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Research Article

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Differences between Purified Ectopic and Normal Alpha Subunits of Human Glycoprotein Hormones

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ABSTRACT "Ectopic" proteins, not distinguished immunologically from the common alpha subunit of the human glycoprotein hormones, were purified approximately 10,000-fold from a gastric carcinoid tumor (A.L.-α) and from tissue culture medium of bronchogenic carcinoma cell lines (ChaGo-α). The purified A.L.-α was homogeneous by sodium dodecyl sulfate (SDS) gel electrophoresis while the purified ChaGo-α showed multiple components, some of which represented aggregated species. In SDS gel electrophoresis, the apparent molecular weights of A.L.-α (15,000) and dithioerythritol-reduced ChaGo-α (13,000) were significantly lower than those of the alpha subunits of human chorionic gonadotropin (hCG-α), luteinizing hormone, follicle-stimulating hormone, or thyroid-stimulating hormone (22,000–23,000). Binding experiments with [^{35}S]-SDS suggested that these apparent differences in molecular weight resulted, at least in part, from diminished binding of the SDS by the normal compared to the ectopic alpha subunits. In gel chromatography, the apparent molecular weights of A.L.-α (27,000) and ChaGo-α (30,000) were slightly higher than those of normal alpha subunits (23,000–24,000). Both A.L.-α and ChaGo-α were not distinguished from hCG-α in ion-exchange chromatography. The composition of A.L.-α was similar to that of hCG-α in 13 amino acids but showed decreased phenylalanine and increased valine; glucosamine was identified in both A.L.-α and hCG-α. Under conditions in which hCG-α combined with the hCG beta subunit (hCG-β) to produce 95% of the expected gonadotropin-binding activity in a rat testis radioreceptor-assay, A.L.-α incubation with hCG-β resulted in only 2% of the expected activity, and ChaGo-α incubation with hCG-β produced no detectable activity. These characteristics of ectopic alpha subunits may reflect abnormalities of neoplastic protein synthesis or carbohydrate attachment which result in polypeptides with chemical and immunologic similarity to normal subunits but with differences in physical and combining properties; alternatively, the ectopic subunits may represent as yet unrecognized alpha precursor forms.

INTRODUCTION

It is now well established that a variety of human malignant tumors can synthesize and secrete polypeptide hormones and other proteins not normally associated with their tissue of origin, resulting in "ectopic" polypeptide syndromes (1-4). The tumor cell product has usually been found to be similar to the product of the normal cell of origin, although in some cases immunologic or physicochemical differences have been noted (4). However, virtually all of the latter studies have been performed with ectopic polypeptides of relatively low purity and usually under conditions where in vivo metabolism could not be excluded as a cause for alterations in the molecule.

In recent years it has been demonstrated that the glycoprotein tropic hormones: follicle-stimulating hormone (hFSH),[1] luteinizing hormone (hLH), thyroid-stimulating hormone (hTSH), and chorionic gonadotropin (hCG) consist of two subunits, a specific beta subunit and an alpha subunit which is nearly identical among the four hormones (5). We have recently described isolated ectopic production of materials immunologically identical to the hCG beta subunit (6) and to the common alpha subunit ("alpha") (7) in

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[1] *Abbreviations used in this paper:* α, alpha subunit, β, beta subunit; hCG, human chorionic gonadotropin; hFSH, human follicle-stimulating hormone; hLH, human luteinizing hormone; hTSH, human thyroid-stimulating hormone; SDS, sodium dodecyl sulfate.

vivo, as well as unbalanced production of hCG subunits in vitro (8).

The present communication describes the purification and partial characterization of ectopic alpha subunits derived from both in vivo and in vitro sources. These studies indicate differences between the ectopic and normal alpha subunits of glycoprotein hormones. A preliminary report of this work has appeared (9).

EXPERIMENTAL PROCEDURE

Materials

Bovine and human serum albumin, ovalbumin, carbonic anhydrase, cytochrome *c*, lysozyme, and chymotrypsinogen A were purchased from Schwarz/Mann (Div. of Becton, Dickinson & Co., Orangeburg, N. Y.). Bovine thyroglobulin was a gift of Dr. Harold Edelhoch, and hCG (ca. 10,000 IU/mg, bioassay and radioreceptor-assay) was a gift of Dr. Jean Hickman. Human placental lactogen, hLH, hFSH, and hTSH were provided by the Hormone Distribution Program of the National Institute of Arthritis, Metabolism and Digestive Diseases, Bethesda, Md. hCG-α and hCG-β were obtained from Dr. Om Bahl or Dr. Robert Canfield, hLH-α and hFSH-α were obtained from Dr. Leo Reichert, and hTSH-α was obtained from Dr. Ione Kourides and Dr. Farahe Maloof. Diatonaceous silica (Celite) (Hyflo-Super Cell) was purchased from Johns-Manville Products Corp. (Denver, Colo.), DEAE-cellulose (DE52 microgranular) was from Whatman Chemicals (Div. of W&R Balston, Maidstone, Kent, England), and cross-linked beaded dextran (Sephadex G-100) and DEAE-Sephadex (A50) were from Pharmacia Fine Chemicals Inc. (Piscataway, N. J.). Recrystallized acrylamide, methylenebisacrylamide, and tetramethylethylenediamine were obtained from Bio-Rad Laboratories (Richmond, Calif.), and recrystallized sodium dodecyl sulfate (SDS) (99%) was from Pierce Chemical Co. (Rockford, Ill.). All other chemicals were analytical reagent grade products purchased from major domestic suppliers.

Methods

Cell culture. The establishment and maintenance of ectopic alpha-producing cell lines derived from a human bronchogenic carcinoma (designated "ChaGo" lines) has previously been described (8, 10). In the current experiments, uncloned ChaGo cells as well as the C-5 clonal strain (8) were grown in 250-ml plastic culture flasks containing 30 ml of a medium composed of 20% (vol/vol) fetal bovine serum and 80% (vol/vol) Roswell Park Memorial Institute Medium 1640 (Buffalo, N. Y.) with penicillin (25 U/ml) and streptomycin (25 μg/ml). Medium was collected during the plateau phase of cell growth (ca .10^7 cells/flask) every 3 days and centrifuged at 1,500 *g* for 20 min to eliminate a small number of floating cells and debris. The supernate was stored at -20°C and subsequently lyophilized.

Radioimmunoassay and radioreceptor-assay. Purified hCG, hCG-α, and ectopic alpha were iodinated with carrier-free ^{125}I (Amersham/Searle Corp., Arlington Heights, Ill.) by a modification (11) of the chloramine-T method (12). The alpha subunit concentration in various samples was determined by a double-antibody radioimmunoassay as previously described (7).

Gonadotropin-binding activity was determined by radioreceptor-assay according to the method of Catt et al. (13). Both purified hCG (Dr. Jean Hickman) and an impure hCG reference preparation (Second International Reference Preparation) were used as standards in these assays; the purified hCG consistently had a specific activity of ca. 10,000 IU/mg by receptor-assay, which agreed well with values determined by a mouse uterine weight bioassay (6).

Combination of alpha and beta subunits. hCG-α as well as ectopic alpha subunits were incubated with hCG-β, and the degree of subunit combination was measured by the appearance of activity in the gonadotropin radioreceptor-assay, using a modification of the method of Reichert et al. (14). Various alpha subunits (45 μg) and hCG-β (68 μg) were incubated at 37°C in 185 μl of 0.01 M sodium phosphate pH 7.4 at a final concentration of 0.62 mg total protein/ml (1.7×10^{-5} M for each subunit)[2] in a 300-μl conical glass vial, capped with paraffin. At zero time and various intervals thereafter, 10-μl aliquots were removed from the incubate with the same calibrated micropipette, diluted in 1 ml cold 0.05 M sodium phosphate pH 7.4 containing 0.1% (wt/vol) ovalbumin, and kept frozen until assayed. Previous studies by us and others (14) had indicated that such a dilution prevented further combination of alpha and beta subunits during the subsequent incubation (2 h at 37°C) required for the receptor-assay. In other control experiments individual alpha or beta subunits were incubated separately under similar conditions. The percent of complete alpha and beta subunit combination, which would have yielded 0.62 mg hCG/ml incubate, was calculated for each time point by measurements in the gonadotropin receptor-assay using purified hCG as standard. Results were calculated as the average of two to three dilutions in duplicate, since the dose-response curves of all samples were parallel to standard hCG.

SDS polyacrylamide gel electrophoresis. Electrophoresis was performed in 5×0.5-cm gels containing 7.4% acrylamide, 0.2% methylenebisacrylamide, 0.01 M sodium phosphate pH 7.0, and 0.2% SDS, using modifications of the methods of Reichert and Lawson (17). Proteins (0.1–20 μg) were usually dissolved in 50 μl 0.01 M sodium phosphate pH 7.0 containing 1% SDS and 1% 2-mercaptoethanol and incubated at 40°C for 1 h. In certain other experiments, samples were dissolved in 0.01 M sodium phosphate containing 1% SDS, 0.05 M dithioerythritol, and 0.001 M EDTA and incubated at 25°C for 4 h with or without the presence of 8 M urea. Samples with urea were dialyzed extensively in a 100-μl equilibrium dialysis cell against an identical solution of SDS, dithioerythritol, and EDTA before electrophoresis. All samples were loaded directly onto gels after the addition of 10–15 crystals of sucrose and 2 μl of 0.06% (wt/vol) bromphenol blue. Electrophoresis was performed at a constant current of 7 mA/gel, and the gels were either stained with Coomassie Blue or cut into 1-mm slices for counting of radioiodinated proteins in a well-type autogamma counter. Gels were destained by immersion in acetic acid : methanol : water (20 : 120 : 280 vol/vol/vol) for 24–48 h. Relative mobilities and apparent molecular weights of various stained bands were calculated with the use of four to seven standard reference proteins according to the method of Weber et al. (18). The

[2] For those calculations the molecular weight of ectopic alpha was assumed to be equal to that of hCG-α; the molecular weight values used for hCG-α (15,000) and hCG-β (22,000) are those calculated from their chemical composition (15, 16).

position of stained bands was usually confirmed by measurement of the absorbancy peak at 500 nm using a Gilford linear gel scanner and recorder (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Other analyses. The apparent molecular weight of various alpha subunits was also determined by gel chromatography on 1.5 × 90-cm Sephadex G-100 columns calibrated with four to five standard proteins. The void volume of columns was determined by the elution peak of bovine [^{125}I]thyroglobulin (mol wt = 660,000), and the total volume was calculated from column dimensions or from a tangent drawn from the descending limb of the inorganic ^{125}I elution peak.

Amino acid analyses of hCG-α (purified by Dr. Om Bahl) and purified ectopic A.L.-α (see below) were performed with a Beckman 120-C two-column analyzer (Beckman Instruments, Inc., Fullerton, Calif.) and automatic digital integrator (Infotronics Corp., Houston, Tex., CRS-210) after hydrolysis of 30-μg samples in 6 N HCl at 110°C for 24 h. The sensitivity of the instrument was increased approximately 10-fold to a level of 1–2 nmole of each amino acid by use of a 4–5-mV resistor pack in the recording assembly. Individual amino acids in the various samples were measured in the range of 2–10 nmol, where the coefficient of variation for each determination was 5–10%. Protein determinations were performed by the method of Lowry et al. (19) using bovine serum albumin as standard. In certain cases protein concentration was also estimated by the absorbancy at 280 nm using either the empirically determined extinction coefficient ($E_{1\,cm}^{1\%}$) of bovine serum albumin, 5.6, or that of hCG-α, 3.7. The latter was determined using a weighed quantity of hCG-α and is in reasonable agreement with the value of 4.3 reported by Morgan et al. for a different preparation (20).

Purification of ectopic alpha. Clinical and laboratory studies documenting the ectopic secretion of alpha-like polypeptides by a gastric carcinoid tumor in vivo (A.L.-α) (7) and by bronchogenic carcinoma cell lines in vitro (ChaGo-α) (8) have previously been published. In the present study ectopic A.L.-α was purified from carcinoid tumor obtained at autopsy, and ChaGo-α was purified from tissue culture medium. The initial extraction step was carried out at room temperature, but all subsequent chromatography steps were performed at 4°C. Tracer amounts of standard ^{125}I-hCG-α were added to the preparations as an internal radioactive standard for each purification step, and various fractions from each step were measured for standard hCG-α radioactivity, ectopic alpha immunoactivity, and absorbancy at 280 nm.

A.L.-α was extracted from 37 g of lyophilized tumor by percolation through 50 g Celite according to the method of Bates et al. (21). The fractions containing immunoactive alpha (57% [vol/vol], ethanol-5% [wt/vol] sodium chloride, total volume 130 ml) were pooled, dialyzed against running distilled water, and lyophilized. The extract was dissolved in 2 ml of 0.15 M sodium chloride-0.01 M sodium phosphate-0.02% (wt/vol) sodium azide pH 7.4, and applied to a 1.5 × 90-cm column of Sephadex G-100 equilibrated with the same buffer and eluted at a flow rate of 3 ml/h. Fractions containing immunoreactive alpha were pooled (37 ml total), dialyzed against 0.04 M Tris-phosphate pH 7.5, and applied to a 1.5 × 30-cm column of DEAE-Sephadex equilibrated with the same buffer. Proteins were eluted from the column at a flow rate of 40 ml/h with a linear 200-ml gradient from 0 to 0.4 M sodium chloride in 0.04 M Tris-phosphate pH 7.5. Fractions containing immunoactive alpha were pooled (42 ml total, Fig. 1, upper panel), concentrated by ultrafiltration (Amicon Corp., Lexington, Mass., UM-10 membrane) to 2 ml, and reapplied to the Sephadex G-100 column used in the initial chromatography step. Fractions containing alpha (20 ml total, Fig. 1, lower panel) were pooled and concentrated to 2 ml by ultrafiltration in 0.01 M sodium phosphate pH 7.4.

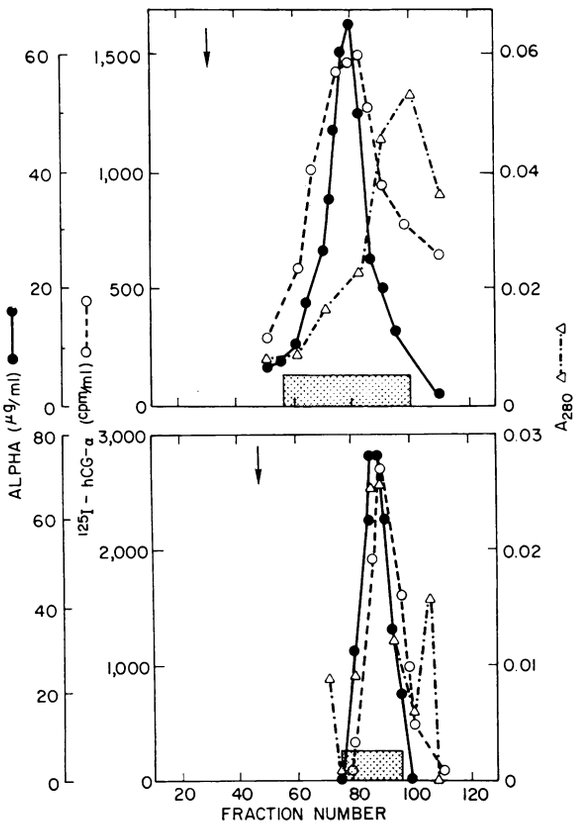

FIGURE 1 Purification of A.L.-α by ion-exchange and gel chromatography. In each step standard ^{125}I-hCG-α was added and cochromatographed with the sample. The unlabeled "alpha" in various fractions was measured by an hCG-α radioimmunoassay (see Methods) at sufficiently high dilutions so that there was no interference from the radioactivity present in the fractions. The shaded areas on each abscissa indicate the fractions pooled and concentrated by ultrafiltration. (Upper panel) DEAE-Sephadex chromatography (step A4, Table I). The column (1.5 × 30 cm) was equilibrated at 4°C with 0.04 M Tris-phosphate pH 7.5 and the sample applied in 37 ml of the same buffer. The arrow indicates the start of elution with a linear 200-ml gradient from 0 to 0.4 M sodium chloride. 1-ml fractions were collected at a flow rate of 40 ml/h. The elution peaks of ^{125}I-hCG-α and A.L.-α corresponded to 0.06 M sodium chloride. (Lower panel) Sephadex G-100 chromatography (step A5, Table I). The column (1.5 × 90 cm) was equilibrated at 4°C with 0.15 M sodium chloride, 0.01 M sodium phosphate, 0.02% (wt/vol) sodium azide pH 7.4, and the sample applied to 2 ml of the same buffer. 1-ml fractions were collected at a flow rate of 3 ml/h. The arrow indicates the void volume of the column.

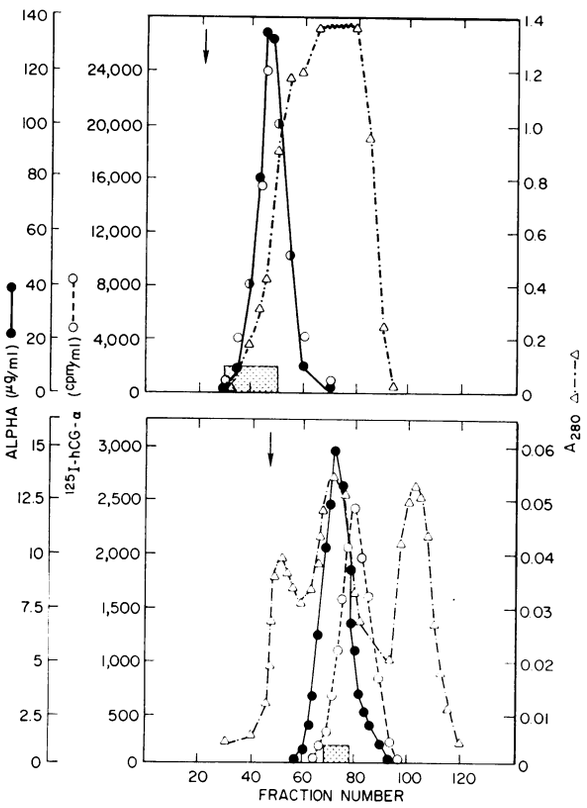

FIGURE 2 Purification of ChaGo-α by ion-exchange and gel chromatography. (Upper panel) DEAE-cellulose chromatography (step B4, Table I). Methods similar to those described in Fig. 1 except that 2-ml fractions were collected. The elution peaks of ^{125}I-hCG-α and A.L.-α corresponded to 0.06 M sodium chloride. (Lower panel) Sephadex G-100 chromatography (step B6, Table I).

ChaGo-α was extracted from 406 g lyophilized tissue culture medium derived from a mixture of a 26-liter pool of both uncloned lines and the C-5 clonal strain, using 508 g Celite for percolation as described above. Pilot experiments with this extract indicated a higher degree of subsequent alpha purification by the use of ion-exchange rather than gel chromatography as the initial step and somewhat higher recovery of alpha by the substitution of DEAE-cellulose for DEAE-Sephadex. Therefore the extract was purified by two successive steps on DEAE-cellulose followed by two successive steps on Sephadex G-100 (Fig. 2), using methods virtually identical to those described above for the A.L.-α purification.

RESULTS

Purification of ectopic alpha; criteria for purity. The results of the purification of the two ectopic alphas are summarized in Table I. Although in each case a several thousandfold purification was achieved, the A.L.-α purification was more efficient and complete primarily because of the greater effectiveness of the Celite extraction step. The final specific immunoactivity of the A.L. preparation (1.1 mg alpha/mg protein) suggested virtually complete purification while that of the ChaGo preparation (0.34 mg alpha/mg protein) suggested incomplete purification.

In SDS gel electrophoresis, the purified A.L.-α preparation showed only one stained band in several different runs of 3-10 μg (Fig. 3A). Only in one experiment, performed after repeated freezing and thawing of the preparation, did a second band of apparent molecular weight 90,000 appear (Fig. 4A, gel 10); this presumably represented an aggregated component. Although the limited supply of purified A.L.-α prevented loading of the gels with large amounts, the appearance of only one band in the 10-μg sample supported the high degree of purity of this preparation. This was particularly true since the main contaminant during the A.L.-α purification migrated in the region of the human serum albumin; if this contaminant displayed the same high avidity for Coomassie Blue as human albumin, one would have expected to detect as little as 1% in the 10-μg A. L.-α sample (Fig. 3B).

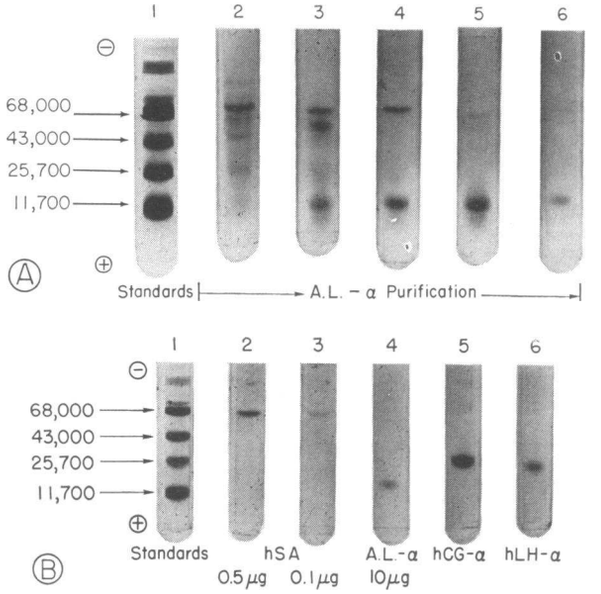

FIGURE 3 SDS polyacrylamide gel electrophoresis of A.L.-α in 7.4% gels as described in Methods. Samples dissolved in 50 μl 0.01 M sodium phosphate pH 7.0 containing 1% SDS, 1% 2-mercaptoethanol, and incubated at 40°C for 1 h before application to the gel. (A) (1) 5 μg each of bovine serum albumin (mol wt = 68,000), ovalbumin (43,000), chymotrypsinogen A (25,700), cytochrome *c* (11,700). (2) 20 μg original tumor (step A1). (3) 20 μg ethanol-saline extract (step A2). (4) 20 μg first Sephadex G-100 eluate (step A3). (5) 20 μg DEAE-Sephadex eluate (step A4). (6) 3 μg second Sephadex G-100 eluate (step A5). (B) (1) 3 μg each of standard proteins as in A1. (2) 0.5 μg human serum albumin (hSA). (3) 0.1 μg hSA. (4) 10 μg second Sephadex G-100 eluate (step A5). (5) 10 μg hCG-α. (6) 10 μg hLH-α.

TABLE I

Purification of Ectopic Alpha Subunits

Step	Total protein*	Total alpha	Recovery	Specific activity	Purification factor
	mg	*mg*	%	*mg alpha/mg protein*	
(A) A.L.-α					
(1) Original tumor	43,300	7.02	100	1.62×10^{-4}	1
(2) Ethanol-saline extract	41.3	3.67	52	8.89×10^{-2}	549
(3) 1st Sephadex G-100	11.2	2.36	34	2.11×10^{-1}	1,300
(4) DEAE-Sephadex	3.45	1.75	25	5.07×10^{-1}	3,130
(5) 2nd Sephadex G-100	0.477	0.88	13	1.84	11,400
	(0.83)‡			(1.06)‡	(6,540)‡
(B) ChaGo-α					
(1) Original medium	208,000	11.7	100	5.63×10^{-5}	1
(2) Ethanol-saline extract	6,020	6.38	55	1.06×10^{-3}	19
(3) 1st DEAE-cellulose	543	4.27	36	7.86×10^{-3}	140
(4) 2nd DEAE-cellulose	19.3	0.821	7	4.25×10^{-2}	755
(5) 1st Sephadex G-100	1.80	0.600	5	3.33×10^{-1}	5,910
(6) 2nd Sephadex G-100	0.30	0.160	1	5.33×10^{-1}	9,470
	(0.47)‡			(3.40×10^{-1})‡	(6,040)‡

* For A.L. based on average of Lowry method and absorbancy at 280 nm, using bovine serum albumin as standard; for ChaGo based on absorbancy only (see Methods).

‡ Protein calculated from absorbancy at 280 nm using hCG-α as standard (see Methods).

Purification of ChaGo-α revealed the appearance after the first gel chromatography step of two principal protein bands on SDS gel electrophoresis, one of apparent molecular weight 13,000 and the other ca. 95,000 (Fig. 4A, gel 6). After a second chromatography on Sephadex G-100 and concentration by ultrafiltration the higher molecular weight form predominated (Fig. 4A, gel 7), despite the fact that the fractions pooled had twice been selected from lower molecular weight areas of the G-100 chromatogram (Fig. 1, lower panel). It therefore seemed likely that the higher molecular weight species was an aggregate of the lower molecular weight form, especially since it was similar to a component which appeared in the highly purified A.L.-α after repeated freezing and thawing.

To determine whether the method of sample preparation before electrophoresis (usually 1 h at 40°C in 1% SDS, 1% 2-mercaptoethanol) influenced the amount of the higher molecular weight form, other methods of preparing samples were studied (Fig. 4B). When ChaGo-α was directly applied to the SDS without any prior reduction or preincubation in SDS all of the protein migrated as the higher molecular weight form (Fig. 4B, gel 3). However when the preparation was incubated for 4 h at 25°C in 1% SDS containing 0.05 M dithioerythritol and 0.001 M EDTA, either with or without the presence of 8 M urea, a major lower molecular weight band (ca. 13,000) reappeared (Fig. 4B, gels 6 and 9). However, other high molecular weight species were still noted on the gels, making assessment of final purity still uncertain. Additional SDS gel studies with ^{125}I-labeled ChaGo-α supported the theory that the lower molecular weight species was the immunoactive monomer (see below).

After application of 10 μg of A.L.-α or ChaGo-α to 7.4% polyacrylamide gels not containing SDS, no bands were detected by Coomassie Blue staining. Preliminary experiments in which similar gels were not stained but were sliced and then eluted with buffer showed very broad bands of alpha immunoactivity consistent with microheterogeneity of charge.

Physicochemical properties of normal and ectopic alpha. Both A.L.-α and ChaGo-α coeluted with ^{125}I-hCG-α (previously shown to coelute with unlabeled hCG-α) during ion-exchange chromatography, suggesting no major differences in charge between the ectopic and normal alpha subunits (Figs. 1 and 2, upper panels). During gel chromatography both ectopic alphas displayed significantly lower elution volumes than the hCG-α tracer, suggesting higher molecular weight (Figs. 1 and 2, lower panels, Table II). However, in SDS gel electrophoresis, both ectopic alphas displayed significantly higher mobility than hCG-α or other standard alpha subunits, suggesting lower molecular weights (Figs. 3 and 4, Table II). Moreover, ^{125}I labeling of both ectopic alphas produced a considerable increase in the apparent molecular weight of the immunoactive com-

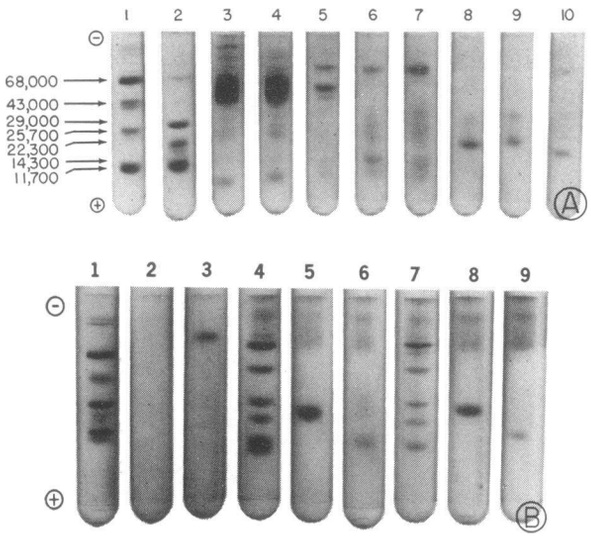

FIGURE 4 SDS polyacrylamide gel electrophoresis of ChaGo-α. Samples preincubated in SDS and 2-mercaptoethanol as described in Fig. 3. (A) (1) 3 μg each of standard proteins as in Fig. 3A1 and B1. (2) 3 μg each of carbonic anhydrase (mol wt = 29,000), human placental lactogen (22,300), lysozyme (14,300). (3) 20 μg original medium (step B1). (4) 20 μg ethanol-saline extract (step B2). (5) 10 μg second DEAE-cellulose eluate (step B4). (6) 10 μg first Sephadex G-100 eluate (step B5). (7) 10 μg second Sephadex G-100 eluate (step B6). (8) 10 μg hCG-α. (9) 10 μg hLH-α. (10) 10 μg purified A.L.-α after repeated freezing and thawing. (B) Effect of other methods of sample preparation before SDS gel electrophoresis (see Methods). Preparation: gels 1–3, no preincubation; gels 4–6, preincubation in 0.01 M sodium phosphate containing 1% SDS, 0.05 M dithioerythritol, 0.001 M EDTA at 25°C for 4 h; gels 7–9 preincubation as in gels 4–6 in the presence of 8 M urea. Samples: gels 1, 4, and 7, 3 μg each of bovine serum albumin, ovalbumin, chymotrypsinogen A, human placental lactogen, cytochrome *c*; gels 2, 5, and 8, 10 μg hCG-α; gels 3, 6, and 9, 10 μg purified ChaGo-α (second Sephadex G-100 eluate).

ponent by SDS gel electrophoresis but not by gel chromatography (Table II).

Because of these disparities in apparent molecular weight between SDS gel electrophoresis and gel chromatography, we considered the possibility that the ectopic alphas might be binding more SDS than standard alpha subunits. This might also explain our observation that attempts to recover alpha immunoactivity from SDS gels by prolonged dialysis or ion-exchange chromatography (22) yielded 30-40% recovery for standard alpha subunits but less than 0.1% recovery for both ectopic alphas. In fact, direct measurements of [^{35}S]SDS (Amersham/Searle Corp.) binding to dithioerythritol-reduced alpha subunits as measured by equilibrium dialysis (23) revealed 0.70 μg SDS/μg protein for hCG-α, 0.91 for A.L.-α, and 1.28 for ChaGo-α, compared to 1.38 for the nonglycoprotein bovine serum albumin.

Amino acid analyses. The highly purified A.L.-α was examined in two separate amino acid analyses; in the second experiment standard hCG-α (O. Bahl) was also subjected to the identical 24-h acid hydrolysis and analysis. These data were each compared statistically to the average of 11 separate amino acid analyses in the literature for different preparations of the four standard human glycoprotein hormone alpha subunits, among which there are no significant differences (24-34). For 13 amino acids A.L.-α was essentially identical to hCG-α and to literature analyses (Table III). The glycine content of A.L.-α was apparently higher than literature analyses of standard alpha subunits; however, our analysis of hCG-α also showed higher glycine content than the literature for reasons that are not clear. Half-cystine content of A.L.-α was apparently lower than hCG-α and literature analyses. Unfortunately, quantification of cysteic acid after performic acid oxidation could not be performed to confirm this finding. In each analysis A.L.-α showed a significantly higher valine content and significantly lower phenylalanine content than hCG-α or literature analyses, the latter two having identical values in both cases. Glucosamine was also identified in both the A.L.-α and hCG-α samples, although the methods did not permit precise quantification.

Combination of alpha and beta subunits. We next studied the ability of hCG-α and purified ectopic alphas to combine with equimolar amounts of hCG-β to produce a gonadotropin active in a rat testis radioreceptor-

TABLE II
Apparent Molecular Weight of Various Alpha Subunits in SDS Gel Electrophoresis and Sephadex G-100 Chromatography

Preparation	Apparent mol wt	
	SDS gel electrophoresis	G-100 chromatography
	daltons	
(A) Standard		
(1) hCG-α	23,000±800*	24,000
(2) ^{125}I-hCG-α	22,000‡	24,000
(3) hLH-α	22,000	—
(4) hTSH-α	22,000	23,000
(5) hFSH-α	22,000	—
(B) Ectopic		
(1) A.L.-α	14,700±400*	27,000
(2) ^{125}I-A.L.-α	22,000‡	24,000
(3) ChaGo-α	13,200±300*,§	30,000
(4) ^{125}I-ChaGo-α	23,000‡	30,000

* Mean ±1 SD, n = 6.
‡ Immunoactive component after iodination.
§ Principal component after dithioerythritol reduction, see Fig. 4B.

assay (Fig. 5). hCG-α (Canfield) and hCG-β (Canfield) combined to yield 95% of the theoretical activity, results similar to those of Reichert et al. (14). However under identical conditions the A.L.-α incubation with hCG-β resulted in only 2% of the expected gonadotropin activity while the incubation of ChaGo-α with hCG-β produced no activity. In other experiments equimolar amounts of ChaGo-α, hCG-α, and hCG-β were mixed and it was shown that there was minimal inhibition of standard alpha-beta combination by ectopic alpha or contaminants in the ChaGo preparation. These data indicate that both ectopic alphas were incapable of combining normally with hCG-β, either because of diminished physical combination or because the resulting alpha-beta dimer was incapable of binding to the gonadotropin receptor.

DISCUSSION

Ectopic alpha subunits highly purified from two different tumors, although similar to standard alpha subunits in immunologic and ion-exchange properties, differ from standard alpha in gel chromatography, SDS gel electrophoresis, and ability to combine with hCG-β. For A.L.-α the purification was sufficiently high (probably $>95\%$) to permit amino acid analysis, which also showed certain differences from standard alpha. Unfortunately, the purification of ChaGo-α was apparently incomplete, primarily because of inefficient purification at the initial extraction step as compared to A.L.-α (Table I). This probably resulted from the fact that the principal contaminants in the ChaGo culture medium were fetuin and other glycoproteins soluble in ethanol-saline, while the main albumin-like contaminant in A.L.-α was probably insoluble during extraction. However, the actual purity of the final ChaGo preparation is still uncertain—the absolute specific immunoactivity values are difficult to interpret since they depend on accurate quantification of total protein, quantitative immunologic identity between ChaGo-α and the hCG-α used as standards in the immunoassay, as well as the absence of aggregated forms of low immunologic potency. For ChaGo-α there were, in fact, considerable amounts of such aggregated forms, which also complicated assessment of purity from SDS gels.

TABLE III

Amino Acid Analyses of hCG-α and A.L.-α Compared to Literature Analyses of Various Human Alpha Subunits

Amino acid*	hCG-α‡ (O. Bahl)	A.L.-α‡ 1	A.L.-α‡ 2	Literature analyses§
Lysine	5.5	6.7	7.5	6.0±0.69
Histidine	4.1	2.8	3.9	3.0±0.35
Arginine	3.0	1.9	4.3	3.6±0.45
Aspartic acid	8.8	8.4	9.5	6.7±0.99
Threonine	8.2	6.6	7.9	8.6±0.87
Serine	8.3	7.9	7.0	8.3±0.92
Glutamic acid	10.0	11.4	10.1	10.2±0.73
Proline	8.0	9.9	6.2	7.7±1.1
Glycine	8.3‖	9.4¶	9.2¶	5.4±0.65
Alanine	7.0	5.6	4.5	5.2±0.79
Half-cystine	7.1	3.9¶	1.3¶	9.4±1.4
Valine	7.8	9.2‖	10.3‖	7.5±0.85
Methionine	1.8	2.7	2.2	3.1±1.1
Isoleucine	1.0	3.0	3.5	2.1±1.2
Leucine	3.0	5.1	6.1	4.9±0.62
Tyrosine	3.6	2.2	2.8	4.0±1.5
Phenylalanine	4.5	3.2¶	3.5¶	4.3±0.18
Glucosamine	+	+	+	+

* All data expressed and literature values recalculated when necessary, as μmol/100 μmol amino acid.

‡ 24 h hydrolysate, no correction for hydrolytic losses; norleucine internal standards yielded 95–104% recovery.

§ Mean ±1 SD; based on 11 separate analyses — 2 of hCG-α, 5 of hLH-α, 2 of hFSH-α, 2 of hTSH-α, usually 20–24 h hydrolysate (24–34).

‖ $P < 0.01$ vs. literature analyses, unpaired t test.

¶ $P < 0.001$ vs. literature analyses.

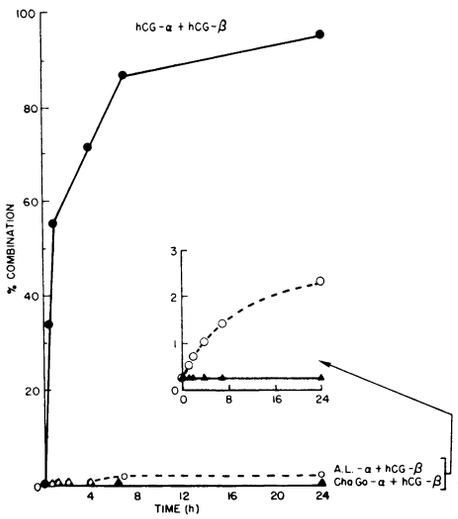

FIGURE 5 Combination of purified hCG-α (closed circles), A.L.-α (open circles) and ChaGo-α (closed triangles) with equimolar (1.7×10^{-5} M) amounts of hCG-β at 37°C to form a gonadotropin active in a rat testis radioreceptor-assay. The percent combination represents the hCG activity observed at each time interval relative to that expected for complete combination of alpha and beta subunits. The insert shows the percent combination for A.L.-α and ChaGo-α on an expanded ordinate scale, the shaded area representing the limits of detection for the assay. Individual subunits incubated separately showed no significant gonadotropin activity at any time interval. Further details of the incubation and receptor-assay are described in Methods.

The difference between ectopic and standard alpha subunits does not appear to be related to the source of the materials or to the method of purification. Although the hCG-α used in these studies had been prepared from urinary hCG, hLH-α, hFSH-α, and hTSH-α had been prepared from pituitary hormones. The ectopic alphas were derived from both tumor tissue and tissue culture medium, the latter under conditions where exogenously added hCG was not degraded (8). Purification of ectopic alphas was achieved with standard techniques of extraction and chromatography without use of pH extremes or denaturants. In this regard techniques used in their purification were actually less harsh than those used to purify standard alpha subunits, such as 8 M urea to dissociate the alpha and beta subunits (25). Yet exposure of ChaGo-α to 8 M urea did not alter its properties in SDS gel electrophoresis (Fig. 4B). Finally, the gel and ion-exchange chromatographic properties of ^{125}I-hCG-α used as an internal standard in each purification step were not altered during copurification with the ectopic alphas.

The possibility that proteolytic enzyme contaminants in the ectopic alpha preparations might have altered their properties is also unlikely. Direct measurement of proteolytic activity by an ultrasensitive hide powder azure assay (35) revealed minimal ($<$ 10 ng trypsin equivalents/ml extract) activity in both preparations even when examined at 37°C. Moreover, proteolytic contaminants which might have been activated during SDS gel electrophoresis (18) were specifically ruled out by two additional experiments. In the first, A.L.-α was preincubated with SDS and mercaptoethanol at 100°C for 5 min, conditions which usually inactivate proteolytic enzymes (18); in the second, both A.L.-α and ChaGo-α were mixed with ^{125}I-hCG-α and neither preparation changed the mobility of the radioactive standard during the gel run.

In gel chromatography both ectopic alphas, particularly ChaGo-α, displayed slightly lower elution volume than hCG-α, suggesting higher molecular weight; yet in SDS gel electrophoresis both ectopic alphas displayed considerably higher mobility than various standard alpha subunits, suggesting lower molecular weight. However, it is known that the true molecular weights of various glycoproteins may not be estimated reliably by gel techniques. For standard alpha subunits our studies and those of others (17) indicate that gel chromatography and SDS gel electrophoresis provide considerable overestimates of molecular weight (22,000–24,000) compared to the true value (14,000–15,000) known from their amino acid and carbohydrate composition (15). In SDS gel electrophoresis part of this abnormal behavior may be explained by our finding of significantly diminished SDS binding, as has been noted for other glycoproteins (23). Moreover, the higher degree of SDS binding shown by the ectopic alphas, possibly related to decreased or different carbohydrate content, may explain their considerably lower apparent molecular weight by this technique.

It is actually unlikely that there are large differences in molecular weight between the ectopic and standard alphas in view of the relatively similar estimates in gel chromatography and in SDS gels after alpha radioiodination which produced a single imunoactive labeled species. Similar polypeptide molecular weights for normal and ectopic alpha was also suggested by the similar composition for 13 amino acids of hCG-α and A.L.-α, although a precise calculation of minimum molecular weight by the method of Nyman and Lindskog (36) was not successful, presumably because of hydrolytic losses for certain amino acids (see below).

The amino acid composition of A.L.-α differed from identically analyzed hCG-α and from literature analyses of various alpha subunits in the content of half-cystine, valine, and phenylalanine. Unfortunately it was not possible to perform hydrolyses at multiple time intervals to correct for hydrolytic losses nor to quantify half-cystine as cysteic acid after performic acid oxidation. Therefore, the significance of the differences in half-cystine remains uncertain, for it is possible that unknown factors may have produced greater destruction of this amino acid in the A.L.-α than in the hCG-α preparation. The differences in valine and phenylalanine appear less likely to be the result of technical factors, as these are stable amino acids and the content of each in hCG-α was in close agreement with literature analyses. Although glucosamine was identified in both A.L.-α and hCG-α, no precise quantitation was made for this or any other carbohydrate moiety. It will be important to confirm these apparent amino acid differences and to perform a complete carbohydrate analysis when larger amounts of purified ectopic alpha are available.

A striking feature of both ectopic alphas was their inability to combine normally with hCG-β to produce a gonadotropin active in a radioreceptor-assay. Under conditions in which hCG-α combined with hCG-β to produce 95% of the expected activity, A.L.-α produced only 2% and ChaGo-α less than 0.5%. Other mixing experiments indicated that this inability was not the result of inhibitors of normal alpha-beta combination. Nor was it likely the result of ectopic alpha self-aggregation which, although present in ChaGo-α, was minimal in A.L.-α. Recent experiments using an independent physicochemical method based on enhancement of 1,8-anilinonaphthalenesulfonate fluorescence, to assess alpha-beta combination (37) confirm these data (Ingham, K., H. Edelhoch, B. Weintraub, and S. Rosen, unpublished observation).

The inability of ChaGo-α to combine with hCG-β may explain the instances of inefficient subunit combination which have been observed in various ChaGo clonal strains in vitro (8). Abnormal alpha-beta combination may also be a factor in the genesis of isolated ectopic alpha secretion; indeed, it has been shown that isolated secretion of immunoglobulin light chains by certain myeloma cell lines may be the result of abnormal heavy-light chain combination with rapid intracellular destruction of uncombined heavy chains (38).

Final elucidation of the structure of these poorly combining alpha forms may ultimately shed light on the structure-function relationships of the subunits of the glycoprotein hormones. Such relationships, including the role of carbohydrate residues, have not yet been clearly established, although it has been shown that carboxypeptidase treatment (39) as well as acylation (40) of alpha subunits inhibits their ability to recombine with beta subunits. Moreover, we have recently observed that certain preparations of putatively "normal" alpha subunits prepared from hFSH, hLH, hTSH, and hCG may have significantly diminished ability to combine with hCG-β (Weintraub, B., B. Stannard, and S. Rosen, unpublished observations). These studies as well as the present one have also demonstrated that such poorly combining forms may still have intact immunologic reactivity.

The biologic significance of the differences between normal and ectopic alpha subunits is unclear and could be simply the result of neoplastic derangements in protein synthesis—either at a transcriptional, translational, or post-translational level, involving attachment of carbohydrate residues—producing polypeptides with chemical and immunologic similarity to normal subunits but with differences in physical and combining properties. Yet it appears significant that two different tumor products, one of in vivo origin and one of in vitro origin, were so remarkably similar. Another possibility is that the ectopic alphas represent precursor forms of normal alpha subunits, whose conversion to the normal form is impaired in the tumor. Although our studies provide no direct evidence for such a precursor hypothesis, there is ample precedent for neoplasms producing precursor forms of polypeptides (4). Immunoactive alpha-like forms of apparently higher molecular weight in gel chromatography have been noted in placental extracts, pregnancy serum, and urine (41, 42), but these have not yet been purified or further characterized, nor has their role as precursors been established. Hopefully, further study of ectopic subunit production will shed light on the events of normal glycoprotein hormone biosynthesis.

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