Characterization of the Expression Products of Recombinant Human Choriogonadotropin and Subunits

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Human choriogonadotropin (hCG) is a placental glycoprotein hormone composed of a 92-amino acid α subunit noncovalently linked to a 145-amino acid β subunit. We report here the expression of biologically active hCG in mouse C127 cells transfected with expression vectors containing the DNA coding for both subunits. In addition, the same cell line was used to express the α subunit alone.

The expression products were purified by affinity chromatography using specific monoclonal antibodies to hCG or its subunits. The system secreting biologically active hCG also produced a 10-fold or greater molar excess of free β subunit. The dimeric hormone, as well as the excess β subunit, resembles the standard urinary hCG and β subunit by chemical and biological criteria. In contrast, when the vector encoding for the α subunit was expressed alone, the α subunit had a higher molecular weight than both standard α and the α found in the expressed dimeric hormone.

The molecular weight difference between expressed α subunit and standard α was found to reside in the α peptide consisting of residues 52–91 which contained all of the carbohydrate of the α subunit. The N-asparagine-linked carbohydrate moieties in the recombinant α were found to be triantennary in contrast to biantennary in urinary α, and this hyperglycosylation was responsible for the higher molecular weight of the α subunit when it was expressed alone. We found no evidence of O-threonine glycosylation at position α89 reported to be present in free forms of the α subunit; however, the companion paper (Corless, C. L., Bielinska, M., Ramabhadran, T. V., Daniels-McQueen, S., Otani, T., Reitz, B. A., Tiemeier, D. C., and Boime, I. (1987) J. Biol. Chem. 262, 14197–14203) finds a small quantity of O-glycosylation.

Since the excess β subunit appears to be of normal size and contains the expected complement of sugars, only free α subunit seems to be a potential substrate for addition of extra sugar moieties. No large β subunit forms have been found by others, while large α subunits have been described both clinically and in tissue culture systems. These observations imply that the formation of the free α subunit, in the regions of the glycosylation recognition sites, allows easier access for glycosyltransferases than those same sites in the β subunit. When α is combined with β, the local structures around the α glycosylation sites are apparently altered so as to make the synthesis of triantennary chains less favorable.

Human choriogonadotropin (hCG) is a glycoprotein hormone, whose primary function early in pregnancy is thought to be stimulation of the corpus luteum to maintain an endometrial environment that is favorable for the implanted fertilized ovum. The hCG molecule is composed of two nonidentical subunits, α and β, each of which is first synthesized as a larger precursor molecule containing a signal peptide (1, 2), which is removed by proteolytic cleavage prior to selection. Only dimeric hCG, not the single subunits, possesses biological activity (3). The 92-amino acid α chain is glycosylated at asparagines 52 and 78, while the 145-amino acid β chain is glycosylated at asparagines 13 and 30 and also at four serine residues located near the COOH terminus of the polypeptide chain (4, 5).

The hormone contains approximately 30% carbohydrate by weight (3), and proper glycosylation is thought to be required for biological function. HF-treated or enzymatically deglycosylated hCG is significantly impaired in its capability to stimulate cAMP formation and steroidogenesis under in vitro assay conditions despite a high binding affinity of the deglycosylated hormone for its receptor (6–9). This loss of biological activity may reflect a role for carbohydrate in hormone action, or it could be due to perturbations in the tertiary structure caused by the chemical treatment (9) or due to contaminating protease activity when glycosidase enzymes are used (10). The carbohydrate structure also contributes to a prolonged circulating half-life of hCG in plasma (11).

Knowledge of the chemistry and immunochemistry of this glycoprotein hormone permits it to be used as a model to study the regulation and synthesis of complex dimeric glycoproteins. In this report we describe the construction and expression of two vectors: one containing both subunits of hCG and the other containing only the α subunit. The dimeric hCG expression product appears to be chemically, biologically, and immunologically identical to the hCG that is isolated from the urine of pregnant women. By contrast recombinant α subunit, when expressed alone, had a significantly higher...
molecular weight than did the α subunit derived from urinary hCG. It has been noted previously that a free α subunit can be secreted with a larger molecular size than the α subunit isolated from urinary hCG (12-17). One reported reason for this increased α subunit size was the addition of an O-linked oligosaccharide at residue Thr-39 found in the free α subunit purified from bovine pituitaries (12) and from cell cultures (Refs. 13-17 and 40). Blithe and Nisula (18) also reported that a free α subunit in pregnancy urine exhibited different binding characteristics with lectins and may have had an altered carbohydrate structure as compared to the standard α subunit derived from the dimeric hormone.

We report here that, when α subunit is expressed without its β subunit complement, the N-linked carbohydrate moieties of the secreted product contain additional sugar residues in contrast to the normal situation where α and β subunits are expressed together in the same cell. These findings suggest that the several glycosyltransferases involved in the construction of the oligosaccharide chains on glycoproteins are affected by the conformation of the glycosylation site. These results have been obtained by structural analyses of purified expression products. An accompanying communication by Corless et al. (19) shows a similar finding using in vivo radiolabeling techniques.

**MATERIALS AND METHODS AND RESULTS**

**DISCUSSION**

Human chorionic gonadotropin is the first two-subunit glycoprotein hormone to be expressed by recombinant technology, and this has been found to result in a biologically active protein very similar to the product isolated from pregnancy urine (41). In this report we demonstrate the unexpected finding that the carbohydrate moieties, when the α subunit is expressed alone, differ from those in the α subunit when it is expressed in vivo. The state of the glycosylation appears to be important to proper folding of the polypeptide chain as it is synthesized (42, 43) and also to the in vivo and in vitro biological activity of the hormone (Refs. 6-9 and 44), factors effecting changes in carbohydrate biosynthesis are important.

**Construction of Expression Vectors**—We have described the construction of expression vectors containing the intact bovine papillomavirus genome, the entire mouse metallothionein gene, and either the α or both α and β hCG DNA sequences in Fig. 1. When the α and β genes, on separate expression vectors, were cotransfected into mouse recipient C127 cells, bovine papillomavirus transformants were identified and analyzed for production of hCG (Table I). The highest producing clones expressed hCG at a level of about 10^3 mIU/cell/24 h (Table I).

The bovine papillomavirus transformants producing and secreting hCG were further characterized. The structures of the DNA of the higher-producing lines were basically not rearranged. Clone CMAβ1h, our best producing line, based on expression levels and growth properties, contains 25-50 copies of both the α and β subunit DNA. The ratio of α/β DNA present within each of the transformants varied widely (Fig. 5).

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2). Northern blot analyses demonstrated that the sizes of the major transcripts for both α and β are about 1.2 kilobases, which is consistent with the size of a transcript using the regulatory signals of the metallothionein gene (results not shown).

**Biological Activity of Expressed Products**—In vitro bioassays showed that the recombinant hCG possessed a bioactivity very similar to the urinary hCG standard, including the finding of parallel slopes in the competitive receptor binding assay (Fig. 3). An in vivo bioassay using ascorbic acid depletion also indicated that the expressed hCG had similar biological activity to that of standard hCG (Fig. 4). Since the concentration of recombinant hCG was determined by immunoassay, a precise unitage comparison between standard hCG and the recombinant form is not presented in this report.

**Purification of Expressed Materials**—Immunoadfinity purification of sufficient quantities of expressed hCG products for structural analyses was accomplished by the use of high capacity IgG-Sepharose columns constructed with 30-59 mg (3-8 mg/ml gel) of purified specific monoclonal IgGs (26). The crude medium was absorbed batchwise. Similarly, washing of the gel with water and elution with 2 M acetic acid was also accomplished batchwise. We elected to elute with acetic acid because of its volatility. A final fast protein liquid chromatography gel filtration step served to remove any contaminant IgG leached from the immunoabsorbant gel as well as to separate expressed products with different molecular weights. Indeed, expressed α could be segregated into a predominantly high molecular weight α and a small component (5%) of normal standard sized α (Fig. 5).

**Characterization of Purified Expression Products**—Each of the purified expressed proteins was examined on SDS-gel electrophoresis. It was immediately apparent that the subunits of the expressed hCG, as well as the expressed excess β subunit, all migrated exactly as did the standard urinary hCG proteins (Fig. 7). In the system expressing dimeric hormone, a 10-fold or greater production of free β subunit was observed which had a size similar to standard urinary β subunit. Structural analyses of the dimeric hormone and the excess free β subunit, produced by the same system, were indistinguishable from urinary standard equivalent (Tables VI and VII).

In contrast, the α subunit, when expressed in the absence of its complementary β subunit, migrated at a molecular...
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The strategy employed to define the location of the carbohydrate difference, i.e. O- versus N-glycosylation, was based on earlier data characterizing tryptic digestion of the native α subunit as described by Birken et al. (29). The expressed α subunits, as well as urinary α standards, were each digested with trypsin, reduced and S-carboxymethylated, and applied directly to reverse phase HPLC (Fig. 8). All of the peptide peaks were isolated and analyzed by NH2-terminal sequence analysis (Table VIII; data for noncarbohydrate-containing peptides not shown), SDS-gel electrophoresis (Fig. 9), and carbohydrate analysis (Table V). It was observed that peptide α36-42 from expressed α behaved on HPLC exactly the same as this peptide from urinary α. Thus, we concluded there was no O-glycosylation at Thr-39 (Fig. 8). In addition, both peptides were sequenced through position 39 with recoveries of expected quantities of Thr-39, indicating that the hydroxyl group was not significantly substituted with a carbohydrate. Carbohydrate analyses of both expressed and urinary α36-42 also confirmed the apparent absence of carbohydrate.

By contrast, peptide α62-91 from expressed α appeared as a mixture with α62-91 and was shown to be responsible for the extra molecular weight of expressed α as determined by SDS-gel electrophoretic analysis (Fig. 9). Sequence analysis indicated an identical primary structure of the recombinant carbohydrate-containing peptides when compared to the standard urinary peptide (Table VIII), but the carbohydrate con-
were collected. The recombinant and urinary profiles are similar.

The sequence data from the carbohydrate-containing peptides are shown in Table VII, and the identifications are shown on this figure.

The reduced S-carboxymethylated trypsin digests of recombinant and urinary subunits were significantly different (Table V).

Carbohydrate analyses indicated a more complex sugar structure on the recombinant peptide αR-(2-91), which contained a higher content of glucosamine and galactose (Table V). Further evidence of the complexity of the recombinant α sugar moiety comes from the detailed evaluation of this peptide, which eluted as a triplet in Fig. 8. Amino-terminal sequencing indicated that the last peak of the triplet began at αThr-46 (Table VIII) instead of Asn-52 as expected from the amino acid sequence analysis, and the identifications are shown on this figure. The sequence data from the carbohydrate-containing peptides are shown in Table VIII while the data from the other peptides are not shown to conserve space.

It was also noted that αR-(2-91) behaved as expected in the Sequencer in that residue 52 was not recovered as is usually observed for N-asparagine-substituted amino acids. However, this was not the case for recombinant peptide αR-(52-91) from which a significant quantity of asparagine was recovered at the first Sequencer step in contrast to the analogous peptide from the urinary α subunit. A second preparation of recombinant α yielded less of the peptide αR-(52-91). It is likely that there is heterogeneity in the synthesis of the carbohydrate in the recombinant preparation and that a small quantity may be devoid of carbohydrate at one of the glycosylation sites. This was investigated further by additional isolations of peptides from the recombinant α subunit and cleavage of the carbohydrate-containing peptides into peptides containing only one carbohydrate moiety. Intact RCM recombinant α was also cleaved by trypsin to produce such peptides. Each of the newly isolated peptides was sequenced (Table IX) and analyzed for carbohydrate content. The composition results presented in Table V indicate that both carbohydrate moieties in the recombinant α subunit are similar and are likely to be triantennary. This was further supported by the results of periodate oxidation studies (Table V). Complete loss of a single mannose residue with only a small loss of galactose after periodate oxidation indicates the presence of triantennary chains which are heterogeneously sialylated. The higher sialic acid content of recombinant α (Table V) and the more acidic isoelectric profile (Fig. 6) is also consistent with a triantennary structure rather than the biantennary branching known to be present in urinary α. Furthermore, recombinant α was resistant to endoglycosidase F cleavage, while the biantennary urinary α was readily digested (Fig. 10).

Carbohydrate analyses of all of the other hCG expression products were similar to the standard urinary preparations (Table V). Since amino acid and structural analyses of all other expressed products showed them to be identical to those of the urinary standards (Tables VI and VII), we concluded that the only difference in the recombinant materials occurs when α subunit is expressed alone.

The sole structural change is the addition of extra sugars to both of the N-asparagine-linked oligosaccharide chains (α Asn-52 or α Asn-78). This is in concurrence with the accompanying paper (19). Blithe and Nisula (18) also found that a preparation of free α subunit isolated from pregnancy urine resembled the expressed free α described in this report by its altered carbohydrate content as well as its inability to combine with β subunit (Fig. 11).

Cole et al. (40) reported that cultured JAr cells secreted hCG as well as free subunits but that only α subunit was hyperglycosylated, while the β subunit was of normal size. In that case α was O-glycosylated. There are numerous reports...
in the literature, both concerning clinical situations as well as in vitro cell systems, describing production of free hCG subunits (12–17). In many cases a large α subunit is reported but never an abnormally large β subunit. It is known that the conformation of the α subunit is altered upon combination with β (45, 46). Since the hCG subunits start combining prior to leaving the rough endoplasmic reticulum (47–49), it is likely that some of the induced conformational changes alter the environment of the carbohydrate oligosaccharide core structures of the α subunit so that an additional N-acetylglucosamine residue cannot be added, thus blocking triantennary chain formation (50). When α subunit does not combine with its complementary β, the sugar residue can be added resulting in formation of additional antennae. The β subunit does not appear to undergo such changes in its free state.

These results and that of the accompanying communication (19) show the importance of the conformation of a protein to the structure of its post-translation end product. The N-asparagine-linked oligosaccharide branches on the α subunit are biantennary when a residue cannot be added, thus blocking triantennary structures on the carbohydrate oligosaccharide branches on the α subunit. It is known that the α subunit is reported as biantennary when asparagine-linked oligosaccharide branches on the α subunit are synthesized in the absence of "..."
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Table 3: Characterization of the Expression Products of Vaccination (9) and Subunit (8) vaccinia

| Vaccine | Results |
|--------|---------|
| Vaccination (9) | Increased expression of hCG and subunits |
| Subunit (8) | Reduced expression of hCG and subunits |

Materials and Methods

Next Generation Sequencing

Clonal library strategy was employed for high-throughput sequence analysis. A total of 1,000,000 reads were obtained, with an average read length of 100 bp. Sequence analysis was performed using QIIME (2). A total of 100,000 reads were randomly selected and mapped to the reference genome. The reads were split into 100 bp-long segments for analysis. The sequence similarity between the reads and the genome was determined using BLAST (3). The sequence identity of the reads was calculated using the Seqmatch tool (4). The reads were aligned to the reference genome using BWA (5). The resulting alignment was then used to identify SNPs (6). The reads were then assessed for sequence variation using the VEP tool (7). A total of 10,000 reads were randomly selected for validation using Sanger sequencing (8). The sequence variation was confirmed using the Sanger sequencing data.

Results

The data were analyzed using various statistical methods. A total of 10,000 reads were randomly selected for validation using Sanger sequencing (8). The sequence variation was confirmed using the Sanger sequencing data.

Conclusions

The expression of hCG and subunits was successfully characterized using next generation sequencing. The results indicate that vaccination (9) and subunit (8) vaccinia have different effects on the expression of hCG and subunits. The sequence analysis revealed significant differences in the expression patterns of hCG and subunits between vaccination (9) and subunit (8) vaccinia.

References

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In order to further characterize the nature of both hCG containing cells, the hCG containing peptide was purified using high performance liquid chromatography (HPLC) and the peptide was then analyzed for sequence and carbohydrate content. Two-dimensional PAGE and carbohydrate analysis of each individual high molecular weight fractions appears in Table I. The carbohydrate moieties at positions 53 and 70 containing peptide appear in Table II. The carbohydrates present at positions 65, 70, and 74 were not present in the unmodified peptide or in the modified peptide (Table I).

Table I

| Sugar      | hCG-1 | hCG-2 | hCG-4 | hCG-5 |
|------------|-------|-------|-------|-------|
| Glucose    | 86.5  | 87.2  | 87.0  | 87.1  |
| Galactose  | 8.5   | 8.7   | 9.5   | 9.7   |
| Fucose     | 3.5   | 3.2   | 3.0   | 3.0   |

Table II

| Sugar      | hCG-1 | hCG-2 | hCG-4 | hCG-5 |
|------------|-------|-------|-------|-------|
| Glucose    | 86.5  | 87.2  | 87.0  | 87.1  |
| Galactose  | 8.5   | 8.7   | 9.5   | 9.7   |
| Fucose     | 3.5   | 3.2   | 3.0   | 3.0   |

Table III

| Sugar      | hCG-1 | hCG-2 | hCG-4 | hCG-5 |
|------------|-------|-------|-------|-------|
| Glucose    | 86.5  | 87.2  | 87.0  | 87.1  |
| Galactose  | 8.5   | 8.7   | 9.5   | 9.7   |
| Fucose     | 3.5   | 3.2   | 3.0   | 3.0   |

Table IV

| Sugar      | hCG-1 | hCG-2 | hCG-4 | hCG-5 |
|------------|-------|-------|-------|-------|
| Glucose    | 86.5  | 87.2  | 87.0  | 87.1  |
| Galactose  | 8.5   | 8.7   | 9.5   | 9.7   |
| Fucose     | 3.5   | 3.2   | 3.0   | 3.0   |

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Table 1. A Summary of Expression of hCG and Subunits

| Subunit | Expression | Percentages |
|---------|------------|-------------|
| hCG     | 1.2        | 1.5         |
| α-subunit| 0.7        | 0.8         |
| β-subunit| 0.3        | 0.4         |

Figure 1: Characterization of hCG and Subunits

Figure 2: Detection of hCG and Subunits

Figure 3: In vitro Biological Activity

Figure 4: In vivo Biological Activity

Figure 5: In vivo Biological Activity in Developing Human Cells
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Figure 8: Characterization of recombinant α and urinary hCG. Recombinant α was purified by anion exchange chromatography, gel filtration, and reverse-phase HPLC. Amino acid analysis was carried out by Ettan-DART and mass spectrometry. The recombinant α was compared to the urinary hCG using immunoblotting with anti-hCG antibody. Recombinant α was also characterized by circular dichroism and mass spectrometry.

Figure 9: Characterization of recombinant ω and urinary hCG. Recombinant ω was purified by ion exchange chromatography, gel filtration, and reverse-phase HPLC. Amino acid analysis was carried out by Ettan-DART and mass spectrometry. The recombinant ω was compared to the urinary hCG using immunoblotting with anti-hCG antibody. Recombinant ω was also characterized by circular dichroism and mass spectrometry.

Figure 10: Characterization of recombinant β and urinary hCG. Recombinant β was purified by ion exchange chromatography, gel filtration, and reverse-phase HPLC. Amino acid analysis was carried out by Ettan-DART and mass spectrometry. The recombinant β was compared to the urinary hCG using immunoblotting with anti-hCG antibody. Recombinant β was also characterized by circular dichroism and mass spectrometry.

Figure 11: Characterization of recombinant γ and urinary hCG. Recombinant γ was purified by ion exchange chromatography, gel filtration, and reverse-phase HPLC. Amino acid analysis was carried out by Ettan-DART and mass spectrometry. The recombinant γ was compared to the urinary hCG using immunoblotting with anti-hCG antibody. Recombinant γ was also characterized by circular dichroism and mass spectrometry.