracts (9). There is an initial peak (A) of material which is not retained by the resin and appears within one column volume of effluent. Three major histone peaks are apparent and these are denoted with the established nomenclature to correspond with their emergence from the column.

If control and glucocorticoid-treated cells are incubated in the presence of radioactive amino acids for 4 hrs at 37°C, the radioactivity profile of control cultures corresponds to the turbidity pattern and shows a similar degree of labeling for all the major histone peaks (Fig. 1). Using the double label technique (^{3}H amino acids in control cultures, ^{14}C amino acids in steroid-treated cultures) it can be seen that the degree of labeling is consistent for each isotope, resulting in a relatively uniform $^{3}H/^{14}C$ ratio except for fractions 70 - 75. At these fractions the ratio increases from a baseline level of 1.10 to 1.39. This reflects a greater incorporation of labeled amino acid into

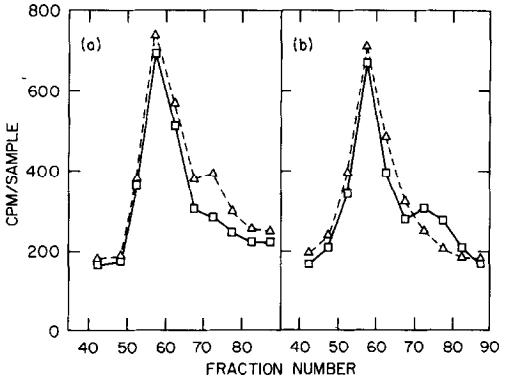

Figure 2: Effect of isotope reversal on labeled amino acid uptake by fibroblast histones. Replicate cell suspensions were incubated in the same manner as in Figure 1 (a) or with the isotopes reversed (i.e. control cultures received ^{14}C amino acids, steroid-treated cultures received ^{3}H) (b). See Figure 1 for legend.

these fractions by control cells relative to glucocorticoid-treated cells. Reversal of isotopes give comparable results (Fig. 2), confirming the decreased incorporation of radiolabeled amino acids in these particular fractions in cell cultures incubated with triamcinolone acetonide.

The time course of this glucocorticoid effect was examined by comparing the total protein nitrogen of both peak H_1 and fraction 70 - 75 (peak x) as a function of duration of steroid treatment (Table I). With respect to peak H_1 for both the control and treated samples the total protein nitrogen did not vary significantly. Similarly, peak x protein was quite similar in all control samples. However, the glucocorticoid-treated samples had total protein nitrogen values for peak x which displayed a steady decrease with longer treatment times. The effect is quite pronounced if the protein nitrogen content of peak x is expressed as a percent of the H_1 histone (Table I).

There is an absolute decrease of peak x, as well as reduced incorporation

Table I: Time course of glucocorticoid-induced effect on protein x. Replicate cell suspensions were incubated with either 10^{-7} M triamcinolone acetonide or 0.1% ethanol for the indicated time. The cells were then harvested and the acid-soluble protein from each sample was prepared and fractionated as described under Materials and Methods. One-half the volume of the tubes from fraction 45 to 85 of each sample was removed and the protein content determined as described using turbidity at 420 nm. The fractions comprising the H_1 and x peaks from each sample were pooled and the total protein nitrogen of each peak was assayed.

Duration of Treatment (hr)	Total Protein Nitrogen, H_1 (µg)		Total Protein Nitrogen, x (µg)		% Total Protein Nitrogen, x/H_1	
	Control	Treated	Control	Treated	Control	Treated
1/2	162.0	166.3	8.8	10.5	5.4	6.3
1	157.5	170.6	10.5	9.8	6.7	5.7
2	166.5	175.5	8.3	7.2	5.0	4.1
3	157.5	171.1	9.0	5.3	5.7	3.1
4	157.3	175.0	9.8	4.9	6.2	2.8
Mean Value	160.2	171.7	9.3	-	5.8	-

of labeled amino acid mixture, between 1 and 2 hour after glucocorticoid exposure. It is not apparent at this time if this effect is a result solely of curtailment of synthesis or whether there is some sort of displacement from chromatin and degradation of this histone protein following glucocorticoid treatment. Under control conditions the peak appears to be relatively stable since a 13 hour chase (data not shown) has no effect on the location or intensity of the radioactive peak.

A number of steroid hormones, including estradiol (3), progesterone (10), and aldosterone (11) have been shown to induce the synthesis of specific proteins in their respective target tissues. It is perhaps significant that all these systems are examples of the anabolic effects of steroids and thus far there has been no evidence to indicate that the same basic mode of action also holds for systems in which the overall response to the steroid is antianabolic. The physiological role of the x protein in fibroblast nucleus cannot be defined at the present time. However, it is tempting to speculate that this single histone protein, which accounts for less than 1% of the total histones, serves a key role in the biological action of glucocorticoids.

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