

The β -subunit of human chorionic gonadotrophin exists as a homodimer

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ABSTRACT

The free β -subunit of human chorionic gonadotrophin (hCG β) is well recognised as a product of many epithelial tumours. Recently, it has been shown that this ectopic production may have a functional relationship to tumour growth. The growth-promoting activity of hCG β may be explained by its structural similarity to a family of growth factors which all contain the same distinct topological fold known as the cystine-knot motif. Since the other members of this family all exhibit their activities as homo- and heterodimers, it is possible that the same may be true for hCG β .

Using size-exclusion chromatography, low stringency SDS-PAGE and matrix assisted laser desorption/ionisation (MALDI) time-of-flight (TOF) mass spectrometry (MS) we have shown that pure preparations of hCG β contain hCG $\beta\beta$ homodimers. Size-exclusion chromatography revealed asymmetric

elution profiles with a forward peak corresponding to the size-exclusion characteristic of a globular protein with an approximate mass of 44–54 kDa and a late shoulder centered around an elution position expected for a globular protein of approximately 29 kDa. Two immunoreactive hCG β species, of approximately 32 and 64 kDa, were clearly resolved by SDS-PAGE and Western blotting. When analysed by MALDI-TOF MS a \sim 23 kDa monomer and a \sim 46 kDa dimer were identified.

Formation of hCG $\beta\beta$ homodimers is consistent with the behaviour of other cystine-knot growth factors and strengthens the inclusion of the glycoprotein hormones within this superfamily. It has yet to be determined whether it is this dimeric molecular species that is responsible for growth-promoting activity of hCG β preparations in tumours.

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INTRODUCTION

Human chorionic gonadotrophin (hCG) is a member of the glycoprotein hormone family, which also includes luteinising hormone (LH), follicle-stimulating hormone and thyroid-stimulating hormone, all existing as $\alpha\beta$ heterodimers. The α -subunit is common to all members of the group and it is the β -subunits which confer the structural and functional identity of the heterodimer. Furthermore, only the intact heterodimer is functional; the free subunits do not exhibit gonadotrophic or thyrotrophic activity. Therefore, any growth factor activity exhibited by the free β -subunit is generally regarded not to occur through the LH/hCG receptor, but via a novel pathway.

The free β -subunit of hCG (hCG β) is widely used as a marker of Down's syndrome (Spencer *et al.* 1992, Cuckle *et al.* 1994) and has been proposed as a prognostic marker for many epithelial tumours (Iles *et al.* 1996). In both situations the expression of hCG β was regarded as having no biological significance. However, it has now been shown that the β -subunit may have its own unique functions. Lunardi-Iskandar *et al.* (1995) and Gillott *et al.* (1996) have shown growth inhibitory and growth stimulatory effects respectively of free hCG β . Recently, elucidation of the crystal structure of hCG revealed a similar topology between hCG β and transforming growth factor β (TGF β), platelet derived growth factor β (PDGF β) and nerve cell growth factor (NGF) (Laphorn *et al.* 1994, Wu

et al. 1994). The feature common to all four molecules is a unique group of three disulphide bridges in the centre of the molecule, the so called 'cystine-knot motif'. It is interesting to note that the cystine-knot growth factors mediate their biological activity through the formation of homo- and/or heterodimers. In view of the recently reported biological activity of hCG β (Gillott *et al.* 1996) and the structural similarities within this group of proteins, we have investigated the possible existence of an hCG $\beta\beta$ homodimer using size-exclusion chromatography, SDS-PAGE and matrix-assisted laser desorption/ionisation (MALDI) time-of-flight (TOF) mass spectrometry (MS).

MATERIALS AND METHODS

Recombinant hCG β subunit, which is expressed in a mammalian cell line, containing N- and O-linked carbohydrate moieties similar to those in authentic hCG β , was purchased from Sigma Chemical Co. (Poole, Dorset, UK). The international reference preparation of free hCG β , CR123, which is derived from splitting intact heterodimeric hCG isolated from pregnancy urine, was a kind gift from Dr Steven Birken (Columbia University, New York, USA) (Storring *et al.* 1980). MALDI matrices α -cyano-4-hydroxycinnamic acid (CHCA), 2,5-dihydroxybenzoic acid (DHB), 3,5-dimethoxy-4-hydroxybenzoic acid (sinapinic acid (SA)) and 5-methoxysalicylic acid (MSA) were purchased from Aldrich Chemical Co. (Gillingham, Dorset, UK) and used without further purification.

Size-exclusion gel chromatography

Recombinant hCG β and CR123 preparation hCG β (100 μ l at 50 μ g/ml in 1% w/v BSA-PBS) were loaded onto a column (630 mm \times 10 mm internal diameter) of Sephacryl S200HR via a Rheodyne (Berkeley, CA, USA) injection loop. The samples were then eluted with 0.1 M ammonium bicarbonate buffer (pH 7.4) by ascending flow at a rate of 0.3 ml/min using a Shimadzu (Kyoto, Japan) LC-6A HPLC pump. Eluted fractions (0.9 ml) were collected into polystyrene tubes containing 100 μ l 10% w/v BSA-PBS. Buffer containing BSA was added to the collection tubes and at injection, in order to reduce the non-specific absorption of the proteins of interest to the column components and fraction vessels.

The column was calibrated using gel-exclusion chromatography globular protein markers (Sigma): Blue dextran (700 kDa – void volume), alcohol

dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa).

Assay for hCG β

Fractions eluting from the size-exclusion column were assayed for total hCG β using an in-house polyclonal RIA. This assay has been described by Iles (1991) and uses a polyclonal antiserum, S424, raised against the β -subunit of hCG, as binder and 125 I-labelled CR123 free hCG β as tracer. This assay recognises the β -subunit of hCG in the heterodimer of intact hCG, as the free subunit and the hCG β degradation product β -core.

SDS PAGE and Western blotting

Electrophoresis was essentially carried out by the method described by Laemmli (1970). Recombinant hCG β samples were separated on 10% acrylamide gels under both reducing and low stringency conditions using the Mighty Small II gel system (Hoefer, Amersham Pharmacia Biotech, St Albans, Herts, UK). Ten-microlitre samples of the recombinant hCG β preparation (100 μ g/ml) were combined with an equivolume of either non-reducing sample buffer (125 mM Tris-HCl, pH 6.8, 20% glycerol, 4% SDS, 0.02% bromophenol blue) or reducing sample buffer (containing 10% β -mercaptoethanol) before separation. The reducing sample was additionally heated for 3 min in a boiling water bath to ensure complete reduction of the thiol moieties; the low stringency sample was not heated. In both cases 14 C-labelled Rainbow Molecular Mass Markers (Amersham Pharmacia Biotech) were subjected to the same conditions as the corresponding sample prior to separation. The separated proteins were transferred to polyvinylidene difluoride nylon membrane (Immobilon-P, Millipore, Sigma) using the Biometra Fastblot B32 semi-dry blotter (Anachem-Scotlab, Luton, Beds, UK), blocked, then probed overnight with monoclonal anti-hCG β 1/07 (Quantum Biosystems Ltd, Cambridge, Cambs, UK) labelled with 125 I by the chloramine-T method of Greenwood *et al.* (1963). Following multiple washes with PBS 0.1% Tween 20 (Sigma) the blot was exposed to Kodak X-OMAT AR imaging film and developed using the Kodak M35 X-OMAT processor (Kodak, Hemel Hempstead, Herts, UK).

MALDI-TOF MS

The matrices selected for this work are used widely in MALDI analysis, SA and a 9:1 (w/w) mixture of DHB with MSA, referred to as super-DHB

(sDHB), being particularly useful for the analysis of glycoproteins (Kusmann *et al.* 1997). In addition, CHCA was chosen because relative to SA or sDHB it has a lower proton affinity (Burton *et al.* 1997) and is therefore more liable to favour dissociation of non-covalent complexes. Matrix solutions were prepared using HPLC grade acetonitrile (Fisher Scientific UK Ltd, Loughborough, Leics, UK) and purified water (Elgastat UHQ, High Wycombe, Bucks, UK). Solutions of SA and CHCA were prepared at 10 g/l in acetonitrile/water (70:30, v/v). sDHB (a 9:1 (w/w) mixture of DHB with MSA) (Karas *et al.* 1993), was prepared at a concentration of 20 g/l in acetonitrile/water (30:70, v/v). Recombinant hCG β and the international reference preparation were prepared to concentrations of 0.8 and 0.4 mg/ml respectively in purified water.

Half-microlitre aliquots of hCG β sample were mixed on the MALDI sample support with 0.5–1 μ l matrix solution and allowed to air dry. Spectra were then generated using a LASERMAT 2000 mass spectrometer (Thermo Bioanalysis Ltd, Hemel Hempstead, Herts, UK). Light from a pulsed nitrogen laser (λ_{\max} =337 nm) was used to desorb ions from the samples and they were accelerated down a 0.5 m drift tube and detected by a micro-channel plate detector. The detector signal was digitised at a sampling rate of 500 MHz and transferred to a personal computer for data analysis. Spectra were generated by summing 20–35 laser shots and were calibrated externally using horse heart cytochrome C (12 360 Da) (Sigma).

RESULTS

Size-exclusion chromatography

The chromatogram of recombinant hCG β revealed an asymmetric elution profile of immunoreactive material consisting of a forward peak eluting at a position corresponding to the Stokes' radius of a globular protein of approximately 44 kDa in mass and a late shoulder approximating to a globular protein of 29 kDa. CR123 β eluted in a similar manner with a forward peak of ~44–54 kDa and a late shoulder of ~29 kDa (Fig. 1).

SDS PAGE and Western blotting

Under low stringency (non-reducing) conditions two protein bands were separated by SDS-PAGE and following Western blotting were identified immunologically as hCG β . The band with the higher molecular mass (hCG β dimer) was exactly double (~64 kDa) that of the lower band corresponding to hCG β monomer (~32 kDa).

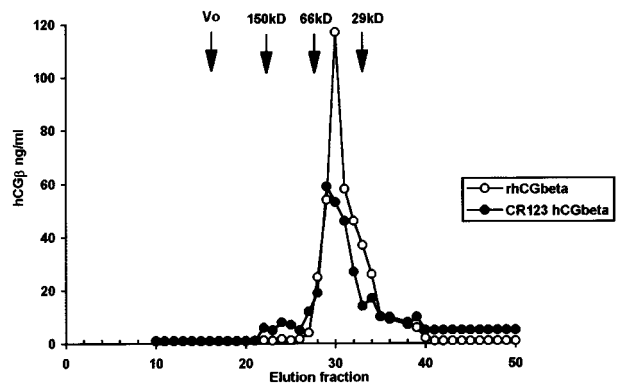


FIGURE 1. Sephacryl S200HR size-exclusion chromatography elution profiles of recombinant and CR123 hCG β preparations. Arrows mark the elution maxima of gel-exclusion chromatography globular protein markers: alcohol dehydrogenase (150 kDa), BSA (66 kDa) and carbonic anhydrase (29 kDa). Void volume (V_o) was determined as the elution maxima for Blue dextran (~700 kDa).

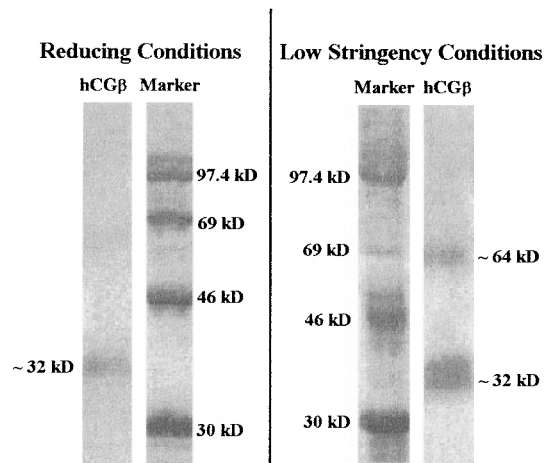


FIGURE 2. Scanned autoradiograph showing a Western blot of separated recombinant hCG β under reducing and low stringency conditions, probed with 125 I-anti-hCG β monoclonal antibody 1/07.

Under reducing conditions the same hCG β preparation gave rise to only one band corresponding to hCG β monomer (32 kDa) (Fig. 2).

MALDI-TOF MS

MALDI-TOF mass spectra of recombinant hCG β analysed in SA, sDHB and CHCA matrices are shown in Figs 3, 4 and 5 respectively. All the spectra were characterised by peaks corresponding to the singly (m/z ~23 kDa) and doubly (m/z ~11.5 kDa) charged ions of hCG β . Spectra generated in sDHB and SA also contain a peak at m/z

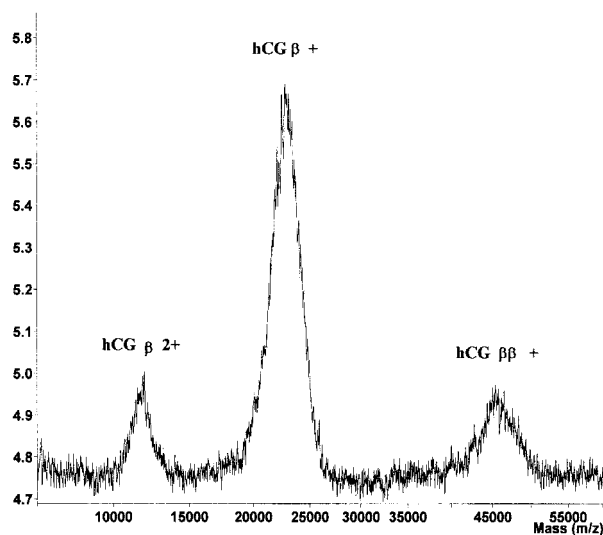


FIGURE 3. MALDI-TOF mass spectrum of recombinant hCG β (0.4 mg/ml) 0.5 μ l with 0.5 μ l SA 10 g/l in acetonitrile/water (70:30), showing peaks corresponding to monomeric doubly charged hCG β (β 2+), monomeric hCG β (β +), and dimeric hCG $\beta\beta$ ($\beta\beta$ +), at approximately 11.5, 23 and 46 kDa respectively.

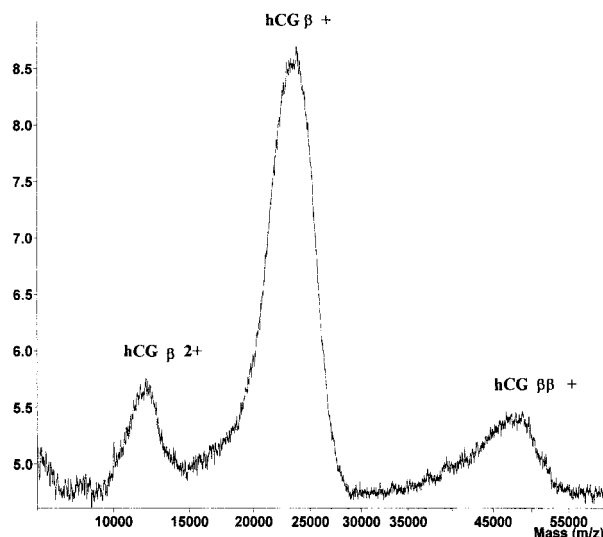


FIGURE 4. MALDI-TOF mass spectrum of recombinant hCG β (0.8 mg/ml) 0.5 μ l with 1 μ l sDHB 20 g/l in acetonitrile/water (30:70), showing peaks corresponding to monomeric doubly charged hCG β (β 2+), monomeric hCG β (β +), and dimeric hCG $\beta\beta$ ($\beta\beta$ +), at approximately 11.5, 23 and 46 kDa respectively.

~46 kDa indicating the presence of a dimer of hCG β . This peak was absent from the spectra acquired using CHCA as the matrix. The signal due to the doubly charged monomer was considerably more intense when CHCA was used as the matrix.

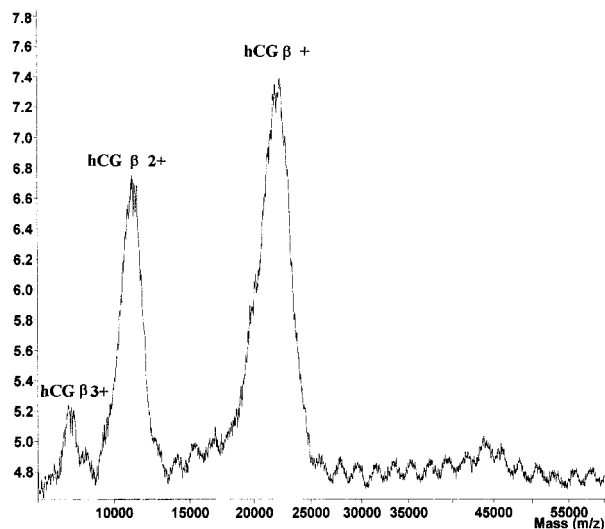


FIGURE 5. MALDI-TOF mass spectrum of recombinant hCG β (0.8 mg/ml) 0.5 μ l with 0.5 μ l CHCA 10 g/l in acetonitrile/water (70:30), showing peaks corresponding to monomeric trebly charged hCG β (β 3+), monomeric doubly charged hCG β (β 2+), and monomeric hCG β (β +), at approximately 7.7, 11.5 and 23 kDa respectively.

In addition, an ion corresponding to the trebly charged hCG β monomer was present in the CHCA spectra. Peaks corresponding to the singly and doubly charged ions of hCG β were observed in the spectrum of the international reference preparation for hCG β (CR123 β) analysed in SA. The singly charged ion of an hCG $\beta\beta$ homodimer was also present, albeit at low intensity (Fig. 6). The microheterogeneous nature of hCG β , due primarily to variations in glycosylation, and in particular sialic acid content, results in the broad peaks characteristic of the generated spectra.

DISCUSSION

The ectopic production of free hCG β is a well-recognised phenomenon in many epithelial tumours (Iles *et al.* 1990). Expression of hCG β by these cancers was originally thought to carry no biological significance, but it is now apparent that this event may significantly affect tumour development given the growth effects described recently (Gillott *et al.* 1996). Similarly hCG β has also been shown to induce regression in the AIDS-related tumour Kaposi's sarcoma (Lunardi-Iskandar *et al.* 1995) and several studies on both *in vitro* and *in vivo* models report similar findings (Harris 1995, Gill *et al.* 1996). However, opinions differ as to the

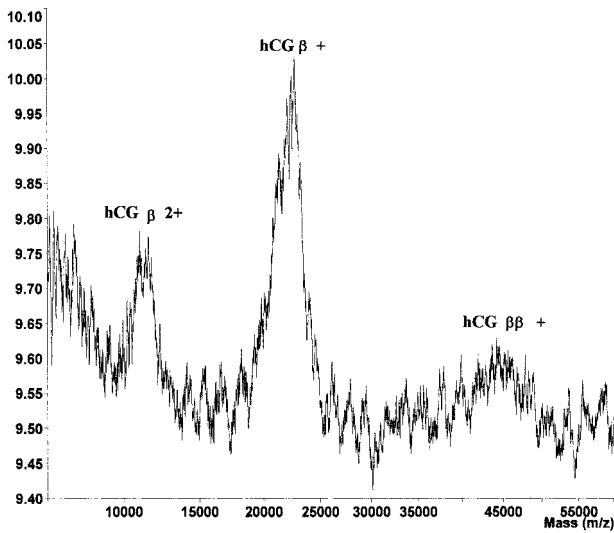


FIGURE 6. MALDI-TOF mass spectrum of CR123 β (0.4 mg/ml) 0.5 μ l with 0.5 μ l SA (acetonitrile/water), showing peaks corresponding to monomeric doubly charged hCG β (β 2+), monomeric hCG β (β +) and dimeric hCG β ($\beta\beta$ +) at approximately 11.5, 23 and 46 kDa respectively.

identity of the actual functional component(s) in the preparations used in these studies; intact hCG (Harris 1995), hCG β (Lunardi-Iskander *et al.* 1995), β -core (Kachra *et al.* 1997), the carboxy-terminal peptide of hCG β (Albini *et al.* 1997) and an hCG associated factor, HAF (Lunardi-Iskandar *et al.* 1998), which may be a ribonuclease inhibitor (Griffiths *et al.* 1997), have all been proposed as the active molecule.

The individual α - and β -subunits of hCG share a distinctive topological folding pattern, known as the cystine-knot motif, with the growth factors TGF β , NGF and PDGF β (Murray-Rust *et al.* 1993, Sun & Davies 1995). Although all these growth factors bind to quite different receptor families, biological activity is achieved only through the formation of either hetero- and/or homodimers. TGF β signalling in particular requires a $\beta\beta$ homodimer and PDGF β forms $\beta\beta$ homodimers which initiate second messenger phosphorylation; PDGF $\beta\alpha$ heterodimers and PDGF $\alpha\alpha$ homodimers are also known to initiate and control kinase activity (Heath 1993). The TGF and PDGF $\beta\beta$ homodimers are bound together 'top to tail' to yield the structure required for receptor binding and activation. This subunit association is very similar to that seen within the hCG heterodimer, in which the carboxy-terminal sequence of the β -subunit wraps around the α -subunit in a 'seatbelt' arrangement (Lapthorn *et al.* 1994). These structural similarities, together

with the recently described growth-promoting activity of hCG β (Gillott *et al.* 1996), suggest that the action may be brought about by an hCG $\beta\beta$ homodimer. It is notable that homodimers of the α - and β -subunits of bovine LH have also been reported (Peng *et al.* 1997).

Furthermore it has been indicated that in high concentrations, β -core, a breakdown product of hCG and hCG β , can also form homodimers (Iles 1991). In view of the very similar folding patterns of hCG β and these growth factors, a possible candidate to mediate the growth factor activities of hCG β is a putative hCG $\beta\beta$ homodimer. Since free hCG β is unable to activate the LH/hCG receptor and stimulate secondary messengers, the reported growth factor activities are assumed to proceed via novel, and as yet unidentified, pathways.

Our data support the existence of hCG $\beta\beta$ homodimers in preparations of hCG β which have previously been shown to exhibit growth factor activities. Intact hCG and free hCG β have always been found to behave unusually on size-exclusion chromatography, eluting close to the void volume and at the expected position of human serum albumin (\sim 70 kDa) on Sephadex G-100 size-exclusion columns (Bell *et al.* 1969, Canfield *et al.* 1971). This phenomenon is due to the extensive glycosylation of hCG and hCG β , which affords them Stokes' radii equivalent to globular molecules of over twice their mass (Butt *et al.* 1994). In this study we used Sephacryl 200HR size-exclusion matrix, which should resolve molecules with a Stokes' radius of approximately 70 kDa in the middle of its chromatographic range (Kardana *et al.* 1991). As can be seen from Fig. 1 the major peak of hCG β eluted at \sim 44–55 kDa but a significant late shoulder eluted at \sim 29 kDa. SDS-PAGE was more definitive with two species being resolved at \sim 32 and \sim 64 kDa respectively. With SDS-PAGE these species were only seen together when separated under low stringency conditions in which the sample was only exposed to SDS, non-reducing conditions and not boiled. Heating alone is sufficient to disrupt the $\alpha\beta$ heterodimer of intact hCG releasing free subunits (Gau *et al.* 1984, Sancken & Bahner 1995) despite the seatbelt on the β -subunit holding the α -subunit in tight association. When stringent conditions of a disulphide reducing agent (β -mercaptoethanol), SDS and heating were employed only the \sim 32 kDa (monomeric) species could be seen (Fig. 2). The differences in assignment of molecular mass of the species between size-exclusion chromatography and SDS-PAGE can be ascribed to the imprecision of these techniques, which can only approximate molecular masses.

To optimise matrix-assisted ionisation in MALDI, the sample is generally mixed with a roughly equivalent volume of matrix, usually a cinnamic or benzoic acid derivative, dissolved in a mixture of acetonitrile and water/0.1% trifluoroacetic acid; the acidic nature of these conditions is generally considered to be sufficient to disrupt quaternary structures in most circumstances (Hillenkamp *et al.* 1991, Cohen *et al.* 1997). Nevertheless, in an earlier study we were able to demonstrate the quaternary structure of the intact hCG heterodimer by MALDI-TOF using an SA matrix (Laidler *et al.* 1995) and in this current investigation we have observed a mass ion akin to that expected of the $\beta\beta$ homodimer in SA and sDHB matrices. Of course, such mass spectral data alone are not unequivocal in showing the presence of the $\beta\beta$ homodimer because proteins may form dimers during ionisation. For that reason, we chose also to analyse the preparations of hCG β using CHCA, which is a more acidic matrix than SA or sDHB. As the subunits of hCG may be separated under very acidic conditions (Morbeck *et al.* 1994), it is reasonable to expect the same to be the case for a $\beta\beta$ homodimer. Recent work relating to the proton affinities of commonly used MALDI matrices demonstrated that CHCA has a lower proton affinity (183 ± 2 kcal/mol), and is therefore a stronger acid, than either DHB or SA (both 204 ± 4 kcal/mol) (Burton *et al.* 1997). On that basis, the peak ascribed to the $\beta\beta$ homodimer, which was observed in the sDHB and SA matrices, would be expected to be attenuated when the CHCA matrix was employed, unless the peak was an artefact due to the ionisation process. Using the CHCA matrix, the peak was so attenuated as for it to be impossible to locate the homodimer. Hence, the contrast in results by MALDI, using different matrices, also supports the presence of the $\beta\beta$ homodimer.

An important factor that influences the stability of the quaternary structure in the heterodimer, and by implication the $\beta\beta$ homodimer, is the large contact surface area between the two interacting subunits; a surface area of approximately 4000 Å² is buried by subunit binding (Laphorn *et al.* 1994, Wu *et al.* 1994). The amount of contact surface area of the heterodimer is in contrast to the area of 600–900 Å² (Novotny *et al.* 1983, Amit *et al.* 1986) of an antibody–antigen complex which is completely dissociated by MALDI sample preparation (Nelson *et al.* 1995).

The quaternary structure of a $\beta\beta$ homodimer could be similar in nature to that of the hCG $\alpha\beta$ heterodimer. The individual subunits are aligned within the heterodimer in a 'head to tail' manner

and are stabilised by a structure, nicknamed the seatbelt, formed when residues 90–110 of the β -subunit wrap around the α -subunit before the formation of a last disulphide bond between residues 26 and 110 of the β -subunit. While formation of the cystine residue 26–110 prior to subunit contact would appear to hinder extracellular subunit association (Huth *et al.* 1993), the seatbelt also presents a barrier to heterodimer dissociation; hCG exists in the circulation at concentrations of 10^{-9} to 10^{-11} M despite an equilibrium constant for subunit interaction of approximately 10^{-6} M (Strickland & Puett 1982, Ryan *et al.* 1988). One may expect a mature $\beta\beta$ homodimer to be more stable than the hCG $\alpha\beta$ heterodimer since its dissociation into hCG β monomers could require two seatbelt-like structures to be reduced rather than one. Should this be the case, it is likely that an hCG $\beta\beta$ species intermediate between the monomer and a fully stable hCG $\beta\beta$ homodimer, containing only one seatbelt, also exists. We would expect such an intermediate species to exhibit a stability similar to that of the hCG $\alpha\beta$ heterodimer.

Since biological activities, not mediated through the LH/hCG receptor, have been described for both the α - and β -subunits it is reasonable to suggest that a novel molecular structure may be involved (Blithe *et al.* 1991, Blithe 1994, Lunardi-Iskandar *et al.* 1995, Gillott *et al.* 1996). The experimental data presented here suggest that a $\beta\beta$ homodimer is a possible mediator of the previously reported growth factor activities. The formation of such dimeric molecular species may arise from post-translational events such as glycosylation, which have, in the α -subunit, been shown to prevent $\alpha\beta$ heterodimer formation (Blithe *et al.* 1991). Similar modifications in glycosylation of hCG β may favour the formation of the $\beta\beta$ homodimer rather than the $\alpha\beta$ heterodimer. It is not yet known if homodimers form *in vivo*, but the concentrations analysed here may be comparable with local tissue concentrations. It would, therefore, be feasible to suggest that a proportion of ectopically produced free hCG β may exist as a homodimer.

It has not yet been determined whether the $\beta\beta$ homodimer described here is responsible for the growth-promoting activities of hCG β . However, the demonstration of an hCG $\beta\beta$ homodimer makes it less likely that the responses seen are due to an interaction with the LH/hCG receptor and reinforce the view that a novel receptor may be involved. In addition, the predisposition of proteins containing the cystine-knot motif to form homodimers suggests that hCG β may possibly form heterodimers with other growth factor subunits,

thereby creating unique molecules with multi-functional roles.

In conclusion, the results from this investigation support the presence of an hCG $\beta\beta$ homodimer. This unique species may be responsible for the novel biological activities recently attributed to the free β -subunit, and could play a key role in tumour growth regulation and metastatic control.

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