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Purification and Characterization of the Glycoprotein Hormone α-Subunit-like Material Secreted by HeLa Cells†

G. Stanley Cox* and Ronald A. Rimerman
Department of Biochemistry, University of Nebraska Medical Center, 42 Street and Dewey Avenue, Omaha, Nebraska 68105
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ABSTRACT: The protein secreted by HeLa cells that cross-reacts with antiserum developed against the α-subunit of human chorionic gonadotropin (hCG) has been purified approximately 30000-fold from concentrated culture medium by organic solvent fractionation followed by ion exchange, gel filtration, and lectin affinity chromatography. The final preparation had a specific activity (by RIA) of 6.8×10^5 ng of α/mg of protein and appeared homogeneous by electrophoresis on reducing/denaturing polyacrylamide gels (SDS-PAGE). Amino acid analysis indicated that HeLa-α had a composition very similar to that of the urinary hCG α-subunit. Peptide fingerprints of the HeLa protein and hCG-α revealed that several of the Tyr-, Met-, and Cys-containing tryptic peptides were held in common, thus identifying the tumor protein as a glycoprotein hormone α-subunit with a primary structure similar to that of hCG-α. However, comparison of hCG-α and HeLa-α demonstrated that the tumor-associated subunit was not identical with its normal counterpart. Only two of the three Tyr-containing tryptic peptides present in hCG-α could be detected in HeLa-α after iodination with ^{125}I. HeLa-α eluted prior to hCG-α during Sephadex G-75 chromatography, but the subunits coeluted when the tumor protein was first subjected to mild acid hydrolysis. The purified tumor protein had an apparent molecular weight greater than that of the urinary α-subunit when analyzed by SDS-PAGE (Coomassie blue staining), and this difference was even greater when a partially purified preparation was examined by an immunoblot technique (Western). Isoelectric focusing of the HeLa and hCG subunits demonstrated that the tumor protein had a lower p*I* (4.7-5.5 compared to 6.5-7.8), and removal of sialic acid by mild acid hydrolysis did not entirely eliminate this difference. Immunoprecipitation and electrophoresis of α-subunit from HeLa cultures labeled with [^{3}H]fucose indicated that the tumor subunit was fucosylated, whereas analysis of hCG-α hydrolysates by HPLC confirmed previous reports that the placental subunit does not contain fucose. HeLa α-subunit was unable to combine with hCG β-subunit to form holo-hCG under conditions where the hCG α-subunit was able to do so. The results indicate that, regardless of whether or not a single α-subunit gene is being expressed in both normal and neoplastic tissues, posttranslational modifications lead to a highly altered subunit in the tumor. The differences observed may be useful in diagnosing neoplastic vs hyperplastic conditions and may lend insight into the mechanism of ectopic hormone production by tumors.

Tumors often produce proteins not characteristic of the cell type from which the cancer originated (Odell & Wolfsen, 1975; Blackman et al., 1978). Such ectopic proteins serve as markers for neoplasia and are used clinically for the diagnosis and monitoring of cancer patients. In general, they are a nonrandom collection of polypeptide hormones, embryonic antigens, and fetal isoenzymes. Not all tumors elaborate ectopic products, though often a particular protein may be more commonly associated with a particular type of cancer. For example, oat cell carcinomas frequently secrete ACTH,[1] bronchial carcinomas often produce parathormone, and pan-

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* Address correspondence to this author.

[1] Abbreviations: ACTH, adrenocorticotropic hormone; ConA, concanavalin A; DTT, dithiothreitol; Fuc, fucose; FSH, follicle-stimulating hormone; Gal, galactose; GlcNAc, *N*-acetylglucosamine; hCG, human chorionic gonadotropin; HPLC, high-pressure liquid chromatography; LH, luteinizing hormone; Man, mannose; α-MG, methyl α-glucoside (methyl α-D-glucopyranoside); NeuNAc, *N*-acetylneuraminic acid (sialic acid); PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethanesulfonyl fluoride; POPOP, 1,4-bis(5-phenyloxazol-2-yl)benzene; PPO, 2,5-diphenyloxazole; RIA, radioimmunoassay; RPMI, Roswell Park Memorial Institute; SDS, sodium dodecyl sulfate; T, tryptic peptide; TPCK, N^{α}-tosylphenylalanine chloromethyl ketone; TSH, thyroid-stimulating hormone.

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creatic carcinomas commonly elaborate hCG.

The molecular basis for ectopic protein production is not known but is thought to occur by gene derepression (Odell, 1977). One approach to understanding this phenomenon is to purify and characterize the ectopic products, comparing their physicochemical properties to those of their normal counterparts. Such comparisons may suggest whether the same gene is being expressed in both the normal and neoplastic cells and may provide insight into possible differences in their routes of synthesis. Furthermore, if dissimilarities in protein structure are observed, these may be useful for differentiating between hyperplastic and neoplastic synthesis.

HeLa is a continuous cell line established from a cervical carcinoma (Gey et al., 1952). It has been recognized for some time that HeLa cultures elaborate an enzyme very similar to the placental isozyme of alkaline phosphatase (Elson & Cox, 1969). More recently, it was found that HeLa cells also secrete a protein that cross-reacts with antiserum directed against the glycoprotein hormone α-subunit (Lieblich et al., 1977). The glycoprotein hormones are a family of proteins comprised of two nonidentical subunits, α and β. These hormones (LH, FSH, TSH, CG) share a common α-subunit but have distinct (though similar) β-subunits, which bestow the biological specificity to the hormones. This paper describes the purification and characterization of the immunoreactive α-subunit-like material secreted by HeLa cells. Comparisons between the tumor protein and hCG α-subunit suggest that HeLa-α is similar to the glycoprotein hormone α-subunit but that it differs from its placental counterpart in several respects.

Experimental Procedures

Materials. Purified hCG, α-subunit, and β-subunit were obtained from the Center for Population Research of the National Institute of Child Health and Human Development (preparation CR-123). Bio-Rad Laboratories was the source of DEAE-cellulose (Cellex-D), acrylamide, Dowex AG-1X8 resin, the HPX-87C HPLC column, and pH 3–10 ampholytes (Bio-Lytes). Sephadex G-50, G-75 superfine, and G-100, QAE-cellulose, ConA–Sepharose, and protein A were from Pharmacia. Worthington Biochemicals supplied TPCK-trypsin, and cellulose thin-layer plates were obtained from Eastman. Sigma Chemical Co. was the source of soybean trypsin inhibitor, α-MG, Glc, Gal, Man, Fuc, PMSF, and Antifoam A. Solvents for thin-layer chromatography and 1,4-dioxane were Fisher certified reagents. Bacterial alkaline phosphatase and calf intestinal alkaline phosphatase were obtained from Bethesda Research Laboratories and Boehringer Mannheim Biochemicals, respectively. New England Nuclear supplied $Na^{125}I$, [^{3}H]fucose, iodo[^{14}C]acetic acid, [^{35}S]-methionine, EN^{3}HANCE spray, and endoglycosidases endo H and endo F, whereas [^{35}S]cysteine and NCS tissue solubilizer were purchased from Amersham.

Cell Culture. HeLa S3 cells were maintained at densities of (3–8) × 10^5 cells/mL in spinner culture in either minimum essential medium (MEM) or RPMI-1640 medium supplemented with 5% calf serum, 0.06% (w/v) L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. Spinner flasks were held at 37 °C in a humidified incubator under 5% CO_2/95% air. Generally, cultures were expanded to 20 L and then harvested by centrifugation. Sodium azide was added to 0.05% (w/v), and the conditioned medium was stored at 4 °C until 100 L was accumulated.

Purification of HeLa-α. Conditioned medium (100-L batches) was concentrated 10–20-fold on an Amicon hollow fiber dialyzer–concentrator with a H1P5 cartridge. The concentrated medium was stirred under a fume hood at room temperature with the slow addition of 1,4-dioxane to a final concentration of 50% (v/v). After the final addition, the mixture was stirred an additional 30 min at room temperature and then centrifuged at 8000*g* for 30 min at 4 °C. The supernatant fluid was removed and dialyzed extensively against running distilled water. Following dialysis, solid $(NH_4)_2SO_4$ was slowly added to 80% (w/v) and stirred about 30 min after solution was complete; no attempt was made to maintain the pH. The resulting precipitate was collected by centrifugation (8000*g* for 30 min), resuspended in 20 mM potassium phosphate (pH 8), and dialyzed against the same buffer.

This preparation (approximately 100 mL) was applied to a column of DEAE-cellulose (4.5 × 55 cm) equilibrated in 20 mM potassium phosphate (pH 8) and then developed first with 1.5 L of equilibration buffer and subsequently with a 3-L linear gradient formed from equal volumes of equilibration buffer and equilibration buffer containing 0.5 M NaCl. This and other chromatographic steps were carried out at 4 °C unless otherwise indicated. Ten-milliliter fractions were collected, and aliquots were assayed for immunoreactive α-subunit and material absorbing at 280 nm. As seen in Figure 1A, most of the HeLa subunit was not adsorbed on the resin and eluted in the application buffer. These fractions were pooled and concentrated by lyophilization.

The material was dissolved in 5 mL of 0.1 M NH_4HCO_3, clarified by centrifugation, and chromatographed on Sephadex G-75 (2.5 × 135 cm) equilibrated in the same buffer. Fractions of 2.5 mL were collected and assayed by RIA and A_{280} (Figure 1B). Fractions containing α-subunit were pooled and concentrated by lyophilization.

After the protein was redissolved in 20 mM Tris·HCl (pH 9.3), it was clarified by centrifugation and applied to a column (3 × 15 cm) of QAE-cellulose equilibrated in the same buffer. Following sample application, the column was developed first with 180 mL of equilibration buffer (20 mM Tris·HCl, pH 9.3) and then with a linear gradient constructed from 400 mL of equilibration buffer in the mixing chamber and 400 mL of equilibration buffer plus 0.6 M NaCl in the reservoir. Fractions of 5 mL were collected and assayed for α-subunit (RIA) and total protein (A_{280}). A representative profile is presented in Figure 1C. In contrast to DEAE-cellulose chromatography, where the HeLa subunit did not adsorb to the resin and eluted in the equilibration/application buffer, the tumor protein was bound to QAE-cellulose and was eluted in the salt gradient as two incompletely resolved components. These were designated A and B (according to their order of elution) and were further purified separately (results not presented). Because no apparent difference in the two fractions was noted during subsequent procedures and because the quantity of purified material was limiting, the A and B components were usually recombined.

Following this ion exchange chromatography, the HeLa protein was concentrated by lyophilization and subjected to lectin affinity chromatography on ConA–Sepharose (Figure 1D). The ConA column (1 × 15 cm) had been washed with 0.1 M sodium acetate buffer (pH 5.6) containing 0.5 M NaCl, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, and 0.02% (w/v) NaN_3 and then equilibrated in 50 mM potassium phosphate buffer (pH 7.4) containing 0.15 M NaCl and 0.02% (w/v) NaN_3. The lyophilized sample was dissolved in the phosphate–saline buffer, clarified by centrifugation, and allowed to slowly run into the resin. Chromatography was at room temperature rather than at 4 °C. When the sample had been applied, the column flow was halted for about 30 min and then resumed by washing the column matrix with one bed volume

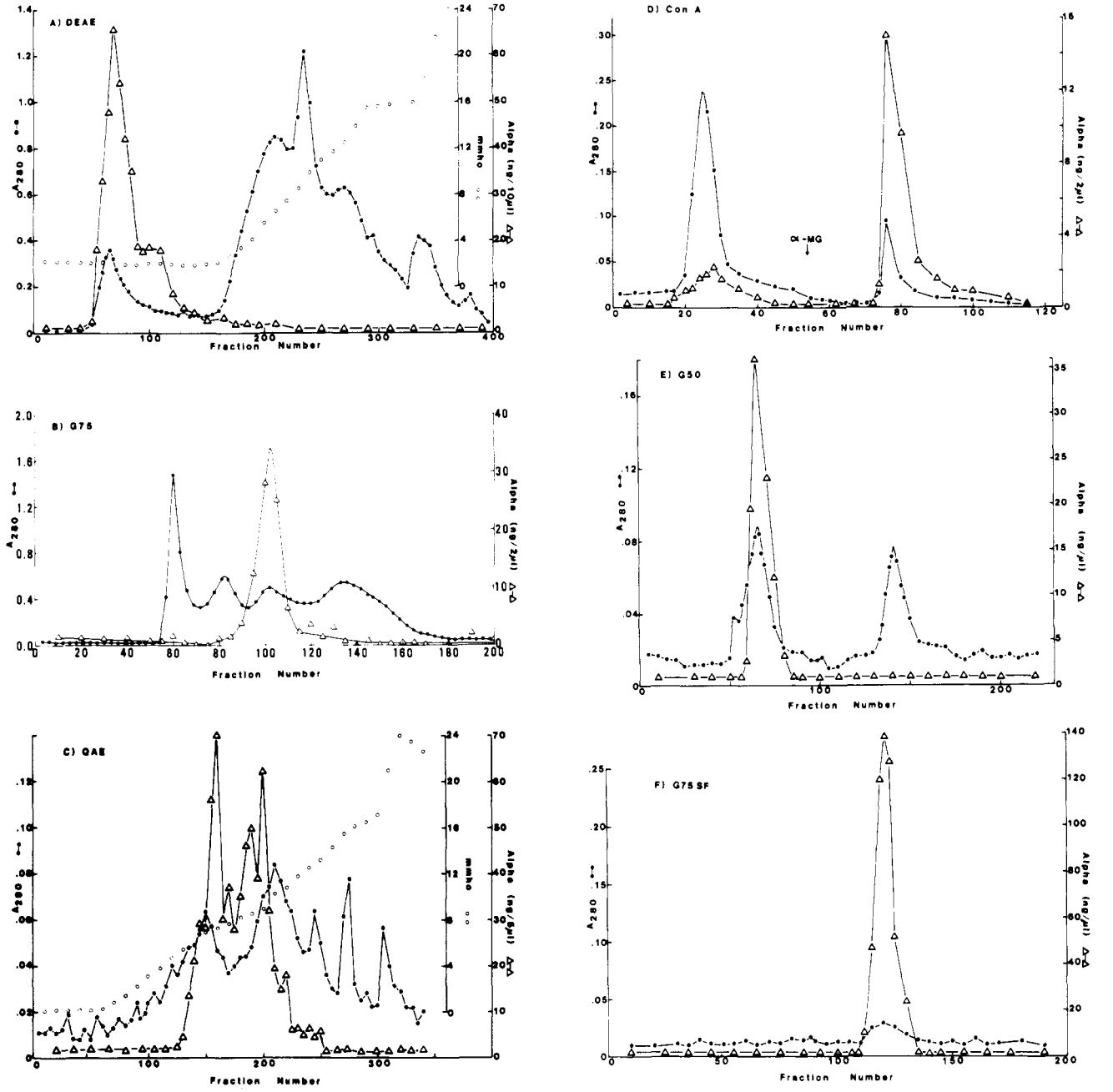

FIGURE 1: Purification of HeLa-α. Conditioned medium was concentrated, extracted with 50% (v/v) 1,4-dioxane, precipitated with solid $(NH_4)_2SO_4$, and sequentially chromatographed on (A) DEAE-cellulose, (B) Sephadex G-75, (C) QAE-cellulose, (D) ConA–Sepharose, (E) Sephadex G-50, and (F) Sephadex G-75 superfine. Conditions of chromatography were as described under Experimental Procedures. Symbols are (△) α-subunit as determined by RIA, (●) total protein as determined by A_{280}, and (○) salt gradient as determined by conductivity.

of phosphate–saline buffer. Material specifically bound to the immobilized lectin was eluted with phosphate–saline buffer containing 0.2 M α-MG (arrow in Figure 1D). Fractions of 1 mL were collected and assayed for α-subunit (RIA) and total protein (A_{280}). The majority of the HeLa protein was specifically bound to the ConA matrix and was subjected to further purification as indicated below. It has previously been demonstrated that both the bound and unbound material retain these elution characteristics when they are rechromatographed on ConA–Sepharose (Cox, 1981c). Nevertheless, the small amount of material eluting in the application buffer was not examined further.

Fractions containing immunoreactive material were concentrated by lyophilization and chromatographed on Sephadex G-50 (1 × 100 cm) equilibrated in 0.1 M NH_4HCO_3 (Figure 1E). Fractions of 0.5 mL were collected and assayed for α-subunit or total protein. The elution volume of immunoreactive HeLa-α was slightly larger than the column void volume; these fractions were pooled and lyophilized.

The solid material was dissolved in 0.5 mL of 50 mM CH_3COOH, clarified by centrifugation, and applied to a column (1 × 110 cm) of Sephadex G-75 superfine equilibrated in 50 mM CH_3COOH. Chromatography was carried out at room temperature rather than 4 °C; development was with 50 mM CH_3COOH; fractions of 0.5 mL were collected and assayed for α-subunit (RIA) and total protein (A_{280}). Appropriate fractions were pooled, lyophilized to dryness, dissolved in water, and lyophilized again. The solid material was taken up in 100 μL of distilled water; small aliquots were assayed for α-subunit by RIA (following dilution) and for total protein according to the method of Lowry et al. (1951).

Radiolabeling and Immunoprecipitation of HeLa-α. Cells were grown to near confluence in 75-cm^2 flasks containing MEM. This was replaced with 10 mL of fresh MEM con-

taining 2.5% dialyzed calf serum and no methionine or cysteine. Individual flasks received 100 μCi of either [^{35}S]-methionine (1020 Ci/mmol), [^{35}S]cysteine (1140 Ci/mmol), or [^{3}H]fucose (60 Ci/mmol). After 24 h, the media were harvested and chromatographed on Sephadex G-25 to remove unincorporated isotope. Fractions corresponding to the void volume were pooled, lyophilized, resuspended in 50 mM Tris·HCl (pH 7.4) buffer containing 0.5 M NaCl, 1% (v/v) Triton X-100, 1 mM PMSF, and 0.02% (w/v) NaN_3, incubated with formalin-treated, heat-inactivated *Staphylococcus aureus* cells (Cowan I strain, ATCC 12598), and clarified by centrifugation (12000*g* for 10 min). This step was included to deplete the samples of labeled material that would bind nonspecifically to IgG (from the calf serum) or to the *S. aureus* cells. Aliquots of 1 mL were incubated with 20 μL of preimmune or anti-α serum at room temperature for 24–48 h. Samples were supplemented with 300 μL of 10% (w/v) *S. aureus* cell suspension, incubated 1 h at room temperature, and centrifuged at 12000*g* for 15 min. The immune complexes were washed twice with 1 mL of 50 mM Tris·HCl (pH 7.4) and 0.15 M NaCl, three times with 1 mL of 50 mM Tris·HCl (pH 7.4), 0.15 M NaCl, 0.5% (w/v) Triton X-100, and 2 M urea, and twice with 1 mL of 10 mM Tris·HCl (pH 6.8). The final pellets were resuspended in a small volume of 8 M urea (for tryptic peptide analysis) or in a solution containing 8% (w/v) glycerol, 4% (v/v) 2-mercaptoethanol, 1.6% (w/v) SDS, and 20 mM Tris·HCl (pH 6.8) (for gel electrophoresis). The solutions were heated at 60 °C for 15 min, clarified by centrifugation, and analyzed as indicated below.

Polyacrylamide Gel Electrophoresis. Radiolabeled samples were immunoprecipitated as described above. After the immune complex was dissociated, bromphenol blue was added, and the samples (300 μL) were boiled for 5 min. An aliquot was counted by liquid scintillation spectrometry, and the remainder was applied to 6 × 130 mm cylindrical gels of 10% polyacrylamide with a 10-mm stacking gel of 3.5% polyacrylamide. The gel buffer and running buffer were as described by Laemmli (1970). After electrophoresis at 2 mA/gel, the gels were sliced mechanically into 1-mm sections, two of which were combined in 10 × 75 mm tubes, covered with 0.5 mL of distilled water, homogenized with a Teflon pestle, and incubated overnight at room temperature. The tubes were centrifuged briefly, and 300 μL of the supernatant was counted by liquid scintillation spectrometry. Analysis of purified subunits was on 1.5-mm slab gels of 3.5% (stacking) and 12.5% (separating) polyacrylamide in the Laemmli (1970) buffer system. Samples (40 μL) were treated as described for tube gels and electrophoresed at 25 mA. Visualization of the protein bands was by Coomassie blue staining.

Western Blot Analysis. The procedure used was a modification of that described by Towbin et al. (1979). Protein samples were electrophoresed at 20 mA through 12.5% polyacrylamide gels containing SDS as described above. When the dye front had migrated 10.5 cm, electrophoresis was terminated, and the slab gel was equilibrated for 30 min in 500 mL of transfer buffer [25 mM Tris base, 192 mM glycine, 20% (v/v) methanol]. Proteins in the gel were electrophoretically transferred to nitrocellulose (Millipore, 0.45-μm pore) at 40 V (100 mA) overnight and then at 63 V (200 mA) for 2.5 h at room temperature. The nitrocellulose membrane was incubated with shaking for 3 h at room temperature in blocking buffer containing 0.05% Tween 20, 0.01% Antifoam A, 0.001% merthiolate, and 5% (w/v) nonfat dry milk. The filter was incubated overnight at room temperature in a plastic sealable bag with 0.1 mL/cm^2 BLOTTO (Johnson et al., 1984) containing anti-α serum (1:1000 final dilution). The filter was washed five times for 5 min in 100 mL of buffer containing 50 mM potassium phosphate (pH 7.4), 0.15 M NaCl, 0.05% Tween 20, 0.02% NaN_3, 0.01% Antifoam A, and 5% (w/v) nonfat dry milk. Following the last wash, the filter was placed in a new bag containing ^{125}I-labeled protein A (10^6 cpm/gel lane) in BLOTTO (0.1 ml/cm^2), and incubation was continued at room temperature for 2.5 h. The filter was subjected to five washes (5 min each) in 200 mL of BLOTTO and three washes (5 min each) in 200 mL of H_2O prior to autoradiography.

Carbohydrate Analysis (HPLC). Purified proteins were hydrolyzed by a modification of the procedure described by Hough et al. (1972). All solutions were made with HPLC-grade water. Samples containing 100 μg of protein [approximately 10 nmol, based on the molecular weight of the polypeptide portion of hCG-α (Morgan et al., 1975; Bellisario et al., 1973)] were mixed with an equal volume of 2 M H_2SO_4 in a 1.5-mL polypropylene microcentrifuge tube. The sample was sealed under N_2 with Teflon tape under the cap and then heated 4 h at 100 °C in a vacuum oven. The hydrolysate was neutralized to pH 6 by the addition of Dowex AG-1X8 resin (HCO_3^- form) and then diluted with 300 μL of water. The liquid was transferred to a clean tube, and the resin was washed twice with 400 μL of water. The sample and washes were combined and passed through a nitrocellulose filter (Millipore, 0.2-μm pore) and then lyophilized and resuspended in 25 μL of water. The samples were injected into a 20-μL loop and pumped at 0.6 mL/min through a Bio-Rad HPX-87C column. Neutral monosaccharides were eluted with water while both column and solvent reservoirs were maintained at 85 °C. Constituents were detected by refractive index and identified by comparison of their retention times to those of purified standards.

Amino Acid Analysis. Approximately 10 nmol of hCG-α (CR-123) and HeLa-α were desalted by chromatography on Sephadex G-25 in 0.1 M NH_4HCO_3 and lyophilized to dryness in acid-washed glass vials. The residues were taken up in 6 M HCl and heated for 24 h at 110 °C under N_2. The hydrolysates were fractionated on a Beckman amino acid analyzer (Model 121MB) and a 2.8 × 370 mm column of Resin AA-10 (Beckman Instruments).

Tryptic Peptide Fingerprints. Lyophilized protein (250 μg of hCG-α plus radiolabeled HeLa-α) was dissolved in 0.4–0.6 mL of 0.5 M Tris-HCl (pH 8.2) containing either 7 M guanidine hydrochloride or 8 M urea, reduced with 10 mM DTT (added as 0.5 M solution) for 2 h at 37 °C under N_2, and then allowed to react with 22 mM iodoacetic acid (added as 0.8 M solution in 0.5 N NaOH) for 2 h at room temperature in the dark. The reaction was quenched with 3 μL of 2-mercaptoethanol followed by extensive dialysis against 0.1 M NH_4HCO_3 at room temperature. The carboxymethylated sample was digested for 5 h at 37 °C under N_2 with TPCK-treated trypsin at a ratio of 1:50 (w/w). The digest was lyophilized, then resuspended in 50 μL of electrophoresis buffer (5% acetic acid, 0.5% pyridine, pH 3.6), and blown dry under a slow stream of N_2. The dried sample was taken up in 5 μL of electrophoresis buffer and applied in 1-μL aliquots to a 20 × 20 cm cellulose thin-layer plate. The plate was sprayed with electrophoresis buffer and covered with a plastic sheet. Electrophoresis was performed at 6 °C with a Savant apparatus at 2000 V for 20 min. The plate was dried in a hood at room temperature and then chromatographed in pyridine/1-butanol/acetic acid/water (10:15:3:12). Following chromatography, the plate was dried at room temperature for 30

Table I: Summary for Purification of the Immunoreactive HeLa-α Protein[a]

fraction	total protein (mg)	total α-subunit (ng)	sp act. (ng of α/mg of protein)	recovery (%)	purification (x-fold)
concentrated media	4.0×10^5	9.1×10^6	23	100	1
dioxane (50%) supernatant	5.5×10^3	4.7×10^6	8.6×10^2	51.6	37
$(NH_4)_2SO_4$ (80%) precipitate	2.0×10^3	4.0×10^6	2.0×10^3	44.0	87
DEAE-cellulose (unadsorbed)	1.4×10^2	1.3×10^6	9.3×10^3	14.3	4.0×10^2
Sephadex G-75	31	1.0×10^63	3.2×10^4	11.0	1.4×10^3
QAE-cellulose	6.4	3.7×10^5	5.8×10^4	4.1	2.5×10^3
conA (bound)	2.5	3.6×10^5	1.4×10^5	4.0	6.3×10^3
Sephadex G-50	1.5	3.5×10^5	2.3×10^5	3.8	1.0×10^4
Sephadex G-75 superfine	0.3	2.0×10^5	6.8×10^5	2.2	3.0×10^4

[a] Details of procedures are given in the text.

min and then in a vented oven at 80 °C for 10 min. Plates were then sprayed with EN^{3}HANCE or a mixture of PPO and POPOP in acetone, dried, and exposed to Kodak XAR film at −70 °C. To detect nonradioactive peptides, plates were sprayed with ninhydrin reagent (1 g of ninhydrin, 0.1 g of cadmium acetate, 10 mL of water, 2 mL of acetic acid, 100 mL of acetone) and then heated at 80 °C for 5–10 min. The method of Yamada and Itano (1966) employing phenanthrenequinone was used to detect those tryptic peptides terminating in Arg; these corresponded to spots 4, 5, and 7 as depicted in Figure 3.

Other Methods. Specific radioimmunoassay (RIA) was used to determine α-subunit levels at all stages of purification and analysis. The tracer was prepared by radiolabeling hCG-α (CR-123) with Na^{125}I by a lactoperoxidase procedure (Roth, 1975). Polyclonal antisera were produced in New Zealand rabbits by standard immunologic techniques using purified hCG α-subunit as the antigen in Freund's complete adjuvant. The immune sera had titers ranging from 1:18 000 to 1:150 000. They exhibited no cross-reactivity with placental lactogen, insulin, or hCG β-subunit and about 2.5% cross-reactivity with intact hCG ($\alpha\beta$ dimer). Details of the assay have been described previously (Cox, 1981a). However, formalin-fixed, heat-inactivated *S. aureus* cells (Cowan I strain, ATCC 12598) were used to precipitate antibody–antigen complexes rather than second antibody as had been used previously.

For the radiologic detection of antigen–antibody complexes on the electroblot membranes (see above), protein A (5–10 μg) was iodinated with Na^{125}I by either lactoperoxidase or chloramine T. Labeled protein was separated from unreacted iodine by Sephadex G-100 chromatography in 50 mM potassium phosphate (pH 7.4) buffer containing 0.1% BSA and 0.02% NaN_3.

Protein concentration was determined by the method of Lowry et al. (1951). Ovalbumin was used as a standard because the color intensity (absorbance at 750 nm per microgram of protein) was closer to that of purified α-subunit than was that of bovine serum albumin.

Results

Purification of HeLa-α. As stated in the introduction, previous studies (Lieblich et al., 1977; Cox, 1981b) have demonstrated parallel dose–response curves for HeLa and hCG α-subunits in radioimmunoassays using polyclonal antisera, suggesting that the two proteins are immunologically very similar. The results presented below support identification of the HeLa protein as a glycoprotein hormone α-subunit yet demonstrate that it is different in both the polypeptide and carbohydrate moieties.

The material secreted by HeLa cells and immunologically cross-reactive with antisera to hCG-α has been purified about 30 000-fold as described under Experimental Procedures. Briefly, conditioned media were concentrated about 20-fold on an Amicon hollow fiber dialyzer–concentrator system. Over 98% of the serum proteins could be precipitated by 1,4-dioxane (50% v/v) with only marginal losses of HeLa-α (Table I). The tumor protein was then purified by sequential chromatography on DEAE-cellulose, Sephadex G-75, QAE-cellulose, ConA–Sepharose, Sephadex G-50, and Sephadex G-75 superfine (see Figure 1 for representative profiles). The final product had a specific activity of 6.8×10^5 ng of α-subunit (RIA)/mg of protein (Lowry) and migrated as a single band on SDS–PAGE (Figure 2) with an apparent molecular weight slightly greater than that of hCG α-subunit. To check for minor contaminants, an aliquot was iodinated with lactoperoxidase, separated from unincorporated ^{125}I by chromatography on Sephadex G-25, and analyzed by SDS–PAGE. Only a single radioactive component migrating with M_r 24 000 was evident (data not presented).

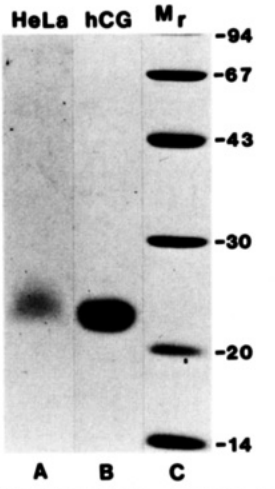

FIGURE 2: Analysis of purified HeLa-α by SDS–PAGE. The purified tumor protein (6 μg) was analyzed on a 12.5% polyacrylamide gel containing SDS in the buffer system by Laemmli (1970). Purified placental α-subunit (10 μg) and molecular weight markers (phosphorylase *a*, M_r 94 000; bovine serum albumin, M_r 67 000; ovalbumin, M_r 43 000; carbonic anhydrase, M_r 30 000; soybean trypsin inhibitor, M_r 20 100; α-lactalbumin, M_r 14 400) were run on parallel lanes. Following electrophoresis at 25 mA for 8 h, the gel was stained and destained. Lane headings indicate the purified HeLa α-subunit (A), urinary hCG α-subunit standard (B), and molecular weight markers (C).

Amino Acid Composition of HeLa-α. The amino acid composition of HeLa-α was determined as described under Experimental Procedures and is presented in Table II, where it is compared to previously published values for the placental α-subunit (Weinbraub et al., 1975; Morgan & Canfield, 1971; Bahl et al., 1972) and an ectopic α-subunit purified from a gastric carcinoid tumor (Weintraub et al., 1975). Of the 15 amino acids determined for HeLa-α, most were in good

Table II: Amino Acid Composition of HeLa-α

residue	hCG-α[a]	HeLa-α[a]	hCG-α[b]	A.L.-α[c]	sequence[d]
Ala	4.7	4.6	7.0	5.0	5
Arg	3.1	2.6	3.0	3.1	3
Asp	5.5	6.8	8.8	9.0	6
Glu	8.6	8.4	10.0	10.8	9
Gly	3.8	3.7	8.3	9.3	3
His			4.1	3.4	3
Ile	1.7	1.5	1.0	3.2	1
Leu	5.0	6.1	3.0	5.6	4
Lys	6.0	4.3	5.5	7.1	6
Met	1.9	2.1	1.8	2.4	3
Phe	3.6	3.8	4.5	3.4	4
Pro	7.0	7.0	8.0	8.0	7
Ser	8.6	7.7	8.3	7.4	8
Thr	8.5	7.5	8.2	7.2	8
Tyr	4.5	2.4	3.6	2.5	4
Val	6.5	5.9	7.8	9.8	7
$^1/_2$-Cys			7.1	2.6	10

[a] This study, duplicate determinations of 24-h hydrolysate. [b] Taken from Swaminathan and Bahl (1970). [c] Taken from Weintraub et al. (1975). A.L.-α refers to an ectopic subunit purified from a gastric carcinoid tumor. [d] Taken from Morgan et al. (1975).

agreement with previously published composition and sequence values for hCG-α and to the composition of urinary α-subunit determined in parallel. These data indicate significant similarity among the α-subunits purified from a gastric carcinoid tumor and continuous cell lines established from cervical and bronchogenic carcinomas. The differences observed must be viewed with some caution, however, since the limiting quantities of purified HeLa-α permitted only a duplicate determination at a single hydrolysis time. A similar determination for hCG-α (CR-123) is presented for comparison to the published composition and as an indication of the experimental error inherent in the present determinations.

Tryptic Peptide Fingerprints of hCG and HeLa α-Subunits. Structural similarity between the normal and tumor proteins was investigated by comparing their tryptic peptide fingerprints. Because amounts of the purified HeLa subunit were limiting, these analyses were performed with radiolabeling techniques. The amino acid sequence of hCG-α (arranged as tryptic peptides starting at the amino terminus) is presented in Table III. It can be seen that by labeling cell cultures with

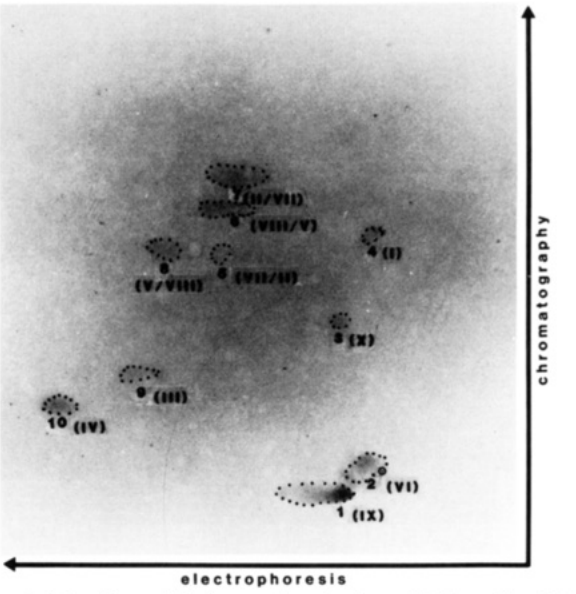

FIGURE 3: Tryptic peptide fingerprint of urinary hCG-α. The hCG α-subunit (CR-123, 250 μg) was reduced, carboxymethylated, digested with trypsin, and analyzed on cellulose thin-layer plates by two-dimensional electrophoresis and chromatography as described under Experimental Procedures. Electrophoresis at pH 3.5 (right to left) and chromatography (bottom to top) are indicated by the arrows, and the origin is designated "o". Peptides were visualized with ninhydrin spray and numbered arbitrarily to facilitate discussion. Spots 3 and 10 have been identified as free serine (TX) and lysine (TIV), respectively.

[^{35}S]cysteine or [^{35}S]methionine, or by iodinating the purified subunits with ^{125}I, it is possible to tag 7 of the 10 tryptic peptides (appropriate residues have been underlined). It is possible that a peptide corresponding to Lys-TV could also be generated, but no evidence for this could be detected in two-dimensional chromatograms of hCG-α stained with ninhydrin. Low yields of this peptide cannot be ruled out, however, and might serve to partially explain the presence of additional, minor spots from radiolabeled HeLa-α (see below). A tryptic peptide fingerprint for standard hCG-α (CR-123) is presented in Figure 3, where the peptides have been detected with ninhydrin and arbitrarily labeled (Arabic numerals).

Table III: Tryptic Peptides of Authentic hCG α-Subunit[a]

TI.	1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33 34 35 NH_2-ALA PRO ASP VAL GLN ASP CYS PRO GLU CYS THR LEU GLN GLU ASN PRO PHE PHE SER GLN PRO GLY ALA PRO ILE LEU GLN CYS MET GLY CYS CYS PHE SER ARG	
TII.	36 37 38 39 40 41 42 ALA TYR PRO THR PRO LEU ARG	
TIII.	43 44 SER LYS	
TIV.	45 LYS	
TV.	46 47 48 49 50 51 THY MET LEU VAL GLN LYS	
TVI.	52 53 54 55 56 57 58 59 60 61 62 63 ASN VAL THR SER GLU SER THR CYS CYS VAL ALA LYS CHO	
TVII.	64 65 66 67 SER TYR ASN ARG	
TVIII.	68 69 70 71 72 73 74 75 VAL THR VAL MET GLY GLY PHE LYS	
TIX.	76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 VAL GLU ASN HIS THR ALA CYS HIS CYS SER THR CYS TYR TYR HIS LYS CHO	
TX.	92 SER-COOH	

[a] Sequence was as determined by Morgan et al. (1975), Bellisario et al. (1973), and Fiddes and Goodman (1979).

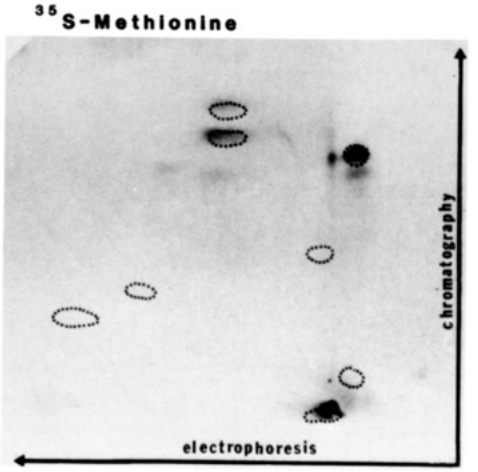

FIGURE 4: Tryptic peptide fingerprint of HeLa-α labeled with [^{35}S]methionine. Cells were labeled with [^{35}S]methionine, and the HeLa α-subunit was immunoprecipitated as described under Experimental Procedures. After the immune complex was dissociated in 8 M urea, 250 μg of hCG-α was added, and the mixture was reduced, carboxymethylated, digested with trypsin, and analyzed on cellulose thin-layer plates by two-dimensional electrophoresis and chromatography as described under Experimental Procedures. Peptides from the tumor protein were visualized by fluorography while those from the placental protein were detected with ninhydrin (broken lines).

Urinary hCG-α was added as carrier whenever radiolabeled HeLa proteins were analyzed, but in some experiments all ten peptides were not visible. The Roman numerals in parentheses refer to the tryptic peptides TI–TX as listed in Table III. These have not been identified unequivocally, but tentative assignments were made on the basis of radiolabeling data (Cys, Met, Tyr), phenanthrenequinone staining (Arg), and migration of standards (Lys, Ser); two numbers indicate a greater degree of uncertainty.

Examination of Met-containing peptides was carried out by incubating HeLa cultures with [^{35}S]methionine followed by immunoprecipitation of the medium with anti-α serum. The immune complexes were washed extensively, solubilized in 8 M urea, reduced, carboxymethylated, digested with trypsin, and analyzed by two-dimensional electrophoresis and chromatography (Figure 4). As seen from the autoradiogram, three prominently labeled peptides were coincident with ninhydrin-positive peptides derived from the urinary α-subunit added as carrier (spots 1, 4, and 6). In addition, three less intense autoradiographic signals were observed that migrated similarly to spots 5, 7, and 8. It is noted that ninhydrin staining did not reveal peptides 5 and 8 in this or in a repetition of this analysis. The primary sequence for hCG-α (Table III) indicates that there should be three or four Met-containing tryptic peptides (TI, TV, TVIII, and Lys-TV). On the basis of our tentative assignments, the results supported this prediction since peptides corresponding to spots 4 (TI), 6 (TVIII), and 8 (TV) were all radiolabeled. The radioactivity in apparent association with spots 1, 5, and 7 cannot be accounted for at present but probably originated from nonspecifically adsorbed material in the immunoprecipitates, or from minor peptides such as Lys-TV. Nevertheless, coincidence of prominent autoradiographic spots (HeLa-α peptides) with ninhydrin spots (hCG-α peptides) argues for extensive similarity in the primary sequences of both subunits.

Tryptic peptides containing cysteine were similarly examined. For the tumor protein, cells were incubated with [^{35}S]cysteine, and the labeled subunit was immunoprecipitated and analyzed as described above. The fingerprint obtained

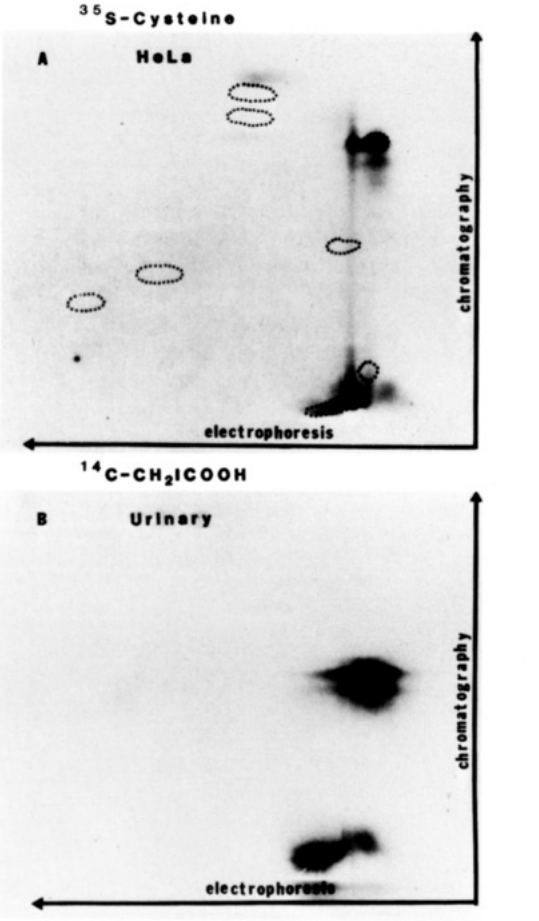

FIGURE 5: Fingerprints of cysteine-containing tryptic peptides from HeLa and hCG α-subunits. (Panel A) HeLa α-subunit was labeled in culture with [^{35}S]cysteine and immunoprecipitated as described under Experimental Procedures and the legend to Figure 4. (Panel B) hCG α-subunit was denatured in 8 M urea, reduced with DTT, and then carboxymethylated with ^{14}C-labeled iodoacetic acid to label the Cys-containing peptides. The reaction was quenched by the addition of excess 2-mercaptoethanol, and unreacted $^{14}CH_2ICOOH$ was removed by dialysis. Both samples (A and B) were digested with trypsin and subjected to two-dimensional analysis as described under Experimental Procedures. Radioactive peptides were detected by fluorography; broken lines denote ninhydrin-positive spots from hCG-α added as carrier.

was compared to that of purified hCG-α that had been reduced and carboxymethylated with $^{14}CH_2ICOOH$ prior to trypsin digestion. The resulting autoradiograms (Figure 5) show that both digests had peptides 1, 2, and 4 in common. These are most likely the three tryptic peptides predicted from the amino acid sequence (Table III and Figure 3). Again, there were additional (though weaker) signals in the HeLa-α digest. The "stutter" of spot 4 was seen for both the HeLa and urinary subunits; it is thought that this may result from heterogeneity of the amino-terminal peptide.

Tyrosine-containing peptides were examined in the experiment illustrated in Figure 6. Purified HeLa-α and hCG-α were labeled with ^{125}I in the presence of chloramine T, digested with trypsin, and fingerprinted as described in the figure legend. Examination of the primary sequence for hCG-α (Table III) indicates that the four Tyr residues occur in three tryptic peptides, and the autoradiogram presented in Figure 6A is consistent with this prediction; these peptides have been numbered 1, 5, and 7 in the figure. As seen in panel B, there were two Tyr-containing tryptic peptides in digests of HeLa-α which migrated identically with peptides 1 and 5 of hCG-α; however, peptide 7 was not detected. The doublet at spot 5

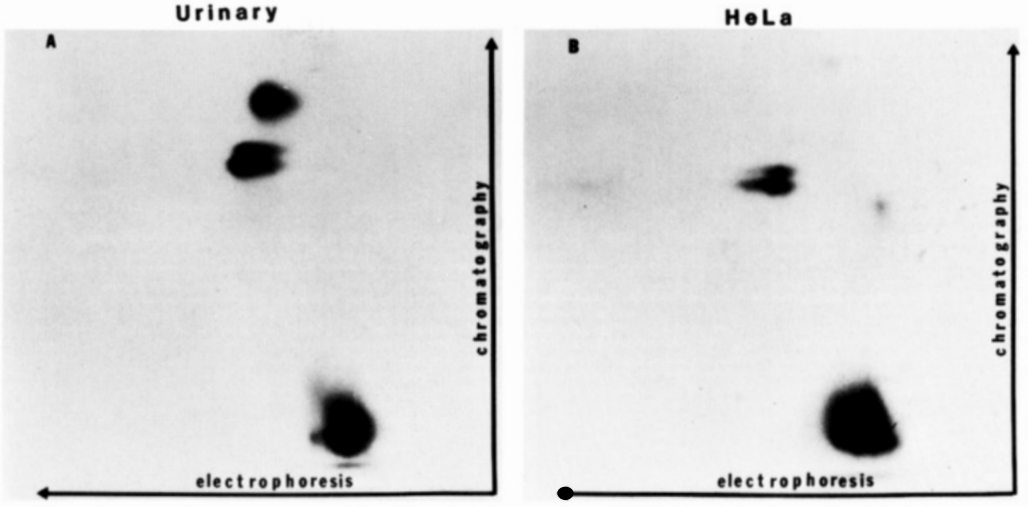

FIGURE 6: Analysis of tyrosine-containing peptides in tryptic digests of HeLa-α and hCG-α. Partially purified HeLa-α and hCG-α (5 μg each) were labeled with 500 μCi of $Na^{125}I$ at room temperature for 2 min in mixtures containing 50 μL of 0.25 M sodium phosphate buffer (pH 7.2), 5–10 μL of protein (5 μg), and 25 μL of chloramine T (4 mg/mL); reactions were terminated by the addition of 100 μL of $NaHSO_3$ (2.5 mg/mL). The entire mixture was applied to a column of Sephadex G-100 and eluted with 0.1 M NH_4HCO_3. Those fractions corresponding to α-subunit were pooled, lyophilized to dryness, immunoprecipitated as described under Experimental Procedures, and resuspended in 7 M guanidine hydrochloride. The samples were supplemented with 250 μg of hCG-α as carrier, then reduced, carboxymethylated, digested with trypsin, and analyzed by electrophoresis and chromatography on cellulose thin-layer plates as described under Experimental Procedures. Radiolabeled peptides were detected by autoradiography.

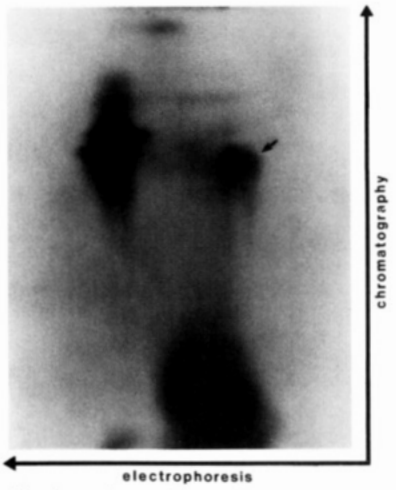

FIGURE 7: Iodination of tryptic peptides in HeLa α-subunit after pretreatment with alkaline phosphatase. Partially purified HeLa-α (3 μg) was incubated 4 h at 37 °C with bacterial alkaline phosphatase (400 units) and calf intestinal alkaline phosphatase (85 units) in 30 μL of a solution containing 50 mM Tris·HCl (pH 8), 1 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 0.5 mM PMSF, 0.1 μg of soybean trypsin inhibitor, and 0.1 μg of aprotinin. The mixture was iodinated with chloramine T, immunoprecipitated with anti-α serum, digested with trypsin, and subjected to bidirectional electrophoresis and chromatography as detailed in Figure 6.

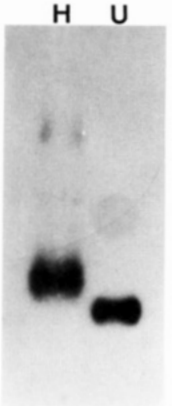

FIGURE 8: Western blot analysis of HeLa and hCG α-subunits. Purified hCG-α (2 μg) and partially purified HeLa-α (3 μg) were electrophoresed in polyacrylamide gels containing SDS and analyzed by an immunoblot technique following electrophoretic transfer to nitrocellulose (for details, see Experimental Procedures). H, HeLa-α; U, urinary hCG-α.

is thought to represent a single peptide; only one spot is present when the protein is iodinated with lactoperoxidase, whereas two appear when iodinations are performed with chloramine T.

Absence of one of the three predicted Tyr-containing peptides in tryptic digests of ^{125}I-labeled HeLa-α is intriguing (Figure 6). On the basis of indirect evidence, we tentatively identify the missing peptide as TII (residues 36–42), although peptide TVII cannot be ruled out. Failure to label this peptide could result if the corresponding Tyr residue were missing, sterically protected, or modified so as to prevent iodination. If HeLa-α contains an O-linked oligosaccharide on Thr^{39} similar to bovine pituitary and choriocarcinoma free α-subunits (Parsons et al., 1983; Cole et al., 1984), it is possible the modification could interfere with iodination of Tyr^{37}. Alternatively, recent evidence from this laboratory has demonstrated that the HeLa α-subunit is phosphorylated (Cox, 1986), and this modification might also alter its susceptibility to iodination. Support for the latter interpretation comes from the appearance of a third iodinated peptide in tryptic digests of HeLa-α when it is labeled subsequent to preincubation with alkaline phosphatase (Figure 7, arrow). The peptide labeled under these conditions migrated to a position different from that expected of the peptide identified as spot 7 in hCG-α (Figure 6A). However, its position was similar to that previously reported for the comparable peptide in bovine pituitary free α-subunit which contains an additional O-linked carbohydrate side chain (Parsons et al., 1983).

Characterization of HeLa-α. As noted in Figure 2, the purified HeLa α-subunit migrated on SDS–PAGE with an apparent molecular weight greater than that of the urinary α-subunit (24 000 vs 22 800). Similarly, Figure 8 shows that when partially purified HeLa-α was electrophoresed through polyacrylamide gels containing SDS, transferred electrophoretically to nitrocellulose (Western blot), and then incubated

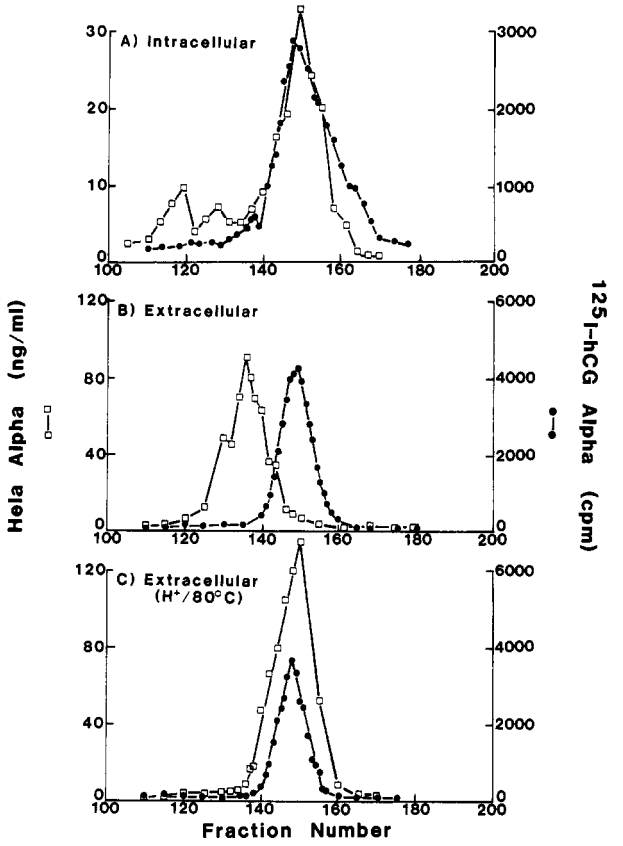

FIGURE 9: Comparison of intracellular and secreted HeLa-α by gel filtration chromatography. Cells were grown in suspension culture, harvested, washed, disrupted in a glass–Teflon homogenizer, and centrifuged at 12000g for 20 min. The supernatant fluid (panel A) or concentrated conditioned medium (panel B) was mixed with ^{125}I-labeled hCG-α (2×10^5 cpm, approximately 2 ng) and chromatographed on Sephadex G-75 superfine. In panel C, a second sample of extracellular HeLa-α was chromatographed following mild acid hydrolysis (75 mM H_2SO_4, 80 °C for 90 min). Elution was with 0.1 M NH_4HCO_3, and appropriate fractions were assayed for HeLa-α by RIA (□) and for the hCG-α standard by solid scintillation spectrometry (●).

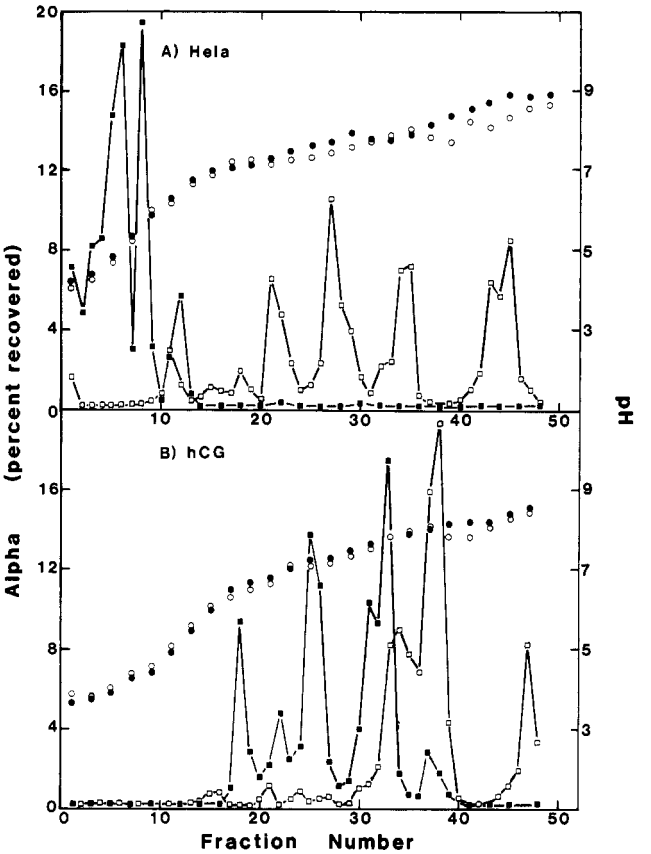

FIGURE 10: Analysis of HeLa and hCG α-subunits by isoelectric focusing. Partially purified HeLa-α (A) and hCG-α (B) were electrofocused on cylindrical gels containing 10% polyacrylamide and pH 3–10 ampholytes both before (■) and after (□) mild acid hydrolysis (75 mM H_2SO_4, 80 °C, 90 min). When the voltage stabilized, gels were fractionated with a manual device and extracted with distilled water at room temperature overnight. The pH (circles) and α-subunit content (squares) of each eluate were determined, where each fraction represents two 1-mm slices.

sequentially with anti-α serum and ^{125}I-labeled protein A, a single radiolabeled species was detected that had an apparent molecular weight significantly greater than that of the urinary α-subunit run in parallel (26 000 vs 23 000). In control experiments, it was demonstrated that the autoradiographic signal was not observed when preimmune serum was used or when filters were incubated with anti-α serum in the presence of 100 μg/mL hCG-α.

In Figure 9 the elution of HeLa-α (open boxes) on Sephadex G-75 superfine is compared to that of urinary hCG-α (closed circles). The tumor protein was detected by RIA while the normal subunit was labeled with ^{125}I and detected by solid scintillation counting. As seen, HeLa-α eluted prior to hCG-α, suggesting it has a greater apparent molecular weight. However, if the tumor protein was acidified (75 mM H_2SO_4) and heated (80 °C for 90 min) before application to the column, it coeluted with the radiolabeled marker (Figure 9C). Because the conditions of mild acid hydrolysis are adequate to remove *N*-acetylneuraminic acid from oligosaccharide side chains (Eagon & Heath, 1977), these data suggest that the tumor subunit is more highly sialylated than the urinary standard. It was demonstrated in a previous publication (Cox, 1981b) that following mild acid hydrolysis the HeLa protein was still bound by *Ricinus communis* lectin, which specifically binds Gal, the penultimate residue in most asparagine-linked complex side chains (Kornfeld & Kornfeld, 1976). Quantities of the purified HeLa protein have not been sufficient to determine NeuNAc directly.

The elution of intracellular HeLa-α from Sephadex G-75 superfine was also compared to that of the secreted protein (Figure 9A). As seen, the majority of intracellular α-subunit emerged from the gel filtration column coincident with the radiolabeled marker; a small amount of immunoreactive material eluted slightly ahead of the urinary standard, similar to the secreted protein. Thus, the majority of intracellular subunit has probably not been fully sialylated.

The normal and tumor subunits were analyzed by isoelectric focusing in polyacrylamide gels. Partially purified HeLa-α and hCG-α were electrofocused in cylindrical gels containing 10% polyacrylamide and pH 3–10 ampholytes. Samples were applied to the top of the gel (anode) and electrophoresed until the voltage stabilized, at which time the gels were fractionated and eluted with distilled water. The pH and α-subunit content of each eluate were determined 20 h later. The results shown in Figure 10 (filled squares) indicate that both subunits were heterogeneous and that the HeLa protein had a lower isoelectric point than the urinary subunit, 4.7–5.5 compared to 6.5–7.8. The pI for both preparations was increased following mild acid hydrolysis (75 mM H_2SO_4, 80 °C, 90 min) as would be expected upon the removal of sialic acid (open squares). However, even after such treatment, many of the tumor subunit forms still focused at lower pHs than the urinary hCG-α forms. Phosphorylation may contribute to this difference (Cox, 1986).

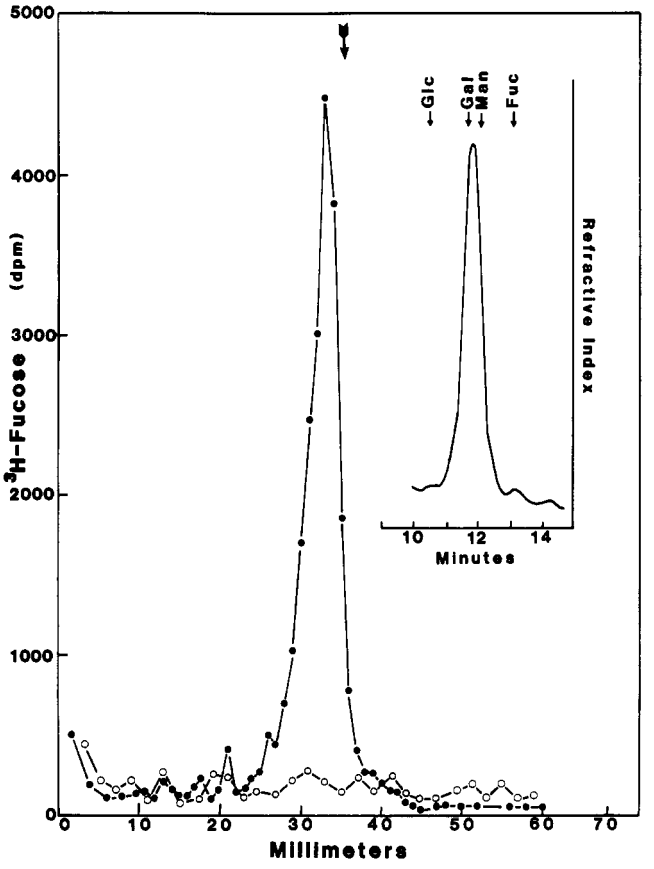

FIGURE 11: Fucosylation of the tumor α-subunit. HeLa cells in a 75-cm^2 flask were labeled for 24 h with [^{3}H]Fuc (10 μCi/mL) in medium containing 1 mM Glc. The secreted subunit was immunoprecipitated as described under Experimental Procedures and electrophoresed on cylindrical SDS–polyacrylamide gels. When the bromphenol blue tracking dye had migrated approximately 12 cm, the gels were removed, sliced into 1-mm sections, digested with NCS as described by the manufacturer, and counted by liquid scintillation. The arrow denotes the position of urinary hCG-α run on a parallel gel. Symbols: (●) anti-α serum; (○) preimmune serum. The inset depicts the neutral monosaccharides present in an acid hydrolysate (see Experimental Procedures) of urinary hCG-α as determined by HPLC (Bio-Rad HPX-87C column, elution with water at 85 °C). Under these conditions, Gal and Man are not separated. Arrows denote elution of the indicated standards.

Fucosylation of HeLa-α. The oligosaccharide moieties of the HeLa protein were also examined. Previous studies have demonstrated that the urinary hCG α-subunit has two asparagine-linked oligosaccharide side chains of the complex type, containing Asn-GlcNAc$_2$-Man$_3$-GlcNAc$_2$-Gal$_2$-NeuNAc$_2$ but not containing Fuc (Kennedy & Chaplin, 1976). The absence of Fuc in the urinary α-subunit was verified in the present study by HPLC analysis of the neutral monosaccharides in acid hydrolysates (Figure 11, inset). In contrast, when medium from HeLa cultures labeled with [^{3}H]fucose was immunoprecipitated with anti-α serum and analyzed by SDS–PAGE, a single peak of radioactivity that migrated slightly slower than urinary hCG-α was observed (Figure 11). This peak was absent when preimmune serum was used. These results indicate that the urinary and HeLa proteins differ in their carbohydrate side chains; the tumor protein is fucosylated while the normal protein is not.

The oligosaccharide side chains of HeLa-α labeled in culture with [^{3}H]fucose were analyzed by gel filtration chromatography (Bio-Gel P-6) following release of the glycan moieties from tryptic glycopeptides by endo H and endo F. This combination of endoglycosidases should remove the carbohydrate chains of both high-mannose and complex types (Elder

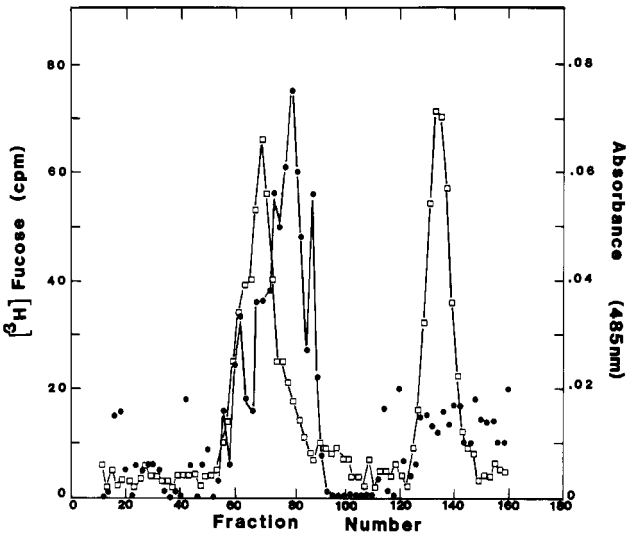

FIGURE 12: Analysis of α-subunit oligosaccharides. HeLa cultures were labeled with [^{3}H]Fuc for 24 h after which the medium was collected, cleared of detached cells by centrifugation, and chromatographed over Sephadex G-25 to remove unincorporated isotope. The α-subunit was immunoprecipitated as described under Experimental Procedures. HeLa-α was released from the immune complex by being heated at 60 °C in a solution containing 8 M urea and 1% 2-mercaptoethanol. After the addition of 1 mg of urinary hCG-α, the mixture was carboxymethylated and digested with TPCK-trypsin (20 μg) as described under Experimental Procedures. The solution was adjusted to contain 0.15 M sodium acetate, 0.05 M Na_2EDTA, 0.5% (w/v) Nonidet P-40, and 0.02% NaN_3 at pH 5.8. To this was added 12.8 μg of trypsin inhibitor (representing a 10% excess of inhibitor over trypsin since 1 mg of inhibitor binds 1.93 mg of trypsin), 5 units of endo F, and 1 μg of endo H. The mixture was incubated for 24 h at 37 °C and then chromatographed on a column (1 × 105 cm) of Bio-Gel P-6 at room temperature in 0.1 M NH_4HCO_3. Alternate fractions of 0.5 mL were either counted by liquid scintillation (□) or analyzed for carbohydrate by the phenol–sulfuric acid test (●), in which the carbohydrate concentration is proportional to color development at 485 nm.

& Alexander, 1982; Trimble et al., 1978). As seen in Figure 12 there were two peaks of radioactivity. One eluted slightly ahead of the heterogeneous oligosaccharides released from the pregnancy α-subunit added as carrier, suggesting the presence of larger carbohydrate groups on the tumor protein. Another eluted close to the column bed volume and did not seem to have a counterpart in the urinary subunit. This peak could represent free Fuc or a disaccharide, which are not separated on this column.

The tryptic peptides which contain Fuc were determined by two-dimensional thin-layer electrophoresis and chromatography. It is difficult to determine from the data presented in Figure 13 whether HeLa-α contains one or two fucosylated peptides, but it seems clear that the radioactivity is associated with spot 1 and/or spot 2, probably corresponding to TVI and TIX, respectively (refer to numbering in Figure 3).

Subunit Recombination. It was of interest to determine whether the purified HeLa α-subunit was competent for combining with hCG β-subunit to form holo-hCG (i.e., αβ dimer). This was examined by radiolabeling 10 μg each of hCG-α and purified HeLa-α with ^{125}I and then incubating these preparations at 37 °C for 48 h with a 10-fold excess (w/w) of hCG β-subunit (CR-123). The reactions were terminated by chilling followed by chromatography of the mixtures on Sephadex G-100. As shown in Figure 14, the ^{125}I-labeled urinary subunit did not elute at the position characteristic of free α-subunit (fractions 60–80) but at a position similar to that of an hCG marker (fractions 35–50). In contrast, the ^{125}I-labeled HeLa protein eluted as the free

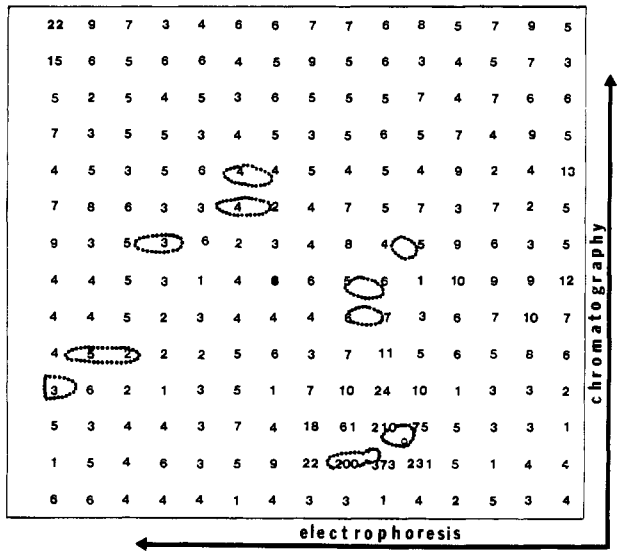

FIGURE 13: Identification of HeLa tryptic peptides containing fucose. A second aliquot of the [^{3}H]Fuc-labeled HeLa α-subunit isolated as described in the legend to Figure 12 was mixed with 200 μg of urinary α-subunit (CR-123) and subjected to two-dimensional tryptic peptide fingerprinting as described under Experimental Procedures. After the peptides were stained with ninhydrin, the cellulose was scraped from the backing in 1.25-cm squares and counted by liquid scintillation spectrometry. Radioactivity (cpm) is indicated by the numbers and position of ninhydrin spots are depicted by the dotted ovals.

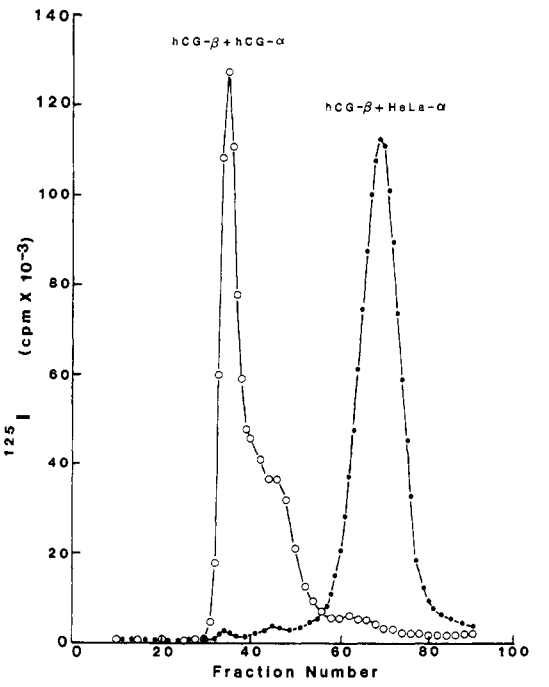

FIGURE 14: Recombination of α- and β-subunits. Purified HeLa (●) and hCG (○) α-subunits (10 μg each) were labeled with ^{125}I in reaction mixtures (40-μL total volume) containing 100 mM sodium acetate (pH 5.6), 500 μCi of $Na^{125}I$ (17 Ci/mg), 5.4 μg of bovine lactoperoxidase, and 29 μM H_2O_2. Following incubation for 2 min at room temperature, the reaction was stopped by the addition of 100 μL of a solution containing 1% (w/v) KI and 16% (w/v) sucrose. Radioiodinated proteins were separated from reactants by gel filtration chromatography. The labeled α-subunits were incubated with 100 μg of hCG β-subunit (CR-123) for 48 h at room temperature in reaction mixtures (100-μL total volume) containing 20 mM potassium phosphate (pH 7.4) and 0.02% NaN_3. Recombination of subunits was assessed by chromatographing the reaction mixtures on Sephadex G-100 in 0.1 M NH_4HCO_3 at room temperature. Aliquots of reaction mixtures were also removed prior to incubation and chromatographed with unlabeled hCG to determine the elution positions of free α-subunit and complete hormone ($\alpha\beta$ dimer).

α-subunit with only minimal material appearing in the region of hCG. Furthermore, removal of NeuNAc from both subunits by mild acid hydrolysis produced very similar profiles (data not presented). This result strongly suggests that the urinary α-subunit is competent for recombining with free β-subunit to form intact hormone (hCG), while the tumor protein is incapable of such association, even after desialylation.

Discussion

The similarity in amino acid compositions and coincidence of several tryptic peptides containing Met, Cys, and Tyr between the HeLa and hCG α-subunits suggest that these proteins share extensive primary sequence homology and argue for identification of the tumor protein as a glycoprotein hormone α-subunit (Figures 2–6). However, the tumor and placental proteins also differ in several respects: (i) Mild acid hydrolysis increases the p*I* (Figure 10) and decreases the apparent molecular weight (Figure 9) of HeLa-α, suggesting the ectopic protein is more highly sialylated than the hCG-α standard CR-123. (ii) The tumor protein contains Fuc in the oligosaccharide side chains while the pregnancy hormone does not (Figure 11). (iii) One of the three predicted Tyr-containing tryptic peptides in HeLa-α could be iodinated only after pretreatment with alkaline phosphatase (Figures 6 and 7). (iv) HeLa α-subunit is incompetent for joining with isolated hCG β-subunit to form holo-hCG under conditions where hCG-α can readily do so (Figure 14). Taken together, these results indicate that the HeLa α-subunit-like material is similar to other free α-subunits previously characterized from bovine pituitaries (Parsons et al., 1983), human choriocarcinoma cells (Cole et al., 1983; Ruddon et al., 1981; Benveniste et al., 1980), and pregnancy serum (Fein et al., 1980). Some of these characteristics are discussed in more detail below.

The specific activity of the purified HeLa protein was 6.8 $\times 10^5$ ng/mg of protein, slightly less than the theoretical 1.4 $\times 10^6$ ng/mg of protein estimated for the hCG subunit on the basis of a carbohydrate content of 30% (Kennedy & Chaplin, 1976). This may indicate that the tumor protein was still impure, even though other bands were not apparent after SDS–PAGE. However, it is likely that the antibodies raised against the urinary α-subunit interact with the normal and tumor proteins to different extents, even though they generate parallel dose–response curves. For instance, it has been noticed throughout these studies that the dose–response curve obtained for HeLa-α following mild acid hydrolysis is shifted to the left compared to that of the untreated subunit (Cox, 1981b), thereby increasing the apparent specific activity by about 2-fold (1.3×10^6 ng of α-subunit/mg of protein). The presence of Fuc (or other modifications) on the HeLa protein and not the placental protein may also retard antibody–antigen interaction, making absolute quantitation difficult.

Our results add to the growing evidence that free α-subunits are more extensively glycosylated than α-subunits dissociated from hCG. Like HeLa-α (Figures 11–13), the α-subunit-like proteins secreted by Chang liver cells and JAr and JEG-3 choriocarcinoma cells contain Fuc (Morrow et al., 1983; Ruddon et al., 1981a; Jones-Brown et al., 1984). Thus, it is possible that one consequence of transformation in these tumors is the elaboration of a fucosyl transferase that recognizes the α-subunit as a substrate. This is interesting in view of the fact that, during the synthesis of hCG in the developing placenta, the β-subunit is fucosylated but the α-subunit is not. The free α-subunits as isolated from bovine pituitaries (Parsons et al., 1983) or secreted by the JAr choriocarcinoma cell line (Cole et al., 1984) contain an O-linked carbohydrate moiety

in addition to the normal N-linked oligosaccharides. This side chain is on Thr^{43} and Thr^{39} of bovine and human free α-subunits, respectively. Several tumor-associated free α-subunits have been characterized as having sialic acid contents higher than those of α-subunits combining with β-subunit to produce holo-hCG. Thus, our findings are consistent with previous observations indicating that neoplastic transformation is often accompanied by a shift toward the synthesis and expression of larger Asn-linked oligosaccharides. The change in size has been attributed to an increase in sialic acid content (Warren et al., 1972; Hunt & Wright, 1985) and to increased β1–6-linked branching (Dennis et al., 1987). Changes in the activity or isozyme profile for galactosyl and fucosyl transferases have also been associated with neoplasia (Bauer et al., 1978; Kessel & Chou, 1976; Suda et al., 1987; Kijimoto-Ochiai et al., 1981; Padolsky & Weiser, 1975).

In previous studies, Mizuochi et al. (1983) purified and characterized intact gonadotropin (αβ heterodimer) from the urine of a patient with choriocarcinoma. The amino acid compositions of the tumor-derived and pregnancy hormones were very similar, but the carbohydrate side chains were different. More than 97% of the sugar chains of choriocarcinoma hCG was devoid of sialic acid, while those of normal hCG were mostly sialylated. The choriocarcinoma hormone contained unusual biantennary complex-type chains as well as mono-, bi-, and triantennary chains, the latter not being found on normal hCG. Additionally, there were twice as many fucosylated side chains in choriocarcinoma as compared to normal hCG.

The apparent molecular weight for HeLa-α is greater than that for hCG-α when determined by gel filtration chromatography (M_r 33 500 for HeLa-α vs M_r 22 700 for hCG-α) and by SDS–PAGE (M_r 24 000–25 200 for HeLa-α vs M_r 22 800 for hCG-α). Crude α-subunit preparations obtained from human Chang liver cells (Morrow et al., 1983), bronchogenic carcinoma cells (Weintraub et al., 1975), choriocarcinoma cells (Benveniste et al., 1979b; Ruddon et al., 1981a; Jones-Brown et al., 1984), a gastric carcinoid tumor (Weintraub et al., 1975), and the bovine pituitary (Parsons et al., 1983) also have apparent molecular weights greater than that of α-subunit released from hCG. This discrepancy in apparent molecular weights can most probably be accounted for by difference in carbohydrate constituents, as it has long been recognized that determination of molecular weight by physical methods produces erroneous results for glycoproteins. Thus, changes in the oligosaccharide moieties attached to the same polypeptide chain would cause significant differences in molecular weight estimates for that glycoprotein (Whitaker, 1963). This is clearly illustrated by the experiments of Fein et al. (1980) in which they showed that chromatography of both eutopic and ectopic (free and combined) α-subunits on Sepharose CL-6B under denaturing conditions (6 M guanidine hydrochloride, pH 3) eliminated the differences in apparent molecular weight determined for these proteins during chromatography under nondenaturing conditions, and their elution positions were coincident with that of a chemically deglycosylated subunit. Removal of the tryptic peptide containing the O-linked oligosaccharide from bovine pituitary free α-subunit results in its coelution with the LH α-subunit (Parsons & Pierce, 1984), and observations from this laboratory suggest that treatment of HeLa-α with mild acid [Figure 9 and Cox (1981b)] or neuraminidase (G. S. Cox, unpublished results) to remove sialic acid is sufficient to effect this change.

The amino acid composition of HeLa-α was compared to that for hCG-α determined in parallel (Table II) or published previously (Swaminathan & Bahl, 1970) and to that of an ectopic α-subunit purified from a gastric carcinoid tumor (Weintraub et al., 1975). We conclude from the results that HeLa-α has an amino acid composition very similar to that of hCG-α, although minor differences cannot be excluded. The differences observed must be viewed with caution since only sufficient HeLa protein was available for analysis at a single hydrolysis time. In addition to minor differences reported previously for A.L.-α (Weintraub et al., 1975), a definitive difference in primary structure has been noted for an ectopic α-subunit-like protein isolated from a patient with undifferentiated carcinoma (Nishimura et al., 1986). Sequencing of the tumor protein uncovered the substitution of Ala for Glu at residue 56. Parsons et al. (1983) also report a slight decrease in Lys content for the free α-subunit-like protein purified from bovine pituitary. In the case of another polypeptide hormone, the sequence of gastrin produced by tumor and nontumor tissues was found to be the same (Boel et al., 1983; Kato et al., 1983).

It has previously been suggested that free α-subunits may represent as yet unrecognized precursor forms (Weintraub et al., 1977), but more recent evidence suggests that the free and combined α-subunits undergo differential posttranslational processing (Dean et al., 1980; Benveniste et al., 1979b; Peters et al., 1984), resulting in end products (rather than precursors) which have very different characteristics. In this regard, Benveniste et al. (1979a) carried out a comparative analysis of the α-subunits of three distinct origins and found that those α-subunits in combination with β-subunit (either in pregnancy hCG or choriocarcinoma hCG) were similar to each other but different from the α-subunit secreted free, the latter exhibiting a higher apparent molecular weight, a lower isoelectric pH, and a decreased ability to recombine with hCG-β.

Separated subunits of the glycoprotein hormones can be recombined to give fully active molecules (Reichert et al., 1969). It is possible that the α-subunits produced in tumors are defective since they appear greatly impaired in their ability to interact with β-subunits in a productive manner (Weintraub et al., 1975, 1977; Benveniste et al., 1979b). HeLa-α also seems to fall into this category as evidenced by the data illustrated in Figure 14. At present, the explanation for this characteristic is not completely understood, but Parsons and Pierce (1984) have shown that removal of the Thr-linked carbohydrate from the bovine pituitary free α-subunit with a mixture of glycosidases results in an α-subunit-like material capable of combining with LH β-subunit. Association of hCG α- and β-subunits probably occurs in the rough endoplasmic reticulum (Peters et al., 1983), where both subunits are sensitive to endo H and probably contain $Man_8GlcNAc_2$ (Ruddon et al., 1980). Peters et al. (1983) have shown that free α-subunit was more extensively processed to endo H resistant forms than was αβ dimer in choriocarcinoma cells, suggesting that the Asn-linked oligosaccharides on free α-subunit may be sterically more accessible than those on combined α-subunit. Similarly, Magner et al. (1984) have demonstrated that the carbohydrate processing of thyrotropin differs from that of free α-subunit and total glycoproteins in microsomal subfractions of mouse pituitary tumor; the rate of mannose trimming is rapid for free α-subunit, moderate for TSH (i.e., αβ dimer), and slow for non-TSH glycoproteins. By analogy, this difference in accessibility between combined and free subunits may permit fucosylation, more extensive sialylation, and phosphorylation of the ectopic free α-subunits.

Our results indicate that, regardless of whether or not a single α-subunit gene is being expressed in both normal and

neoplastic tissues (Boothby et al., 1981), posttranslational modifications lead to a highly altered subunit in the tumor. The differences observed may be useful in diagnosing neoplastic versus hyperplastic conditions and may lend insight into the mechanism of ectopic hormone production by these tumors.

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Characterization of High Molecular Weight Transforming Growth Factor α Produced by Rat Hepatocellular Carcinoma Cells†

Noreen C. Luetteke,*,‡ George K. Michalopoulos,‡ Joaquin Teixidõ,§ Reid Gilmore,§ Joan Massagué,§ and David C. Lee‖

Department of Pathology, Duke University Medical Center, Durham, North Carolina 27710, Department of Biochemistry, University of Massachusetts Medical School, Worcester, Massachusetts 01605, and Department of Microbiology and Immunology and Lineberger Cancer Research Center, The University of North Carolina, Chapel Hill, North Carolina 27514

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ABSTRACT: In addition to the mature 50 amino acid transforming growth factor α (TGFα), some transformed cells appear to produce multiple higher molecular weight forms. The structure and derivation of most of these larger soluble TGFα species remain to be established. We previously reported that a chemically induced rat hepatocellular carcinoma cell line, JM1, secreted acid-stable proteins which bind to epidermal growth factor receptors and stimulate DNA synthesis in primary cultures of normal adult rat hepatocytes. Purification and characterization of these hepatoma-derived growth factors have indicated their relationship to TGFα. Two EGF-competing activities of apparent M_r 30K and 10K were separated by gel filtration of concentrated JM1-conditioned medium and further purified by ion-exchange chromatography and reverse-phase HPLC. Both growth factors were detected by a radioimmunoassay specific for TGFα. Western blotting with antibodies to the 50 amino acid TGFα revealed that the lower molecular weight factor comigrated with the synthetic 6-kDa rat TGFα. The higher molecular weight TGFα appeared on immunoblots as a diffuse band of 18–21 kDa, which converted to the mature 6-kDa form upon digestion with elastase, confirming a precursor–product relationship. However, the 18–21-kDa proteins did not react with antibodies directed against the carboxy-terminal cytoplasmic segment of the transmembrane TGFα precursor. Enzymatic deglycosylation of the 18–21-kDa TGFα species by sequential removal of sialic acids and O- and N-linked carbohydrate reduced the molecular weight to 11K. The size and soluble nature of this polypeptide suggest that it represents the extracellular domain of the transmembrane TGFα precursor. In vitro translation of JM1 mRNA, enriched for the TGFα mRNA by hybridization selection, yielded a protein of 17 kDa which was immunoprecipitated by antibodies directed against the carboxy-terminal regions of the mature TGFα and the TGFα precursor. In the presence of microsomes, the primary translation product converted to 20 kDa, which has previously been shown to be due to N-linked glycosylation. These data demonstrate that JM1 hepatoma cells release the mature TGFα plus larger processing intermediates which are proteolytically derived from a common transmembrane glycoprotein precursor.

Transforming growth factor (TGFα) is a single polypeptide of 50 amino acids which acts as a mitogen for mesenchymal and epithelial cells [for a review, see Derynck (1986)]. Rat TGFα shares 34% amino acid sequence identity, but no immunological cross-reactivity, with mouse EGF (Marquardt et al., 1984; De Larco & Todaro, 1978). Both EGF and TGFα bind with comparable affinity to a common 170-kDa receptor and activate the intrinsic tyrosine kinase, leading to receptor autophosphorylation, internalization, and down-regulation (Massagué, 1983). However, unlike EGF, TGFα synthesis is most prevalent and abundant in tumor cells and cells transformed by retroviruses, oncogenes, or chemicals. Recently, expression of TGFα mRNA and/or protein has also been detected in certain normal tissues and cultured cells including bovine anterior pituitary cells (Samsoondar et al., 1986), rat maternal decidua (Han et al., 1987), and human epidermis and primary keratinocytes (Coffey et al., 1987).

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* Address correspondence to this author at the Department of Pathology, Duke University Medical Center, Box 3432, Durham, NC 27710.
‡ Duke University Medical Center.
§ University of Massachusetts Medical School.
‖ The University of North Carolina.

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