Expression, characterisation and immunoassay of recombinant marmoset chorionic gonadotrophin dimer and β-subunit

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Abstract

A specific and sensitive ELISA for measuring marmoset chorionic gonadotrophin (mCG) in culture medium, urine and plasma was developed using a polyclonal antibody raised against recombinant mCG, tagged with six histidine molecules (rmCG-6His), as the capture antibody. A well-characterised monoclonal antibody (518B7), which was generated against bovine luteinising hormone (bLH) and has been shown to detect CG and LH in Callithricid monkeys, was biotinylated and used as the secondary antibody. Purified rmCG, calibrated against human CG (hCG; CR127) by bioassay, or the β-subunit (rmCGβ), quantified from amino acid analysis and carbohydrate analysis, was used as the standard. The assay was able to detect CG activity in medium collected from cultured marmoset embryos before attachment and through to the trophoblastic vesicle stage, plasma and urine collected from pregnant marmosets, marmoset placenta and pituitary homogenates. The assay was validated and its performance compared with a bioassay based on MA10 cell response to CG, with hCG as the standard. The sensitivity was 103 pg/ml (5 pg/well) of rmCGβ and 476 pg/ml (24 pg/well) of the heterodimer rmCG. The mean recovery of standard added to embryo culture medium, marmoset urine and plasma was 104, 112 and 92% respectively. The intra- and interassay variation was less than 10 and 16% respectively. The low cross-reactivity with cynomolgus monkey and baboon LH, their β-subunits, cynomolgus monkey and baboon follicle-stimulating hormone and hCG suggests that the assay is specific for mCG.

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Introduction

Chorionic gonadotrophin (CG) is a placentally derived glycoprotein hormone which is made up of an α- and β-subunit (Pierce & Parsons 1981). CG and related members of this group of hormones, including luteinising hormone (LH), follicle-stimulating hormone (FSH) and thyroid-stimulating hormone, have unique α- and β-subunits and share a common α-subunit. The most well characterised biological function of CG is its ability to extend the life of the corpus luteum and the associated progesterone production via receptors present on the luteinised cells. Interdiction of CG (via active or passive immunisation) or progesterone (using progesterone receptor antagonists) terminates ongoing pregnancy, by preventing implantation from taking place (Hearn 1979). Moreover, peripheral levels of CG are an important clinical indicator of ongoing healthy pregnancies, while falling or low levels suggest abnormal embryonic implantation and/or development.

Given the necessity for appropriate levels of production of CG by the early primate embryo for the maintenance of a viable pregnancy, little is known of the ontogeny and regulation of its expression and secretion at peri-implantation. There are a number of studies at the molecular level, which have described some of the transcription factors required for tissue-specific expression of the α- and β-subunits of human CG (hCG) (Jameson & Hollenberg 1993 and references therein). Few studies have looked at regulation of CG at the cellular level, to establish the role of endogenous factors (cytokines, growth factors), particularly at peri-implantation. This is primarily due to the many ethical constraints on the use of human embryos for research purposes and to a lack of suitable species-specific probes to allow the use of non-human primates for such studies. These problems have been avoided to some degree through the use of in vitro cultured chorioiarcinoma cell lines such as JEG-3 and primary placental cell cultures, which have shown an ability of some endometrial-derived factors to influence CG expression (Oerbauer et al. 1988, Guilbert et al. 1991, Ren & Braunstein 1991). These cell lines, while providing useful information, may not provide a true indication of the role of such factors in regulating CG expression at the early embryonic stages. First, there may be differences in the receptor populations between the cultured placental cells and the cells of the early primate embryo. Secondly,
CG-expressing cells in the early embryo may be influenced by neighbouring embryonic cells and such influences cannot be accounted for in the isolated cultured placental cell lines.

In recent years, the common marmoset (*Callithrix jaccus*) has proven to be a useful model for the investigation of early embryo development, assisted reproductive technology and general reproductive biology. The marmoset has also been used for many years to study CG expression through pregnancy and in the early cultured marmoset embryo (Hearn et al. 1988a,b). In these studies an in vitro bioassay using freshly isolated mouse Leydig cells was used to monitor the CG levels. This assay, developed by Ziegler et al. (1986), is very sensitive and able to detect hCG levels stated to be as low as 100 pg/ml (Seshagiri et al. 1995). While these methods of analysis are sensitive, they are also expensive and very labour intensive. Specific antibodies to mCG offer the potential for a rapid sensitive immunoassay. Heterologous antibody RIAs for mCG using polyclonal antisera have been described by Chambers & Hearn (1979) and Ziegler et al. (1987). More recently a mouse monoclonal antibody (518B7) directed to the ß-subunit of mCG (Matteri et al. 1987, Ziegler et al. 1993, Rosenbusch et al. 1994, Simula et al. 1995). Ziegler and co-workers (1993) went on to develop an RIA, which is a simple competitive rapid sensitive immunoassay. Our laboratory has recently cloned and expressed in mammalian cells the ß- and ß-subunits of mCG (Simula et al. 1995). In the present study we describe (1) the expression and purification of recombinant 6-histidine-tagged mCG (mCG-6His), rmCG and the ß-subunit (rmCGß) protein, (2) the production of a specific rabbit polyclonal anti-mCG antibody and (3) the subsequent development and validation of a sensitive two-site ELISA.

**Materials and Methods**

**Construction of 6-histidine tagged mCGß cDNA**

The cDNA for mCGß was altered by PCR to incorporate the coding sequence for six consecutive histidine residues (sequence of downstream primer with double underline) to its C-terminus. PCR was conducted under the following conditions. To a final volume of 50 µl was added 100 nmoI mCGß cDNA, 5 µl 10 × reaction buffer, 15 pmol each primer (upstream, 5' - CAC GGGCCCGGGGAGCAGCAACCAAGGATG-3'; downstream, 5' - ATCAAGCTTAGTGATGTTG-3'), 2·5 U Taq/Pwo DNA polymerase mix (Expand Long Template PCR System, Boehringer-Mannheim, Mannheim, Germany), 1·75 µl 10 mM dNTP mix, water to 50 µl, and overlaid with 50 µl mineral oil. The tubes were placed in an automated PCR thermal cycler (Corbett Research, Sydney, NSW, Australia). After an initial denaturation at 94 °C for 3 min, a 35-cycle programme was initiated with cycle parameters of 94 °C for 30 s and 68 °C for 2 min, followed by a final extension time of 5 min at 68 °C. The expected PCR product was purified and ligated into pBluescript SK+ (Strategene, La Jolla, CA, USA) using the available ApaI/HindIII sites present on the amplification primers (underlined above). Clones shown to have the correct insert DNA were sequenced (AmpliCycle Sequencing Kit; Perkin-Elmer, Foster City, CA, USA) to confirm the integrity of the entire modified mCGß cDNA (mCGß-His).

**Expression of mCG dimer and mCG-6His dimer in CHO.K1 cells**

A ß-actin promoter-driven mammalian cell expression vector containing the mCGß, mCGß or mCG-6His cDNAs were constructed as described previously (Simula et al. 1995). Plasmid DNA (2 µg) of the mCGß vector was co-transfected with an equal molar ratio of either the mCGß or mCGß-6His vector, using the calcium phosphate co-precipitation method. The conditions for the isolation of recombinant CHO.K1 cell clones and subsequent scale-up of the culture volumes were as previously described (Simula et al. 1995).

**Purification of mCG-6His**

Medium collected from cultures expressing mCG-6His was clarified by centrifugation and either stored at −20 °C or concentrated to one-tenth its volume by ultrafiltration, using a spiral-wound membrane cartridge (10 kDa molecular mass cut-off; Amicon Inc, Beverly, CA, USA). The concentrate of 2–5 litres of culture medium was diluted three times with deionised water; 10 × PBS, pH 8, and 500 mM imidazole were then added to give a final concentration of 50 mM phosphate, 300 mM NaCl and 1 mM imidazole. The concentrate was loaded on to a 10 ml column of Ni–nitrilotriacetate (Ni-NTA) resin (Qiagen Inc., Chatsworth, CA, USA), pre-equilibrated with 50 mM phosphate, pH 8, containing 300 mM NaCl. The column was washed with 100 ml equilibrating buffer containing 30 mM imidazole; mCG-6His was then eluted with 250 mM imidazole at a flow rate of 0·5 ml/min. Fractions were assessed for immuno-reactive mCG using an RIA based on the monoclonal antibody 518B7, donated by Dr Jan Roser, Department of Animal Science, University of California, Davis, CA, USA.
Purification of mCGβ protein

Medium (5–10 litres) collected from cultures expressing mCGβ was clarified, concentrated and diluted with deionised water as above and the pH adjusted to 5·5 with acetic acid. The concentrate was loaded on to a SP–Sepharose Fast Flow cation exchange column (12 × 5 cm; Pharmacia Biotech, North Ryde, NSW, Australia) equilibrated with 20 mM sodium acetate (pH 5·5) and eluted with a stepwise gradient of NaCl. The eluate, in 10 ml fractions, was collected into tubes containing 50 µl 1 M Tris (pH 9·0) and the presence of immunoreactive mCGβ was determined by RIA as described above. Sodium phosphate (500 mM, pH 7·4) and Tween 80 were added to the pooled immunoreactive fractions to a final concentration of 50 mM and 0·01% respectively. The sample was then subjected to immunoaffinity chromatography on an anti-bovine LH–Sepharose column pre-equilibrated with 50 mM sodium phosphate, pH 7·4. The column was prepared by coupling the monoclonal antibody 518B7 to N-hydroxysuccinimide (NHS)-activated Sepharose (Pharmacia Biotech) according to the manufacturer’s protocol. Briefly, 35 mg antibody dissolved in 5 ml coupling buffer (0·2 M NaHCO3, pH 8·3, 0·5 M NaCl), was loaded on to a 5 ml column of NHS-activated Sepharose and allowed to stand for 30 min at room temperature. Unbound antibody was washed out of the column and any excess active groups were deactivated according to the supplier’s protocol. After being loaded, the column was washed with 100 ml elution buffer and then eluted with 3 M ammonium thiocyanate or 100 mM glycine–HCl, pH 2·2. Immunoreactive fractions were pooled, concentrated and desalted using Macrosep centrifugal concentrators (10 kDa molecular mass cut-off) and lyophilised. A third chromatography step was employed to remove low molecular mass immunoreactive contaminants. After affinity chromatography, pooled fractions containing mCGβ were reconstituted in 0·1% trifluoroacetic acid (TFA) and loaded on to a reverse-phase HPLC column (Waters; Delta C4; 8 × 100 mm), pre-equilibrated with 0·1% TFA. mCGβ was eluted at 1 ml/min using a linear gradient of 0–40% acetonitrile over 80 min.

Partial purification of rmCG dimer

Medium from cultures of CHO cells expressing biologically active rmCG were concentrated by ultrafiltration as described above for mCG–6His, diluted with an equal volume of 100 mM phosphate buffer, pH 7·0, containing 0·1% NaN3 and 0·1% Tween 80. The medium was then subjected to immunoaffinity chromatography as described for the purification of mCGβ. The column was eluted with 100 mM glycine hydrochloride, pH 2·2. The immunoreactive fractions eluted were pooled, diluted three times with 500 mM phosphate buffer, pH 7·0 (loading buffer) and loaded at 1 ml/min on to a phenyl–Sepharose HP column (Pharmacia Biotech), which had been pre-equilibrated with the same buffer. The column was washed with 10 column volumes of the loading buffer and eluted with a linear gradient from 100% loading buffer to 100% 10 mM phosphate, pH 7·0 containing 20% isopropanol over 20 column volumes. The dimer was monitored by the MA 10 bioassay which is a modification of the assay developed by Ascoli (1981) (Simula et al. 1995).

Electrophoresis

Purification of rmCG-6His, rmCGβ and rmCG was monitored by discontinuous SDS–PAGE in 12% gels under reducing and non-reducing conditions by the method of Laemmli (1970). Protein bands were visualised by silver staining (Silver Staining Kit; Bio–Rad Laboratories, Regents Park, NSW, Australia) and molecular masses were determined by comparison with protein standards (low molecular mass calibration kit; Pharmacia Biotech) or subjected to Western blotting.

Western blotting and immunostaining

The proteins resolved by SDS–PAGE were transferred to a nitrocellulose membrane (0·4 µm; Bio–Rad Laboratories) by the procedure described by Towbin et al. (1979), at 100 mA overnight. The membrane was incubated with 5% low–fat dried milk in 100 mM phosphate buffer, pH 7·5, with 150 mM NaCl and 0·1% Tween 20 for 1 h at room temperature. A further incubation was performed with monoclonal antibody 518B7 (diluted 1:5000) for 1 h at room temperature, followed by incubation with anti-mouse IgG horseradish peroxidase conjugate (Bio–Rad Laboratories; diluted 1:5000) for 1 h at room temperature. Membranes were washed (5 × ) with the above phosphate-buffered saline–Tween 20 solution after each incubation. Detection was achieved with enhanced...
Antibody production

Recombinant mCG-6His protein recovered after immo-
obilised metal ion affinity chromatography on a Ni–NTA column was calibrated against hCG in the RIA described above. A New Zealand White rabbit was immunised by subcutaneous multiple-site injection. A primary injection of approximately 3000 pmol immunogen in complete Freund’s adjuvant was followed by booster injections of approximately 1000 pmol immunogen in incomplete adjuvant every 3–4 weeks. The rabbit was bled 14 days after each immunisation and titres were determined by the ability of serial dilutions of serum to bind radiolabelled purified mCGβ.

Development of a mCG-specific ELISA

Preparation of biotinyl-anti-LHβ monoclonal antibody (518B7) raised against bovine LH was biotinylated using a Biotin Labeling Kit from Boehringer–Mannheim (Sidney, NSW, Australia) (cat. no. 1418165). The antibody and N-biotinoyl-L-aminocaproic acid-N-hydroxysuccinimide ester (biotin–7-NHS) were mixed in a molar ratio of 1:10 and incubated at room temperature, with mixing, for 2 h. Non-reacted biotin was separated according to the kit protocol. Preparation of microtitre plates: 100 µl (0·9 µg) affinity-purified anti-rabbit IgG (Silenus Laboratories) in coating buffer (50 mM carbonate/bicarbonate buffer, pH 9·6, containing 0·01% thimerosal) was aliquoted into each well of the microtitre plate (MaxiSorp; Nunc, Roskilde, Denmark) and incubated overnight at 4 °C. The microtitre plate was subsequently blocked with 260 µl (0·26 µg) BSA in coating buffer for 1 h at room temperature. The plate was washed three times with 260 µl wash buffer (50 mM PBS, pH 7·4, with 0·05% Tween 20; PBS-Tw) followed by the addition of 100 µl polyclonal mCG antiserum (R64) diluted 1:2000 in assay buffer (50 mM PBS, pH 7·4, with 0·05% BSA) and incubated for 2 h at room temperature. The plate was washed three times with PBS-Tw, 50 µl of sample or standard rmCG/ rmCGβ ranging from 0·01 to 24 ng/ml and 50 µl biotinylated antibody 518B7 (diluted 1:2000) were added and the mixture was incubated overnight at 4 °C. The plate was washed three times with PBS-Tw, 100 µl (2·5 U) streptavidin peroxidase (50 mU/ml; Boehringer–Mannheim) was added and the mixture incubated for 30 min at room temperature. Plates were washed three times with PBS–Tw and twice with deionised water; 100 µl substrate (substrate buffer: 100 mM sodium acetate buffer with 1 mM EDTA, pH 5; substrate solution: 10 ml substrate buffer with 25 µl hydrogen peroxide and 250 µl 10 mg/ml 3,3′,5,5′-tetramethylbenzidine (Boehringer Mannheim) in dimethyl sulphoxide) was added and the mixture incubated for approximately 10 min. The reaction was stopped by the addition of 100 µl H2SO4 (500 mM) and the absorbance was measured at 450 nm with a spectrophotometer. Cross-reactivity of related molecules was tested, on a molar basis, against the following gonado-

Embryo/trophoblastic vesicle culture

Embryos were collected from marmoset monkeys using a non-surgical procedure previously described by Thomson et al. (1994) and cultured through to the trophoblastic vesicle stage using a modification of the method reported by Summers et al. (1987). Briefly, embryos were cultured in Alpha Modification of Eagles medium (α-MEM; Trace Biosciences, Castle Hill, NSW, Australia) in a humidified atmosphere of 5% CO2 and 95% air at 37 °C. The medium was supplemented with 10% heat-inactivated foetal calf serum (Trace Biosciences), 2 mM l-glutamine (Gibco-BRL Life Technologies, Grand Island, NY, USA), 5000 U/ml penicillin G and 5000 µg/ml streptomycin sulphate (CSL Biosciences, Parkville, Vic., Australia), 25 µg/ml transferrin (Sigma, Castle Hill, NSW, Australia) and 25 µg/ml insulin (Sigma). The embryos were cultured individually in 1 ml medium in four-well plates (Nunc), allowed to hatch and attach to the plate. The medium was changed after the first day of culture and subsequently every third day.

Gel filtration chromatography of trophoblastic vesicle culture medium

Medium was collected from the culture of marmoset trophoblastic vesicles by the method described by Summers et al. (1987). Chromatography was performed on a Superdex 200 Hi load 16/60 gel filtration column (Pharmacia Biotech), in ammonium bicarbonate (100 mM), at a flow rate of 0·5 ml/min. Fractions (1 ml) were lyophilised three times. On the first two occasions fractions were reconstituted in deionised water and then finally in PBS.

Preparation of pituitary and placental extracts

Marmoset pituitaries and placenta were immediately frozen in liquid nitrogen at the time of collection and then stored at −80 °C. Subsequently, the tissues were homogenised in 50 mM ammonium bicarbonate containing
the protease inhibitors benzamidine hydrochloride (5 mM) and phenylmethylsulphonyl fluoride (PMSF; 1 mM). The homogenates were centrifuged at 13 000 \( g \) for 30 min and the supernatants desalted using a microconcentrator with a 10 kDa molecular mass cut-off membrane (Microcon; Amicon Inc., USA). The extracts were stored lyophilised before being reconstituted in assay buffer for testing of parallelism.

Mass spectrometry, amino acid analysis and carbohydrate analysis of mCG\( \beta \) was performed by Dr Andrew Pound, at the Protein Chemistry Core Facility, Columbia University College of Physicians and Surgeons, New York, NY, USA.

**Amino acid analysis**

Purified rmCG\( \beta \) was hydrolysed at 110 °C for 24 h with 6 M HCl on a Picotag work station (Waters, Milford, MA, USA) and analysed with an Applied Biosystems 420A Derivatiser coupled to an on-line 130A (reverse-phase HPLC) separation system.

**Carbohydrate analysis**

Purified rmCG was hydrolysed with 4 M TFA at 100 °C for 3 h, lyophilised and redissolved in 16 mM NaOH. The neutral and amino sugars were separated on a Carbopac PA1 (4 × 250 mm) anion-exchange column (Dionex, Sunnyvale, CA, USA) using an isocratic gradient of 16 mM NaOH at a flow rate of 1 ml/min. The sugars were detected amperometrically on a Dionex ED40 pulsed amperometric detector equipped with a gold electrode.

**Sialic acid analysis**

The recombinant \( \beta \)-subunit was hydrolysed for 1 h at 80 °C in 0.1 M TFA and analysed on an ion exchange column as described above using an isocratic gradient of 150 mM NaOH in 100 mM sodium acetate for 10 min at 1 ml/min. The sialic acid was detected using an ED40 electrochemical detector (Dionex).

**Statistical analysis**

Parallelism was demonstrated by first performing a linear analysis on the dilution curve of each hormone. The resulting \( \beta \) values from all the regression analyses, which represent the slope, and its standard error were subjected to Student’s \( t \)-test to show if there was any significant difference between the dilution curves, with \( P<0.05 \) accepted as significant. The mean recoveries of mCG from various spiked matrices were tested against the expected values using the paired \( t \)-test.

**Results**

**Purification of mCG-6His**

mCG-6His was purified by immobilised metal ion affinity chromatography as described in Materials and Methods. More than 77% (77–92%) of the immunoreactivity loaded was recovered in the fractions eluted with 250 mM imidazole (Fig. 1). Two major bands at molecular masses of 57 and 34 kDa were visualised by silver staining when the immunoreactive material was subjected to SDS–PAGE (Fig. 2, lane a). Western blot analysis showed these two bands to have affinity for the bovine LH antibody 518B7 (Fig. 2, lane b), indicating the presence of both the heterodimer and the \( \beta \)-subunit, with the majority of the staining being at the lower molecular mass band. Further analysis by SDS–PAGE, under reducing conditions, revealed two bands with molecular masses of 57 and 34 kDa (not shown), indicating a contaminant at the higher molecular mass.
Purification of mCGβ

The initial chromatography of medium from mCGβ-expressing cells on SP-Sepharose resulted in 29 and 66% of mCGβ immunoreactivity being eluted with 100 and 150 mM NaCl respectively (Fig. 3a). Fractions eluted with 150 mM NaCl were pooled and subsequently subjected to immunoaffinity chromatography. Elution of the column with either 3 M ammonium thiocyanate or 100 mM glycine–HCl, pH 2·2, resulted in the recovery of 58% of mCGβ immunoreactivity loaded (Fig. 3b) and an overall increase in purity of greater than 1000-fold (Table 1a). SDS–PAGE and silver staining revealed a single band at 32 kDa (Fig. 4, lane a). Purification of some batches of medium resulted in a minor contaminant at 23 kDa (Fig. 4, lane b), which was removed by a third purification step involving reverse-phase chromatography. The purity was further confirmed by mass spectrometry, which showed one major peak representing a protein with a mean molecular mass of 21,288 Da. The carbohydrate analysis revealed the following mean molar ratios of sugars per protein molecule: fucose 1·3, galactosamine 0·9, glucosamine 8·5, galactose 4·0, mannose 8·1 and sialic acid 2·8. The low levels of galactose and sialic acid suggest heterogeneous oligosaccharides. The glucosamine and

Figure 2 Lane a, silver-stained gel after non-reducing SDS–PAGE of pooled fractions containing immunoreactive CG eluted from the immobilised metal ion affinity chromatography column described in Fig. 1. Lane b, Western blot analysis of the same sample as in (a) using the anti-bovine LH antibody 518B7 for immunostaining as described in Materials and Methods. The silver-stained bands at 57 and 34 kDa in lane (a) correspond to the two immunoreactive bands developed in (b).

Figure 3 (a) Profile of medium from mCGβ-expressing CHO cells chromatographed on a SP-Sepharose column (12 × 5 cm), equilibrated with 20 mM sodium acetate, pH 5·5, and eluted with a stepwise gradient of NaCl at a flow rate of 5 ml/min. (b) Immunoaffinity chromatography of pooled CG immunoreactive fractions eluted with 0·15 M NaCl from previous chromatography step. The affinity column had an anti-bovine LH antibody (518B7) coupled to Sepharose, equilibrated with 50 mM sodium phosphate, pH 7·4, and eluted with 3 M ammonium thiocyanate at a flow rate of 1 ml/min.
galactosamine contents indicate the presence of two N-linked and one O-linked oligosaccharide respectively. The high amount of mannose is probably the result of hydrolysis byproducts, as the mass spectrometry results discount the presence of any impurities.

Partial purification of rmCG dimer

Immunoaffinity chromatography of medium collected from the culture of CHO cells expressing mCG resulted in 83% of immunoreactivity binding to the column and subsequently being eluted with 1 mM glycine–HCl, pH 2·2 (Fig. 5a). The pool of immunoreactive fractions collected and subjected to hydrophobic interaction chromatography was resolved into two peaks as shown in Fig. 5b. Only the second peak was found to be bioactive and isolation of these fractions resulted in a 400-fold purification of the dimer (Table 1b). Analysis by Western blotting and immunodetection showed the first peak, which did not bind to the column, to have a major band at 32 kDa, corresponding to the $\beta$-subunit, and the second peak to have only one major band at 56 kDa (Fig. 6), which under reducing conditions resulted in one band at 29 kDa (not shown).

mCG-6His antibody production

A limited amount of material at the time allowed us to immunise only one rabbit with purified mCG-6His. The animal produced antiserum (R64) that bound $^{125}$I-rmCG$\beta$, with the highest titre being obtained 49 days after the primary injection. A final dilution of 1:15 000 resulted in the binding of 51% of tracer. This bleed was used to develop an immunoassay for mCG.

mCG ELISA

Optimal dilutions of 1:2000 for the antibody R64 and 1:2000 for the biotinylated antibody 518B7 were determined by chequerboard titration of the two antibodies (results not shown).

Table 1 Purification of recombinant mCG and mCG$\beta$ from CHO cell medium

<table>
<thead>
<tr>
<th>Protein* (mg/ml)</th>
<th>CG activity (pmol/ml)</th>
<th>Specific activity (pmol/mg protein)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) mCG$\beta$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original medium</td>
<td>0.509</td>
<td>7.015**</td>
<td>13.78</td>
</tr>
<tr>
<td>SP-Sepharose</td>
<td>0.595</td>
<td>46.3**</td>
<td>77.81</td>
</tr>
<tr>
<td>(0.15 M fraction)</td>
<td>518B7-Sepharose eluate</td>
<td>0.214</td>
<td>3929**</td>
</tr>
<tr>
<td>(b) mCG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Original medium</td>
<td>6.7</td>
<td>636***</td>
<td>205</td>
</tr>
<tr>
<td>518B7-Sepharose eluate</td>
<td>3.9</td>
<td>53 406***</td>
<td>13 694</td>
</tr>
<tr>
<td>Phenyl-Sepharose (fractions 35–44)</td>
<td>0.028</td>
<td>2338***</td>
<td>83 500</td>
</tr>
</tbody>
</table>

*Measured by Bio-Rad protein assay kit based on Bradford dye-binding procedure; **measured by RIA; ***measured by MA10 bioassay.
The recombinant β-subunit was initially purified, quantified from amino acid analysis and initially used as the standard. The recombinant heterodimer (rmCG) was then purified, quantified by the bioassay and subsequently used as the standard. Serial dilutions of rmCG, rmCGβ, rmCG-6His, pituitary and placenta extracts, pregnant marmoset urine and plasma and medium collected from cultured marmoset trophoblastic vesicles resulted in regression lines with slopes that were not statistically different ($P > 0.05$) (Fig. 7a). Specificity tests performed with rmCG, baLH, baLHβ, moLH, moLHβ, baFSH, moFSH and hCG showed cross-reactivity, on a molar basis, measured at 30% signal to noise ratio (i.e. the concentration of hormone that resulted in an optical density reading, above background, of 30% of the maximum optical density obtained with rmCGβ) to be 23, <0.07, 2.3, <0.07, 0.21, <0.07, <0.07 and <0.007% respectively compared with rmCGβ (Fig. 7b). The least detectable dose, defined as the concentration that results in a signal of three standard deviations higher than the mean of the replicates containing only assay buffer, was 10.3 pg/ml (0.5 pg/well) for mCGβ and 234 pg/ml (11.7 pg/well) for intact mCG. However, the lowest concentration with a coefficient of variation less than 10% was 103 pg/ml (5.2 pg/well) for mCGβ and 476 pg/ml (24 pg/well) for mCG. The interassay variation measured in culture medium containing mean levels of 1.6, 11.6 and 18.25 ng/ml rmCG was 16, 13.5 and 9.8% respectively. The mean recoveries of rmCG spiked in triplicate at three different concentrations (range 2.38–11.9 ng/ml) in marmoset plasma, marmoset urine and embryo/trophoblastic vesicle culture medium were 92% (85–100%; $P = 0.0827$), 112% (103–123%; $P = 0.1499$) and 104% (89–118%; $P = 0.1499$) respectively. CG levels measured by ELISA in 27 medium samples collected from trophoblastic vesicle cultures correlated well with levels determined by bioassay (Fig. 8a; correlation coefficient 0.9672). Further proof of CG detection was obtained by analysis of trophoblastic culture medium subsequent to gel filtration. Immunoreactivity, measured by the ELISA, and bioactivity, measured by the MA10 bioassay, of the fractions revealed a common peak

**Figure 5** (a) Immunoaffinity purification of mCG from medium of CHO cells expressing both mCG and mCGβ. The column was the same as that used for purification of mCGβ (Fig. 3b), except elution was with glycine-HCl, pH 2.2. (b) Separation of rmCG from its β-subunit on phenyl-Sepharose after immunoaffinity purification.

**Figure 6** Immunoblot of fractions eluted from phenyl-Sepharose column (Fig. 5b). Pooled fractions 3–12 (lane 2) and 37–43 (lane 1) were subjected to SDS-PAGE under non-reducing conditions and transferred to a nitrocellulose membrane. Immunostaining was achieved with anti-bovine LH antibody 51887 as described in Materials and Methods. The immunoreactive peak not bound to the phenyl-Sepharose column of molecular mass 32 kDa (lane 1) corresponds to rmCG and the bound peak of molecular mass 56 kDa (lane 2) corresponds to the rmCG dimer.
of activity corresponding to an apparent molecular mass of 53 kDa (Fig. 8b).

Embryo/trophoblastic vesicle culture

Four marmoset embryos from the late morula to the hatched blastocyst stage were cultured through to the trophoblastic vesicle stage. mCG was detected in the medium of all embryos before attachment and up to 48 days in culture, with the maximum level measured immediately after attachment (Fig. 9).

Discussion

We have previously described the structure of the CG molecule from the marmoset monkey as being composed of an α- and β-subunit, with a similarity to LH while possessing a C-terminal extension like hCG. In the present study, we expressed mCG as the free β-subunit or as the dimer. This has allowed us to characterise the amino acid and carbohydrate composition of the β-subunit as well as quantify both the dimer and β-subunit for biological immunoassay use. We have previously shown by N-terminal amino acid analysis that the protein structure of mCGβ was virtually identical with that predicted from the gene structure (Simula et al. 1995). The carbohydrate composition suggests the presence of two N- and one O-linked carbohydrates. However, from its nucleotide sequence mCGβ has two O-linked and two N-linked glycosylation signal sequences. Clearly these findings need to be compared with authentic urinary and placental mCG. The presence of CG immunoactivity in the pituitary is intriguing; while cross-reactivity with LH is

Figure 7 (a) Dilution curves of rmCG, rmCGβ, rmCG-6His, marmoset pituitary and placenta extracts, trophoblastic vesicle culture medium and pregnant marmoset plasma and urine. (b) Dose–response curves of purified rmCG, rmCGβ, baLH, baLHβ, moLH, moLHβ, baFSH, moFSH and hCG (CR127). The cross-reactivities, on a molar basis, were estimated at 30% of maximum response. O.D., Optical density.
very likely, recent data from our laboratory using PCR suggest substantial mRNA expression of the CG gene (Gameau et al. 1995). hCG expression in the human pituitary has been described (Chen et al. 1976), but there is no independent evidence that the marmoset pituitary expresses a separate gene for LH. We have previously hypothesised that mCG may serve as mLH when expressed in the pituitary gland (Simula et al. 1995, 1996).

In this paper we report the successful expression of mCG-6His by CHO cells in culture. The subsequent purification by immobilised metal ion chromatography provided a relatively pure mixture of rmCG-6His and rmCGβ-6His with what appeared to be a single contaminant of molecular mass 57 kDa identified by SDS–PAGE under reducing conditions. This may explain the discrepancy between the results of the silver staining and immunostaining. Alternatively, the dimer may not have been completely dissociated, as reported for mCG isolated from trophoblast culture medium under similar conditions (Saunders et al. 1987). Although we were limited by the quantity of recombinant material, one rabbit was immunised and subsequently produced a polyclonal antibody (R64), which appeared to be specific to mCG, as shown by its very low cross-reactivity with baLH, moLH, baFSH, moFSH and hCG. The availability of the non-specific monoclonal antibody 518B7, together with the polyclonal antibody R64, allowed us to develop a sandwich ELISA, which detects both the mCG and its β-subunit. The immunoassay had a higher affinity for the free β-subunit when compared with the dimer but this is common when polyclonal antibodies are used in CG immunoassays. β-subunit assays have been widely used in clinical diagnoses, although they have now been

Figure 8 (a) Correlation of CG levels (ng/ml) measured with the ELISA and the MA10 cell bioassay in 27 trophoblastic vesicle culture samples. (b) Gel filtration of marmoset trophoblastic vesicle culture medium (1 ml of 3 × concentrate) on a Superdex 200 (16/60) column, equilibrated and run with 0·1 M ammonium bicarbonate. Fractions of volume 1 ml were collected at a flow rate of 0·5 ml/min and assayed for CG using the ELISA and the MA10 cell bioassay.

Figure 9 mCG secretion during in vitro development of marmoset embryos. Embryos were placed in culture as a hatched blastocyst (T3), a medium expanded blastocyst (T4), an early blastocyst (B1) and a late morula-early blastocyst (M1). The arrows indicate the time of attachment.
superceded by monoclonal antibodies binding to defined epitopes. The current assay will prove valuable for studies in embryonic secretion of CG and pregnancy related values.

Previous assays for mCG have been based on bioactivity (Seshagiri & Hearn 1993, Simula et al. 1995) and were tedious to perform or were relatively non-specific or insensitive (Ziegler et al. 1993). We have validated the current assay, indicating that it is relatively accurate, precise, sensitive and specific in all biological fluids so far studied.

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