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NATURE OF THE DIFFERENCE IN APPARENT MOLECULAR WEIGHTS BETWEEN THE ALPHA SUBUNIT OF URINARY HUMAN CHORIONIC GONADOTROPIN AND THE ALPHA PROTEIN SECRETED BY HELA CELLS

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Summary

The glycoprotein hormone alpha subunit secreted by HeLa cells has an apparent molecular weight greater than that of urinary chorionic gonadotropin alpha as determined by chromatography on Sephadex G-75 superfine. The difference in gel elution patterns between these proteins is not observed when the HeLa protein is first incubated under conditions employed to remove sialic acid (53 mM H_2SO_4, 80°C, 90 min). Because the modified tumor protein produces a dose-response curve parallel to those of the unmodified tumor protein and the urinary hCG subunit, and is readily bound by ricin-agarose, it is concluded that the antigenicity of the protein has not been greatly altered and that the bulk of the carbohydrate side chains remain intact. These observations suggest that the greater apparent molecular weight of HeLa alpha compared to urinary hCG alpha may be due to an increase in the sialic acid content of the tumor protein.

Introduction

Human chorionic gonadotropin is a glycoprotein hormone composed of two nonidentical subunits, alpha and beta. Although normally synthesized in the developing placenta, it now seems to be well established that several types of tumors are also capable of expressing hCG[1] (1). The complete hormone and excess alpha subunit have been detected in cultures of choriocarcinoma cells (trophoblastic tumor lines), where they represent eutopic gene products (2,3). That is, they are proteins normally expressed by this cell type. More recently alpha and beta have been detected in the culture

[1] Abbreviations used: hCG, human chorionic gonadotropin; MSH, melanocyte stimulating hormone; RIA, radioimmunoassay; MEM, minimum essential media.

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media of several cell lines such as ChaGo (4-7) and HeLa (8,9), which are derived from non-trophoblastic tumors. In these instances, hCG and its subunits represent ectopic gene products, those not normally expressed by the cell type from which the tumor originated.

Lieblich et al. (9) have shown by gel filtration chromatography that the alpha-like protein secreted by HeLa cells elutes with an apparent molecular weight greater than that of the iodinated urinary hCG-alpha used as a marker. It has been proposed that this ectopic alpha may represent a precursor to the mature form of the hormone (9), thus suggesting that the structural gene for alpha was derepressed as a result of tumorogenesis but that the processing enzymes for proper precursor trimming were not. This communication is directed at further characterization of the high molecular weight HeLa protein and suggests that the difference in molecular weights between it and urinary hCG-alpha can probably be attributed to their carbohydrate moieties.

Materials and Methods

Materials. Crude human chorionic gonadotropin was purchased from Organon, Inc. (West Orange, NJ), purified, and dissociated into subunits as described previously (10). The isolated alpha subunit (CR-123) was also obtained from the Center for Population Research of the National Institute of Child Health and Human Development (NIH). Sodium butyrate was purchased from Matheson Coleman and Bell, and Sigma Chemical Co. supplied D-galactose. New England Nuclear was the source of Na ^{125}I for protein iodinations. Sephadex G-75 superfine was obtained from Pharmacia and ricin-agarose was supplied by Miles Laboratories or Sigma. Culture media and serum were obtained from Grand Island Biological Company. Culture flasks were from Corning.

Cell culture. HeLa S3 cells were maintained in spinner culture in Eagle's minimal essential medium (MEM) with spinner salts and supplemented with 6 mM L-glutamine, 6% calf serum, 80 units/ml of penicillin, and 80 µg/ml of streptomycin sulfate. Cells were plated in MEM into 75 cm^2 flasks at a density of about 10^5 cells/ml and grown at 37°C under a humidified atmosphere of 95% air and 5% CO_2 until cells were nearly confluent, generally two or three days. At that time media was replaced and where indicated, butyrate was added. After incubation (72 h) the media was collected, centrifuged to remove any detached cells, and chromatographed as described below.

Alpha radioimmunoassay. Purified alpha was iodinated by the lactoperoxidase method as described by Roth (11). Monomeric hormone was obtained by chromatography of iodination reaction mixtures on Sephadex G-100. The labeled protein used had an average specific activity of 40-95 µCi/µg.

Incubation mixtures for radioimmunoassay contained 0.1-0.5 ml of culture media or 1 ml of appropriate column fractions, rabbit anti-alpha serum (1:10,000 final diluton), ^{125}I-labeled alpha (approximately 80,000 cpm), and 50 mM potassium phosphate buffer (pH 7.4) containing 0.1% bovine serum albumin to a final volume of 1 ml. Mixtures were incubated at room temperature for 16-20 hr at which time 0.2 ml of a 10% (w/v) mixture of formalin treated, heat inactivated, Staphylococcus areus (Cowan I strain) was added and incubation continued at room temperature for 30 min. The immune conjugates were collected by centrifugation and washed once with 1 ml of cold 50 mM potassium phosphate buffer (pH 7.4) containing 0.9% (w/v) NaCl. The wash solution was combined with the original supernatant and counted along with the pellets in a well-type manual solid scintillation counter.

Acid hydrolysis of HeLa media. The method employed was a slight modification of that described by Eagon and Heath (12). Concentrated sulfuric acid was added to samples to a final concentration of 53 mM and heated in a water bath at 80°C for 90 min. They were cooled on ice and then neutralized with concentrated NaOH using the phenol red in the medium as an indicator. Samples were chromatographed immediately or stored frozen after dialysis against 50 mM potassium phosphate buffer (pH 7.4) containing 0.9% (w/v) NaCl.

Chromatography on Sephadex G-75 superfine. HeLa media samples (generally 3 ml), containing iodinated hCG-alpha (about 2 x 10^5 cpm), were applied to a 2.5 x 135 cm column of Sephadex G-75 superfine and eluted at 4°C with 0.1 M NH_4HCO_3 containing 0.02% NaN_3 at a flow rate of approximately 12 ml/h. Fractions of 2.5 ml were collected; these were assayed for urinary hCG-alpha by solid scintillation spectrometry and for HeLa alpha by RIA (1 ml aliquot). For molecular weight analyses, the column void volume and bed volume were determined from the elution volumes of blue dextran and phenol red, respectively. Elution of molecular weight markers was monitored by their absorbance at 280 nm.

Results and Discussion

Difference in apparent molecular weights of HeLa and urinary hCG-alphas. Figure 1 shows the relative elution positions of urinary hCG-alpha and HeLa alpha on a calibrated column of Sephadex G-75. The apparent molecular weights of the placental and HeLa proteins are 22,700 and 33,500 daltons, respectively.[2] These estimates are in reasonably good agreement with those reported by Lieblich et al. (9).

It has previously been demonstrated that a large molecular form of β-MSH associated with ectopic ACTH-producing tumors could be converted to the size of mature hormone by mild trypsinization, thus suggesting a precursor-product relationship between these two forms (13). Attempts in

[2] It is noted that the apparent molecular weight of hCG alpha is somewhat greater than that calculated from its amino acid and carbohydrate composition, M_r 14,900 (19). Such discrepancies are often observed when determining molecular weights of glycoproteins by gel filtration (20).

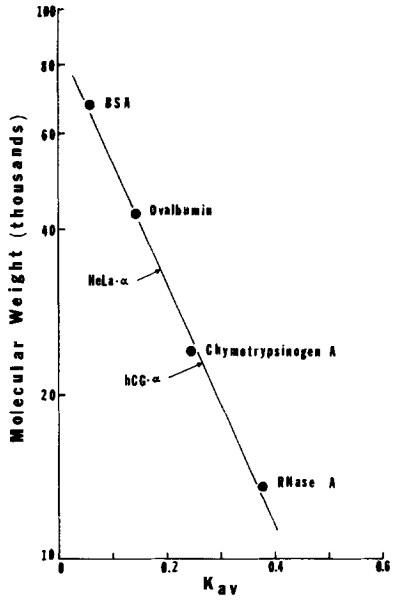

Figure 1. Molecular weight estimation of HeLa alpha by gel filtration. A mixture of iodinated hCG-α (~2 x 10^5 cpm) and HeLa media was chromatographed on a calibrated column (2.5 x 135 cm) of Sephadex G-75 superfine as described in Methods. Molecular weight markers were bovine serum albumin (BSA), M_r 68,000; ovalbumin, M_r 43,000; chymotrypsinogen A, M_r 25,000; and ribonuclease A, M_r 13,700. HeLa α was determined by RIA and urinary hCG-α was located by scintillation counting. On the abscissa, K_{av} is defined as $\frac{V_e-V_o}{V_t-V_o}$, where V_e is the sample elution volume, V_o is the column void volume, and V_t is the column bed volume.

this laboratory to convert the high molecular weight HeLa protein to a size characteristic of urinary alpha by mild proteolysis have thus far been unsuccessful. No change in the Sephadex elution profile could be observed until high concentrations of trypsin and long incubation times were employed; under these conditions the gel filtration profile suggested general degredation, not precursor conversion, since a mature-sized form did not accumulate (C. B. Jesse and C. S. Cox, umpublished data).

Effect of mild acid hydrolysis on the elution of HeLa alpha. Since mild proteolysis did not affect the elution profile of HeLa alpha, possible differences in the carbohydrate side chains of the tumor and placental proteins were examined. Conditioned media was collected from HeLa S3

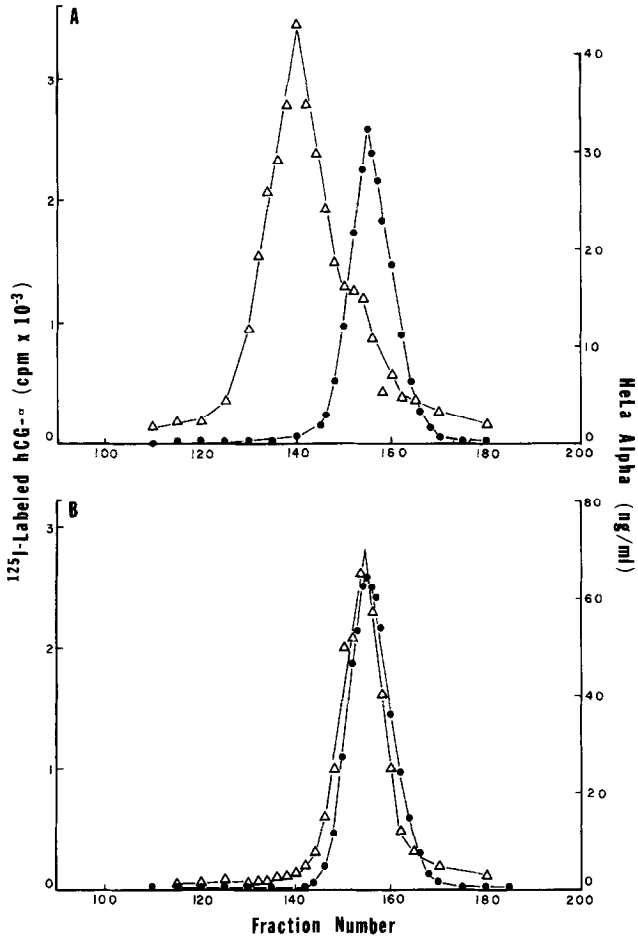

Figure 2. Effect of mild acid hydrolysis on the G-75 elution profile of HeLa alpha. Media from HeLa S3 cultures was concentrated by $(NH_4)_2SO_4$ precipitation and divided into two aliquots. One was chromatographed directly on G-75 superfine (A) while the other was chromatographed following mild acid hydrolysis (B) as described in Methods. In B, the radiolabeled marker was added after the HeLa media was cooled and neutralized. Symbols: ●—●, ^{125}I-labeled hCG-alpha marker; Δ—Δ, HeLa alpha (RIA).

cultures and concentrated by ammonium sulfate precipitation (90% saturation). The precipitates were collected by centrifugation, dialyzed against 0.1 M NH_4HCO_3, and chromatographed on G-75 superfine as described in Methods. As seen in Fig. 2A, the major peak of immunoreactive material (HeLa alpha) eluted ahead of the radioactive tracer (hCG alpha). However, when the culture media was first subjected to mild acid hydrolysis (see Methods),

the elution profiles of HeLa and hCG subunits were virtually indistinguishable as shown in Fig. 2B. Identical results were obtained regardless of whether the iodinated marker was alpha purified from commercial hCG in this laboratory or the subunit (batch number CR-123) distributed through the Center for Population Research of the Institute for Child Health and Human Development (NIH).

Gel filtration of HeLa alpha synthesized in response to sodium butyrate. Several laboratories have reported the induction of hCG and free subunits by mM concentrations of sodium butyrate in several cell lines (8,14,15). Hence, it was of interest to examine the gel filtration characteristics of this induced protein. Figure 3A shows the elution profile of alpha in HeLa media collected after 72 h of cell growth in the presence of 3 mM sodium butyrate. Under these conditions the alpha concentration in media from treated cells was 10- to 20-fold greater than that from control cultures. As seen, the immunoreactive alpha eluted ahead of the placental hCG-alpha marker, in a manner similar to that observed for the HeLa protein from uninduced cells. Likewise, the induced protein was shifted to a position of lower apparent molecular weight upon mild acid hydrolysis (Fig. 3B). Thus, the HeLa subunit from butyrate-induced and uninduced cultures chromatographed coincident with the urinary protein after incubation under conditions used to remove sialic acid from glycoproteins (12).

Characterization of HeLa alpha following mild acid hydrolysis. Parallel dose-response curves in the radioimmunoassay for the tumor protein before and after the acid hydrolysis indicate that its interaction with the antibody had not been grossly altered (Fig. 4A). Moreover, both proteins produced curves parallel to that of urinary alpha. This suggests that the HeLa protein was not bound to another component since binding of hCG-alpha to even the beta subunit produces a non-parallel response in the alpha RIA.

The hexose residue in the penultimate position (next to sialic acid) at the non-reducing end of the asparagine-linked carbohydrate side chains

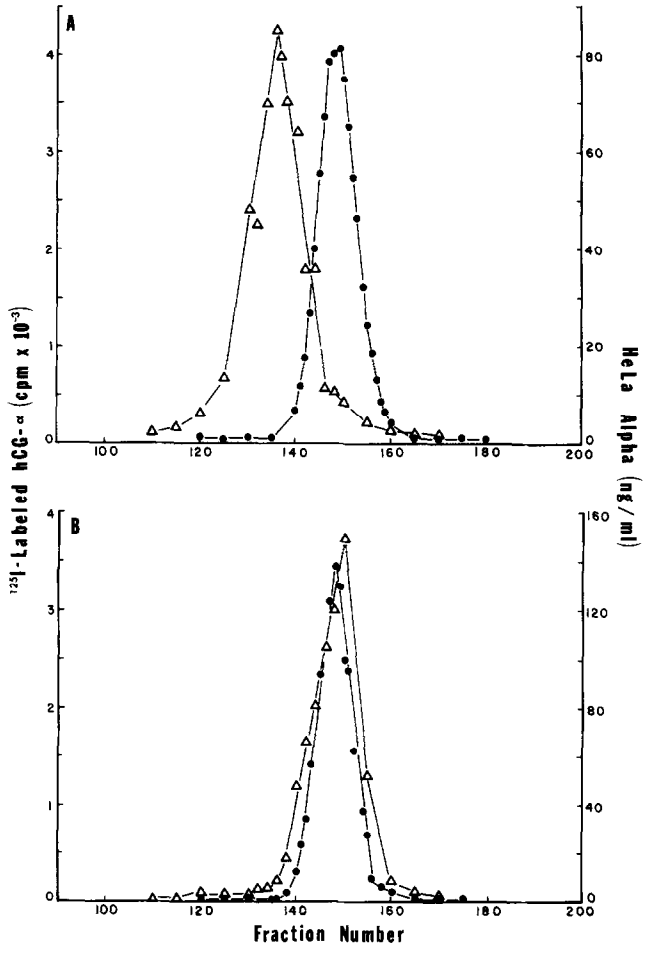

Figure 3. Gel filtration of butyrate-induced HeLa alpha. Media was collected from HeLa cultures exposed for 72 hr to 3 mM sodium butyrate and chromatographed on G-75 superfine without prior concentration as described in Methods. A) Media chromatographed with no prior treatment. B) Media chromatographed following mild acid hydrolysis (see Methods). In B, the ^{125}I-labeled urinary marker was added after the HeLa media was cooled and neutralized. Symbols: •—•, ^{125}I-labeled hCG-alpha standard; Δ—Δ, HeLa alpha (RIA).

of hCG-alpha is D-galactose (16-18). Thus, the complete retention of acid-treated HeLa alpha by ricin, a lectin which specifically binds D-galactose, indicates that the bulk of the carbohydrate side chains were not removed by the acid treatment (Fig. 4B). Control experiments have shown that only about 15% of the untreated HeLa protein will bind to ricin-agarose whereas greater

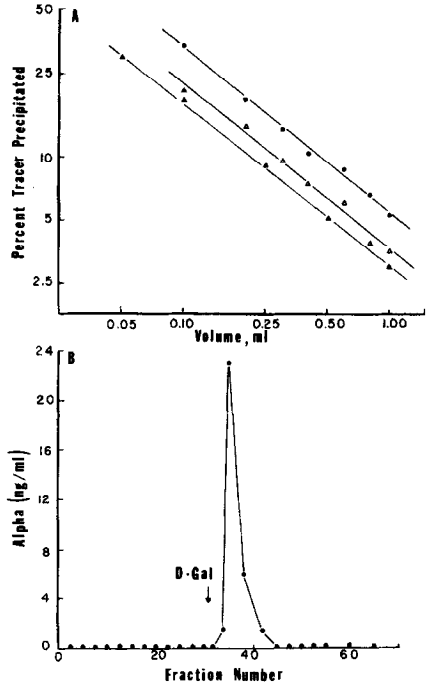

Figure 4. Characterization of HeLa alpha following mild acid hydrolysis. A) Dose-response curves were obtained by adding increasing volumes of either untreated alpha (●—●) or alpha following acid hydrolysis (Δ—Δ) to the standard RIA reaction mixture. The samples used were pooled fractions 135-137 (Fig. 3A) and pooled fractions 149-151 (Fig. 3B) for the control and acid-treated samples, respectively. Urinary hCG-alpha (▲—▲) at a concentration of 250 ng/ml is presented for comparison. B) Fractions 149-151 (Fig. 3B) were pooled and an aliquot was applied to a column (1 x 12 cm) of ricin covalently coupled to agarose and previously equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 0.9% (w/v) NaCl and 0.02% (w/v) NaN_3. After the sample had run in, the flow was turned off for 30 min. The column was then developed by first washing with 50 mM potassium phosphate buffer (pH 7.4) containing 0.9% (w/v) NaCl and then with the same buffer also containing 0.2 M D-galactose (arrow). One-ml aliquots were assayed for alpha.

than 75% will bind following mild acid hydrolysis.[3] These results are in agreement with the known property of sialic acid residues to hinder the binding of ricin to the underlying galactose residues in the oligosaccharide side chains (12).

It is of interest that a recent report by Fein et al. (21) indicates that immunoreactive hCG and free alpha subunit contained in first trimester

[3] Author's unpublished observations.

pregnancy serum and serum from two patients with hormone-secreting tumors initially chromatographed on Sephadex G-100 with apparent molecular weights greater than radiolabeled urinary hormone markers, but chromatographed with molecular weights similar to those of the urinary standard following treatment with a mixture of exoglycosidases. This would suggest that the hormones from the various sources examined were different primarily in their content of carbohydrate.

The present results suggest that the difference in apparent molecular weights between the alpha subunits of urinary hCG and HeLa S3 may be based on differences in their sialic acid content, though relative contributions from increased carbohydrate mass or changes in protein conformation cannot be evaluated. Thus, it seems unlikely that the HeLa protein represents a precursor form of the alpha subunit in the general sense, as previously suggested (9). However, small differences in the polypeptide structures of the two proteins unresolved by the chromatography employed cannot be ruled out. Direct measurements of sialic acid in the two proteins must await purification of the tumor subunit.

Acknowledgements

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