A Single Amino Acid Substitution in an Ectopic $\alpha$ Subunit of a Human Carcinoma Choriogonadotropin*

(Received for publication, April 8, 1986)

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Human choriogonadotropin [hCG] has two dissimilar noncovalently associated subunits, designated $\alpha$ and $\beta$. An ectopically secreted hCG $\alpha$ subunit that fails to associate with the $\beta$ subunit and displays an anomalously high molecular weight on molecular sieve chromatography but not on sodium dodecyl sulfate-polyacrylamide gel electrophoresis has been sequenced. A single substitution of Glu$^{56}$ by Ala$^{56}$ has been found in the altered subunit. No evidence for conformational differences between normal and ectopic $\alpha$ could be found using circular dichroism or intrinsic fluorescence as measures of secondary and tertiary structure, respectively. Hydropathy profiles as determined by the method of Kyte and Doolittle (Kyte, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132) predicted, however, that the hydrophilic segment, Thr$^{54}$-Ser$^{55}$-Glu$^{56}$-Ser$^{57}$-Thr$^{58}$, becomes an extension of the preceding hydrophobic segment when Glu$^{56}$ is substituted with Ala. This solitary hemoglobin S-like mutation may lead to an altered tertiary structure, self-dimerization, or an alteration in glycosylation that could be responsible for the ectopic $\alpha$ subunit's failure to associate with the $\beta$ subunit.

The human placental hormone hCG$^1$ is a glycoprotein synthesized in trophoblastic placental cells 10–12 days after conception. This hormone is necessary for maintenance of the fetus during the first trimester of pregnancy. It binds to a membrane receptor on the ovarian corpus luteum and stimulates adenylate cyclase, which results in the increased synthesis of CAMP and consequently progesterone. The steroid is required for its abnormal properties. The two most commonly offered explanations postulate that the ectopically produced material is an incompletely processed precursor or that differences in glycosylation exist between the normal and abnormal material (7–12).

To establish that this $\alpha$-like material is indeed derived from the normal $\alpha$ protein and to explore the molecular nature of any differences, we describe in this paper the amino acid sequence of an ectopic $\alpha$ subunit. This protein is secreted by a patient with undifferentiated carcinoma of the femoral region. It has previously been shown that this polypeptide (TM-$\alpha$) displays an apparently greater molecular weight as estimated by molecular sieve chromatography but does not differ significantly from normal $\alpha$ upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9). We demonstrate here that a single Glu to Ala substitution has occurred in the altered material while the rest of its primary structure is unaltered. We hypothesize that this subtle change may lead to an aggregation of the altered $\alpha$ subunit, an alteration in glycosylation, or an altered tertiary structure that can account for its abnormal properties.

**EXPERIMENTAL PROCEDURES**

Purification of the Ectopic $\alpha$ Subunit—Ectopic $\alpha$ subunit, TM-$\alpha$, was extracted from the patient’s urine by the kaolin-acetone method (10) and purified on DEAE-cellulose and Sephadex G-25 columns (14). hCG, batch CR-123, was supplied by the Center for Population Research (National Institutes of Child Health and Human Development).

**Reduction and Carboxamidomethylation**—The ectopic $\alpha$ subunit, 1 mg, was incubated in 100 $\mu$l of 0.5 M Tris (pH 8.5), 8 M urea, and 4% mercaptoethanol for 30 min at 25°C and then mixed with 8 mg of iodoacetamide in 40 $\mu$l of 0.5 M sodium hydroxide. After a 15-min incubation in the dark, the mixture was desalted on Sephadex G-25 and lyophilized. The carboxamidomethylated ectopic $\alpha$ subunit was dissolved in 500 $\mu$l of 1% ammonium bicarbonate (pH 8.2), incubated with 100 $\mu$g of endopeptidase Arg-C (Boehringer Mannheim) for 8 h at 37°C and digested with an additional 100 $\mu$g of the enzyme for another 8 h. The digest was lyophilized and fractionated on a reverse phase C4 high performance liquid chromatography column (Bakerbond, 5-μm, Baker Chemical Co.) with a linear 2-h gradient from 0.5 M acetic acid, adjusted to pH 4.0 with pyridine, to 40% 1-propanol in the same buffer. Peptide fragments were collected and subjected to sequencing with a gas-phase protein sequenator. (ABI 470A) using the NVAC program. Phenylthiohydantoin-derivatives were identified by reverse phase high pressure liquid chromatography (15).

**Spectroscopic Analysis**—The circular dichroic spectra of the hCG $\alpha$ and the ectopic $\alpha$ subunits were measured in a 2-mm cell with JASCO J-500A spectropolarimeter. Fluorescence spectra were measured with an SLM 4000 spectrophotometer. Experiments were conducted at protein concentration 0.1 mg/ml.

**RESULTS AND DISCUSSION**

There are three arginines in the $\alpha$ subunit of normal hCG, at positions 34, 42, and 67. Digestion of normal $\alpha$ subunit by an extensive variety of transformed cells (6). It is generally accepted that this material is closely related to the $\alpha$ subunit although its exact nature is unknown. In most cases, ectopic $\alpha$ appears to have a significantly higher molecular weight as determined by molecular sieve chromatography than the normal $\alpha$ subunit (7–12). This apparent increase in size is frequently not confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, however. This presumably altered material usually fails to form native hormone when incubated with the $\beta$ subunit (7–12). The nature of the structural differences between the $\alpha$-like material and the normal $\alpha$ subunit is unknown. The two most commonly offered explanations postulate that the ectopically produced material is an incompletely processed precursor or that differences in glycosylation exist between the normal and abnormal material (7–12).
The Amino Acid Sequence of a Carcinoma hCG α Subunit

with endopeptidase Arg-C produced the expected four fragments of 34, 8, 25, and 25 residues. The yields were approximately 65% according to measurements of the peak areas. The ectopic α subunit also produced four fragments upon the same enzymatic digestion. The amino acid sequences of the first, second, and fourth fragments of the ectopic α subunit were identical to those of the normal hCG α subunit except that the 3 amino-terminal residues were missing from the first fragment (Fig. 1). This is consistent with the frequently encountered heterogeneity of the α subunit resulting from proteolytic removal of several amino-terminal residues (16). The normally glycosylated Asn values and Asn did not appear as phenylthiohydantoin-Asn in sequencing, suggested that they are glycosylated in ectopic α as well. The third fragment of the ectopic α subunit was not, however, identical to the corresponding normal fragment. The glutamic acid residue in position 56 of the normal protein is replaced by an alanine in the ectopic polypeptide (Fig. 1). The yields of the relevant amino acids in positions 54–58 for both normal and the ectopic protein are presented in Table I. This was repeated three times. The same result was obtained when undigested ectopic α was sequenced once to residue 67. The glutamic acid at position 56 is conserved in the bovine, porcine, equine, mouse, rat, and human sequences (5). The Glu to Ala substitution can be achieved by a single A to C base change assuming the GAG Glu codon assignment given in the published cDNA and gene sequences for the normal human α subunit of hCG (17). The existence of a single copy of the α gene per haploid complement (18, 19) argues strongly that the presence of this alteration is due to a mutational event or genetic polymorphism and not to the presence of an additional gene. Fortuitously, Glu falls within a HindII restriction site that will permit screening for the general presence of genetic alterations at this position.

We also examined the far UV circular dichroism and intrinsic fluorescent spectrum of both proteins to test for the possibility of conformational differences. The circular dichroic spectra were identical within experimental error, indicated no significant difference in secondary structure between the proteins. Both α subunits also gave similar tyrosine fluorescence emission with no evidence for tryptophan fluorescence, as expected. Thus, to the extent that the 3 tyrosine residues present provide a monitor of α tertiary structure, the two different subunit forms cannot be distinguished. Secondary structure prediction by the method of Chou and Fasman (20) produced no suggestion of conformational differences. Hydrophobicity profiles as determined by the method of Kyte and Doolittle (21), however, predicted that the hydrophilic segment Thr-Ser-Glu-Ser-Thr became an extension of the preceding hydrophilic segment upon the Glu to Ala substitution. Such an alteration could be important for interactions with the β-subunit.

Despite the similar amino acid sequence and secondary and tertiary structure between the two polypeptides, dramatic structural and functional differences apparently exist. This abnormal protein exhibits an apparently greater size by molecular sieve chromatography (9), but not on sodium dodecyl sulfate-polyacrylamide gel electrophoresis and cannot combine with the β subunit to form native hormone. To confirm these observations, we compared the molecular sieve elution pattern of α, ectopic α and α-β dimer under identical conditions (Fig. 2). As expected, the ectopic α subunit, at 49 kDa, behaves as if its size were much closer to that of α-β dimeric hormone (56 kDa) than the normal α-polyepitope of 26 kDa. It was also confirmed that sodium dodecyl sulfate-polyacrylamide gel electrophoresis of α and ectopic α under reducing conditions suggests similar molecular weights. Under nonreducing conditions, however, evidence for higher molecular weight material in the ectopic preparation is clearly present (Fig. 2).

One simple hypothesis is that the Glu to Ala substitution in the ectopic subunit results in aggregation of the molecule. This is consistent with the molecular sieve and gel electrophoresis data. The presence of a higher molecular weight under native conditions which is abolished when the protein is denatured is characteristic of aggregation phenomena. The 49-kDa size seen with TM-α when compared to 26 kDa for normal α under the same conditions suggests dimer formation. The presence of higher molecular weight material under nonreducing conditions suggests the possible presence of disulfide bonds in any aggregate. The Glu to Ala substitution is reminiscent of the Glu to Val substitution found in hemoglobin S leading to aggregation of this protein (22) and of dimer formation in Bence-Jones proteins (23). Chemical modificati-
tion studies suggest that carboxylic groups on the α subunit are involved in subunit association (24). The conserved nature of Glu$^{66}$ is consistent with this assignment. The apparent hydrophobicity of Thr$^{34}$-Ser$^{55}$-Ala$^{66}$-Ser$^{37}$ in ectopic α would explain the failure of altered α to recombine with β subunits into native hormone. It appears most likely that the single substitution in TM-α is itself responsible for the atypical aggregation. The missing three amino-terminal amino acids are not necessary for subunit interactions or receptor binding activity (25). Glu$^{66}$ is also only 1 residue from a glycosylation site and 3 away from a normally glycosylated Asn residue. Although carbohydrate has also been shown to be unimportant in subunit interactions and receptor recognition (26), the proximity of Glu$^{66}$ to a glycosylation site suggests abnormal glycosylation as a second possible abnormality. This is consistent with differences in lectin binding behavior between this ectopic protein and normal α previously described (9). Free α-like material from bovine pituitaries which fails to interact with the β subunit is known to be glycosylated abnormally at Thr$^{35}$ (12). The corresponding threonine residue in our ectopic α was sequenced correctly, however, indicating that it was not glycosylated.

One must ask to what extent this alteration is a general occurrence in ectopically secreted α subunits. The common observation of the increased size of this material (7–12) suggests aggregation or atypical glycosylation itself may be a common finding, but it seems likely that a variety of primary structural alterations could produce similar effects. A general screening for this particular potential polymorphism made possible by loss of a restriction site at this position could answer this question.

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