Immunochemical characterization of two thyroid-stimulating hormone β -subunit epitopes

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The epitopes of human thyroid-stimulating hormone (hTSH) recognized by two murine monoclonal antibodies (MAbs), designated MAb 279 and MAb 299, have been characterized. These MAbs are highly specific for the β -subunit of TSH. The epitope recognized by MAb 279 appears to be completely conserved between bovine and human TSH and partially conserved in the porcine species. The TSH β -subunit epitope recognized by MAb 299 is only partially conserved between the human, bovine and porcine species. Both MAbs are capable of inhibiting the binding of TSH to its receptor in a TSH radio-receptor assay, indicating that the epitopes either coincide or are located close to the TSH β -subunit receptor-binding sites. The

carbohydrate moieties of the TSH β -subunit appear to play little or no role in the epitope recognition by MAb 279 or MAb 299 while the integrity of the disulphide bonds are essential. The epitopic recognition may also involve lysine residues, as determined by the immunoreactivity with both MAbs following citraconylation of TSH. In addition, the amino acid sequence region between residues bTSH β 34–44 could be excised by trypsin digestion of bovine TSH β (bTSH β) without eliminating epitopic recognition by either MAb. These results provide further insight into the relationship between the structure of the TSH β -subunit epitopes and location of the receptor-binding sites.

INTRODUCTION

Thyroid-stimulating hormone (TSH) is a member of the glycoprotein hormone (GPH) family which also includes follitropin (FSH), lutropin (LH) and chorionic gonadotropin (CG). These hormones are structurally related, with each GPH consisting of a common α -subunit which is non-covalently associated with a hormone-specific β -subunit [1]. The three-dimensional structure of human CG (hCG) was very recently established by X-ray crystallography to 3 Å resolution and molecular modelling studies indicate that the structures of the other GPHs may be essentially the same [2,3]. Prior to the elucidation of the X-ray crystal structure of hCG, a variety of techniques have been employed to gain insight into the three-dimensional structure and epitopic topography of each of the GPHs. With respect to TSH, the relative spatial relationships of epitopes have been determined [4,5], the surface accessibility of particular sequence regions has been investigated using anti-peptide antibodies [6,7] and the location of receptor-binding sites has been investigated using synthetic peptides [8,9]. The TSH–receptor interaction has also been probed using monoclonal antibodies (MAbs) [10–12] and the involvement of the carbohydrates in the TSH β -subunit epitopes has been studied [13,14]. However, no studies have yet identified regions or specific amino acid residues within the TSH β -subunit which comprise the epitopes for MAbs raised to the intact TSH or its subunits. Hence, this paper reports investigations into the binding and partial structural characteristics of the epitopes recognized by two TSH β -subunit-specific MAbs.

EXPERIMENTAL

Hormone preparations

Bovine (b) TSH and LH were purified from bovine pituitary

glands as described previously [15]. The bTSH α - and β -subunits were purified from this preparation by reverse-phase HPLC on a Bakerbond C₁₈ column (25 cm $\times$ 0.46 cm i.d., 300 Å pore size, 5 μ m particle diameter) (J. T. Baker, Phillipsburg, NJ, U.S.A.) using 0.1% trifluoroacetic acid with elution performed with a linear 60 min gradient of 0–50% acetonitrile. Residual α -subunit or intact hormone was then removed from the β -subunit preparations by gel-filtration chromatography on a Superdex 75 column (30 cm $\times$ 1 cm i.d.) (Pharmacia, Uppsala, Sweden) using NH₄HCO₃ as eluent. Crude bTSH (1 i.u./mg) and porcine TSH (pTSH) (5 i.u./mg) were purchased from Sigma Chem. Co. (St. Louis, MO, U.S.A.). The human TSH (hTSH) was obtained from the Human Pituitary Advisory Committee (Canberra, Australia), recombinant hFSH was kindly donated by Dr. Peter Schofield of the Garvan Institute of Medical Research (Darlinghurst, NSW, Australia), hCG (4723 i.u./mg) was purchased from Calbiochem Corp. (La Jolla, CA, U.S.A.). The concentration of each protein tested in the various assays or used for modification was determined by amino acid analysis using the Picotag method (Millipore) [16] performed as described previously [17].

Monoclonal antibodies

All of the MAbs employed in these studies were obtained from Bioclone Australia Pty. Ltd. (Sydney, Australia) and were raised as ascites fluid in Balb/c mice by immunization with hTSH or, in the case of MAb 334P, with hLH. All MAbs were shown, by solution-phase double antibody assays, to be of IgG₁ subclass except MAb 265P which was of IgG_{2a} subclass. The IgG of those MAbs with the 'P' designation was purified from the mouse ascites fluid by Protein A–Sepharose affinity chromatography.

Abbreviations used: TSH, thyroid-stimulating hormone; LH, luteinizing hormone (lutropin); CG, chorionic gonadotropin; FSH, follicle-stimulating hormone (follitropin); GPH, glycoprotein hormone; MAb, monoclonal antibody; IRMA, immunoradiometric assay; STI, soybean trypsin inhibitor; h, human; b, bovine; p, porcine.

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Iodination of bTSH and bTSH β

Bovine TSH was iodinated to a specific radioactivity of 30 mCi/mg using a modification of the lactoperoxidase (EC 1.11.1.7) method [18] then affinity purified with bTSH receptors as described previously [19]. Bovine TSH β was similarly iodinated to a specific radioactivity of 62 mCi/mg.

Iodination of MABs

Protein-A affinity-purified preparations of MAb 279 and MAb 299 were iodinated to an average specific radioactivity of 32–42 mCi/mg using the 'Iodobead' method (Pierce, Rockford, IL, U.S.A.). Briefly, each MAB (5 μ g) was diluted in a total volume of 100 μ l of 100 mM potassium phosphate buffer, pH 7.0, and one iodobead added. The iodination was initiated by the addition of 0.5 mCi (5 μ l) of Na¹²⁵I (Amersham, Bucks., U.K.). Following incubation at room temperature for 15 min with continuous mixing, the reaction mixture was applied to a Sephadex G-25 (fine) (Pharmacia) column (4 cm $\times$ 0.8 cm i.d.) pre-equilibrated in phosphate-buffered saline (PBS) to remove unbound radio-iodide. The radiolabelled protein fraction was then stored at 4 °C for use in the immunoradiometric assay.

Enzyme-linked immunosorbent assay (ELISA)

The proteins were diluted in 40 mM NaHCO₃/Na₂CO₃, pH 9.3, and adsorbed to the wells of a 'Maxisorp' microtitre plate (Nunc, Denmark) for 16 h at 4 °C. The plate was then washed with PBS containing 1 mM MgCl₂ and 0.05 % (v/v) Tween-20 (PBS/MgCl₂/Tween) and excess binding sites blocked by incubation with 50 μ l/well of 1 % (w/v) BSA in PBS containing 1 mM MgCl₂ (PBS/MgCl₂) for 30 min at 37 °C. After washing, 50 μ l of MAb 279P or MAb 299 (diluted 1:2500 in PBS/MgCl₂) was added to the wells, and the plate incubated at room temperature for 90 min. Unbound MAB was removed by washing, then 50 μ l of horseradish-peroxidase-labelled rabbit anti-(mouse IgG) antibody (Dakopatts, Denmark) diluted 1:1000 in PBS/MgCl₂/Tween, was added. Following a 90 min incubation, the plates were washed and 100 μ l of substrate [0.1 % (w/v) 2,2'-azinobis-(3-ethylbenzthiazolinesulphonic acid) (Sigma Chemical Co.) plus 10 μ l of 3 % H₂O₂] in sodium acetate buffer, pH 4.5, was added and incubated at room temperature for 20 min. The reaction was stopped by the addition of 50 μ l of 30 mM NaF and the absorbance at 414 nm was then determined using a Titerfek Multiscan Plate Reader.

Radio-immunoassays (RIA)

Unlabelled GPH proteins in increasing concentrations, tracer (approximately 50000 c.p.m./tube) and MAb 279 or MAb 299 (both diluted 1:2500, i.e. to 50 % maximal binding of the tracer in the absence of a competitor) were incubated in a total volume of 150 μ l of 20 mM Tris/HCl, 154 mM NaCl, 0.1 mg/ml BSA, pH 7.4, for 2 h at 37 °C. The separation of bound from free hormone was achieved following the addition of 50 μ l of rabbit γ -globulin (5 mg/ml) (Grade B; Calbiochem, San Diego, CA, U.S.A.) and 200 μ l of 25 % (w/v) polyethylene glycol 6000 (both precooled to 4 °C), in assay buffer. The radioactivity of the precipitate was then counted. For most GPH protein antigens, assays were performed in triplicate and the results presented as a mean percentage bound $\pm$ S.D.

Immunoradiometric assay (IRMA)

MAbs 279P, 299, 435P, 165P, 273 were diluted 1:10 in 40 mM NaHCO₃/Na₂CO₃ buffer, pH 9.3, and adsorbed to the wells of a

microtitre plate (Nunc) at 50 μ l/well. After 4 h incubation, the plate was washed with PBS/MgCl₂/Tween and excess binding sites blocked as for the ELISA. Human TSH (1 μ g) was then added to each well in 50 μ l of PBS/MgCl₂ and incubated for 90 min. Unbound hormone was removed by washing and approximately 100000 c.p.m. of ¹²⁵I-labelled MAb 279P or MAb 299P was added in 50 μ l of PBS/MgCl₂/Tween and incubated for 90 min. Unbound ¹²⁵I-MAB was then removed by washing and the radioactivity bound to the wells determined. Non-specific binding was determined in the absence of the hTSH antigen. Results are expressed as a percentage of specific binding over non-specific binding, i.e. $(T-N)/N \times 100\%$ where T = test binding (c.p.m.) and N = non-specific binding (c.p.m.).

Receptor-binding inhibition assay

To determine whether MAb 279 and MAb 299 could inhibit the binding of ¹²⁵I-bTSH to its receptor, approx. 10000 c.p.m. of radio-iodinated bTSH was pre-incubated for 1 h at 37 °C in the presence of either MAb 279 or MAb 299 (approx. 10⁻⁷ M–10⁻⁹ M) in a total volume of 100 μ l of 20 mM Tris/HCl buffer, pH 7.4, containing 50 mM NaCl, 1 mM EDTA and 0.5 mg/ml BSA. Negative controls in which either buffer only, or a non-TSH specific MAB (MAb 334) were substituted for MAb 279 and MAb 299 were also included. A bovine thyroid membrane preparation (20 mg equivalent/50 μ l), prepared according to the procedure of Stanton and Hearn [19], was then added and the incubation continued for a further 1 h at 37 °C with constant mixing. Receptor-bound ¹²⁵I-bTSH was then separated from free hormone by filtration through 0.45 μ m pore diam. cellulose acetate filters (Sartorius Australia Pty. Ltd.) and the radioactivity of the filters counted. Non-specific binding of ¹²⁵I-bTSH to the thyroid membranes was determined concurrently by the incubation of the thyroid membrane preparation and tracer in the presence of a receptor-saturating concentration (3.33 $\times$ 10⁻⁶ M) of unlabelled bTSH for 1 h at 37 °C.

Reduction and alkylation of the disulphide bonds of bTSH

The disulphide bonds of two samples of bTSH (10 μ g each) were fully reduced by incubation in 70 μ l of 200 mM Tris/HCl, pH 8.0, containing 6 M guanidine hydrochloride, 2 mM EDTA and 20 μ g of dithiothreitol (Boehringer Mannheim, Germany) [dithiothreitol:protein ratio was 2:1 (w/w)] at 37 °C for 3 h under a N₂ atmosphere with occasional mixing. Alkylation was then initiated by the addition of 40 μ g of iodoacetic acid (puriss grade, Fluka, Buchs, Switzerland) or 40 μ g of iodoacetamide (Sigma Chem Co.), both in 70 μ l of 1.0 M Tris/HCl, pH 7.9. The alkylation was allowed to proceed at 4 °C for 30 min, in the dark, followed by 60 min at room temperature. The reaction was stopped and the protein recovered by precipitation with 100 % methanol.

Deglycosylation of bTSH

Native bTSH (1 mg) was chemically deglycosylated with anhydrous hydrogen fluoride (HF) (10 ml) based on the method of Manjunath and Sairam [20] in a Kelf/Teflon reaction apparatus (Peptide Institute, Osaka, Japan) and the cleavage allowed to proceed for 1 h at 0 °C. The HF was then evaporated off and the sample left overnight under vacuum at room temperature. The deglycosylated bTSH preparation was then recovered with three washes, each of 0.5 ml of 0.2 M NaOH, and neutralized with HCl. Following lyophilization, the sample was resuspended in 25 mM NH₄HCO₃ and stored at -20 °C.

Citraconylation of bTSH

Citraconylation of native bTSH was achieved using a modification of the method of Atassi and Habeeb [21]. Bovine TSH (290 μ g) was dissolved in 200 μ l of 0.1 M $\text{Na}_4\text{P}_2\text{O}_7$, pH 9.0, and a total 60-fold molar excess (based on the lysine content of bTSH) was added as eight 5 μ l aliquots of citraconic anhydride (Sigma Chem. Co.) (diluted 1:4 in 100% ethanol) 30 min apart. The pH was checked after each addition of citraconic anhydride and maintained at approx. pH 8.5 by the addition of 5 M NaOH. The mixture was incubated for a further 2 h at room temperature then centrifuged in a microfuge for 3 min. The supernatant was then applied to a column (4 cm $\times$ 0.8 cm i.d.) of Sephadex G-25, pre-equilibrated with 100 mM NH_4HCO_3 and the protein fraction collected and stored at -20°C . A sample (approx. 10% of total) was deblocked by incubation in 8 vol. of 0.1% (v/v) trifluoroacetic acid for 4.5 h at room temperature. The deblocked sample was then lyophilized and resuspended in 100 mM NH_4HCO_3 and stored at -20°C .

Trypsin digestion of bTSH β

Bovine TSH β (12 μ g) was dissolved in 50 μ l of 100 mM NH_4HCO_3 , 2 mM CaCl_2 and *N*-tosyl-L-phenylalanine chloromethyl ketone-treated trypsin (EC 3.4.21.4; Worthington Biochemical Corp, NJ, U.S.A.) [trypsin:protein ratio was 1:20 (w/w)] dissolved in 10 μ l of 1 mM HCl was added. Digestion was allowed to proceed for 20 h at room temperature with constant mixing, and aliquots (2.5 μ g and 1 μ g) were removed for testing in the TSH-RIA and SDS/PAGE (reduced and non-reduced) respectively. In each case, tryptic digestion was stopped by the addition of soybean trypsin inhibitor (STI; Boehringer Mannheim) [STI:trypsin ratio was 2:1 (w/w)] in 10 μ l of 100 mM NH_4HCO_3 . The remaining sample was lyophilized, resuspended in 100 μ l of 0.1% (v/v) trifluoroacetic acid and applied directly to a polybrene-treated glass-fibre disc which was placed in an Applied Biosystems gas-phase protein sequencer (model 470A) and sequenced for three cycles to determine the location of the trypsin cleavage sites in the bTSH β sequence.

SDS/PAGE analysis

Proteins were separated on 12.5% polyacrylamide gels under non-reducing and reducing conditions with a stacking gel of 5% polyacrylamide according to the method of Laemmli [22], and were silver-stained using the Wray procedure [23]. Samples were diluted in sample buffer and incubated at 100°C for 3 min before loading on to the gel. The reduced samples were prepared by boiling in sample buffer containing 28 mg/ml dithiothreitol. Electrophoresis was performed overnight with a constant current of 7.5 mA.

RESULTS

Immunoreactivity of MAb 279 and MAb 299 with GPHs and subunits

The immunoreactivities of MAb 279 and MAb 299 with the various GPHs were assessed by solution-phase RIA and ELISA. The ED_{50} values (i.e. concentrations of competitors required to inhibit binding of tracer by 50%) for all RIAs are summarized in Table 1. The MAb 279 bound to bTSH and hTSH with equal affinity, while the affinity of MAb 299 was almost 5-fold greater for hTSH than bTSH. Both MAbs were also able to recognize pTSH although with binding affinities significantly lower than those observed for either hTSH or bTSH. The affinity of MAb 279 was determined to be approx. 2-fold higher for free bTSH

Table 1 ED_{50} values for the various antigens tested in an RIA

Results are the means ($\pm$ S.D.) of $n = 3$ assays performed with triplicate data points. For samples indicated with asterisks the result is the mean ED_{50} value ($\pm$ S.E.M.) of $n = 1$ assay performed with duplicate data points.

Antigen	$\text{ED}_{50} \pm \text{S.D.}$	
	MAb 279 [$10^9 \times \text{concn. (M)}$]	MAb 299 [$10^8 \times \text{concn. (M)}$]
hTSH	4.8 ± 1.1	0.9 ± 0.1
bTSH	4.6 ± 0.8	4.2 ± 0.9
bTSH β	2.5 ± 0.3	1.9 ± 0.2
pTSH	16.0 ± 0.7	6.6 ± 0.6
bLH	> 127	> 127
hFSH (recombinant)	> 127	> 127
hCG	> 127	> 127
Deglycosylated bTSH	11.0 ± 1.0	7.7 ± 0.3
Citraconylated bTSH*	110 ± 10	> 127
Citraconylated and deblocked bTSH*	9.0 ± 0.5	12.0 ± 2.0
Trypsin-digested bTSH*	10.0 ± 1.5	6.5 ± 0.3

β -subunit than for the intact bTSH or hTSH. Conversely, the affinity of MAb 299 for intact hTSH was approx. 2-fold greater than for free bTSH β -subunit, which in turn was approx. 2-fold greater than for bTSH. These results were all similarly reflected in the corresponding ELISA results (data not shown).

Neither MAb 279 nor MAb 299 bound to recombinant hFSH or hCG and only MAb 279 displayed a very low cross-reactivity for bLH, which was only distinguishable in the ELISA at very high bLH concentrations (data not shown). No immunoreactivity was observed between MAb 279 or MAb 299 with the free bovine α -subunit.

Effect of MAb 279 and MAb 299 on bTSH receptor binding

The effect of the pre-binding of bTSH to either MAb 279 or MAb 299 on the interaction of TSH with its receptor on thyroid membranes was determined by radio-receptor assay. Both MAbs were able to reduce the binding of radio-iodinated bTSH to its receptor in a concentration-dependent manner (Figure 1) with MAb 279 being slightly more potent in terms of the degree of inhibition. Fifty percent inhibition of tracer-receptor binding was observed at a concentration of 1.5×10^{-9} M for MAb 279 and 4.2×10^{-9} M for MAb 299 (Figure 1). MAb 334, which was employed as a negative control, displayed no inhibition of the receptor binding of bTSH (Figure 1), indicating that the inhibition observed for MAb 279 and MAb 299 was not a result of a non-specific interaction between the receptor or GPH and murine IgG molecules. This inhibition of TSH-receptor binding indicates that the epitopes recognized by MAb 279 and MAb 299 are both located within or close to a receptor-binding site of bTSH.

IRMA

The relative spatial arrangement of the epitopes for MAb 279 and MAb 299 on the TSH β -subunit was assessed using intact hTSH in an IMRA. Human TSH was utilized in this experiment as a number of the other control MAbs employed (e.g. MAb 165P and MAb 273) did not cross-react with bTSH (data not shown). Both MAb 279 and MAb 299 were employed as the adsorbed (coating) and the detection (iodinated) MAbs. A number of other hTSH-specific MAbs were also used as adsorbed

Figure 1 Inhibition of bTSH receptor binding by MAb 279 and MAb 299

^{125}I -bTSH was preincubated with MABs for 1 h then bovine thyroid membranes containing the TSH receptor were added and incubated for a further hour. Receptor-bound ^{125}I -bTSH was separated from unbound ^{125}I -bTSH by filtration through cellulose acetate filters. MAb 334 (raised against hLH) was included as a negative control. The saturability of the receptors by bTSH in the radio-receptor assay is also shown.

Table 2 IRMA using both ^{125}I -MAb 279 and ^{125}I -MAb 299 as detection antibodies

Results represent ^{125}I -MAB binding to hTSH whereby the amount (c.p.m.) specifically bound was expressed as a percentage of the non-specifically bound counts. The non-specific binding was determined in the absence of antigen. A pair of MABs was considered to be sterically compatible (+) if the amount bound (c.p.m.) was 30% higher in the presence of antigen than in its absence using the criterion adopted by Schwarz et al. [26].

Coat MAb	Detection MAb	
	^{125}I -MAb 279	^{125}I -MAb 299
299	349 (+)	4 (—)
279	19 (—)	6 (—)
435P	1485 (+)	901 (+)
165P	81 (—)	372 (+)
273	401 (+)	0.6 (—)

antibodies to provide a more detailed analysis of the relative locations of the epitopes. When MAb 279 was used as the detection antibody, it was able to bind to hTSH which was prebound to MAb 299, indicating that these two MABs recognize distinct epitopes (Table 2). Interestingly, when MAb 299 was used as the detection antibody, it was inhibited from binding to the hTSH-MAB 279 complex.

The different specificities of MAb 279 and MAb 299 were further emphasized by their differential binding to hTSH with the other capture MABs employed in the IRMA (Table 2). For example, MAb 279, but not MAb 299, was able to bind simultaneously with MAb 273 to hTSH, while MAb 299, but not MAb 279, was able to bind simultaneously with MAb 165P to hTSH. Both MAb 279 and MAb 299 bound to hTSH when the GPH was prebound to MAb 435P, a hTSH α -subunit-specific MAB.

Figure 2 SDS/PAGE of bTSH β and trypsin-digested bTSH β

The samples were electrophoresed on a 12.5% separating gel under non-reducing (lanes 1–3) and reducing conditions (lanes 4–6). Detection was by silver staining. Lanes 1 and 4, bTSH β ; lanes 2 and 5, trypsin-digested bTSH β ; lanes 3 and 6, trypsin and soybean trypsin inhibitor. The molecular-mass standards used were: BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), myoglobin (17.3 kDa) and cytochrome *c* (11.7 kDa).

Effect of reduction and alkylation of the disulphide bridges of bTSH on the binding of MAb 279 and MAb 299

Reductive alkylation (with iodoacetic acid or iodoacetamide) of bTSH was used to determine whether the epitopes for MAb 279 and MAb 299 were dependent on the native conformation of the hormone. SDS/PAGE under non-reducing conditions of the reduced and alkylated preparations indicated that both subunits were fully reduced (data not shown). The immunoreactivity of the fully reduced and alkylated bTSH preparations for both MABs was completely abolished in the ELISA (data not shown) indicating that the epitopes recognized by MAb 279 and MAb 299 are dependent on the disulphide bonds of the bTSH β -subunit being intact.

Effect of deglycosylation of bTSH on MAb 279 and MAb 299 binding

Bovine TSH was chemically deglycosylated by treatment with anhydrous HF to determine whether the epitopes for MAb 279 or MAb 299 involved the carbohydrate moiety of bTSH β . SDS/PAGE of the deglycosylated preparation yielded two bands of apparent molecular mass 12600 Da and 10600 Da compared with 17100 Da and 13500 Da for the α - and β -subunits respectively of native bTSH, indicating that the carbohydrates were cleaved from both subunits. The immunoreactivity of the deglycosylated bTSH preparation for MAb 279 and MAb 299 was essentially preserved following chemical deglycosylation with only a 1.8-fold (MAb 299) or 2.5-fold (MAb 279) decrease observed in binding compared with native bTSH as determined by RIA (Table 1). These results were similarly reflected in the ELISA studies where no or only a small decrease of binding to

Table 3 N-terminal sequencing of trypsin-digested bTSH β

The yields (pmol) of amino acids following three cycles of N-terminal sequencing of trypsin-digested bTSH β are shown with the bold numbers referring to the yields of the amino acid residues which are either located at the N-terminus of bTSH β or constitute the peptides formed by trypsin digestion (see Table 4).

Cycle	Amino acid ...	Asp	Asn	Ser	Gln	Thr	Gly	Glu	Ala	His	Tyr	Arg	Met	Val	Trp	Phe	Ile	Lys	Leu	Pro
1		57	0.1	36	0	20	25	8	31	18	89	14	8	28	4	128	0.1	9	119	0
2		26	1	9	5	0	17	9	111	15	36	4	4	174	1	58	5	6	13	4
3		17	21	7	0	43	8	11	18	12	19	2	7	30	0.5	4	88	0	144	6

Table 4 Deduced major trypsin cleavage sites of bTSH β

The yields of the N-terminal sequence of bTSH β , Phe¹-Cys²-Ile³, were used as a guide to the total amount of sequenceable tryptic fragments expected on each Edman cycle. As more than one fragment sequence may contain the same amino acid residue at a particular sequencing cycle, comparison of the yields obtained on each cycle with those of the N-terminal sequence enabled the identification of such an occurrence. For example, the yield of 174 pmol of valine on cycle 2 (Table 3) was approximately double the yield of Phe¹ on cycle 1, hence two fragments must contain a valine at position 2, therefore 50% yield was arbitrarily assigned to each sequence.

	Residue 1	Residue 2	Residue 3	Sequence position	Tryptic peptide*
Sequence 1	Phe	(Cys)	Ile	β 1-3	β T-1a
Yield (pmol)	128	N.D.	88		
Sequence 2	Asp	Val	Asn	β 35-37	β T-5
Yield (pmol)	57	87†	21		
Sequence 3	Leu	Phe	Leu	β 40-42	β T-6
Yield (pmol)	119	58	72†		
Sequence 4	Tyr	Ala	Leu	β 45-47	β T-7
Yield (pmol)	88	111	72†		
Sequence 5	His	Val	Thr	β 70-72	β T-10a
Yield (pmol)	18	87†	43		

* Refer to Figure 3 for locations of peptides. † Yield = 50% of the total yield for that amino acid.

the deglycosylated hormone compared with the native bTSH was observed for both MABs (data not shown).

Effect of citraconylation of bTSH on MAB 279 and MAB 299 binding

The involvement of lysine residues in the bTSH β -subunit epitopes for MAB 279 and MAB 299 was investigated by citraconylation of bTSH. This modification resulted in a derivative with very low (4.1%) immunoreactivity with MAB 279 and no immunoreactivity with MAB 299 at the highest concentration tested in the RIA (Table 1). Similar results were obtained in the solid-phase ELISA assay (data not shown). The immunoreactivity for both MABs was recovered (58% in terms of ED₅₀ values for MAB 279, 35% for MAB 299) following removal of the citraconyl group(s) by mild acid hydrolysis (Table 1).

Effect of trypsin digestion of bTSH β on MAB 279 and MAB 299 binding

The involvement of positively charged amino acid residues in the epitopes for MAB 279 and MAB 299 was further investigated using exhaustive trypsin digestion of the disulphide-intact bTSH β -subunit. Immunoreactivity decreased to 25% (in terms of

Figure 3 Comparison of the tryptic cleavage sites of native TSH β and hCG β

The tryptic peptides correspond to the cleavage sites determined by Liao and Pierce [40] for reduced and alkylated bTSH β . The arrows indicate the tryptic cleavage sites of native TSH β determined in the present study or native hCG β determined by Birken et al. [36].

ED₅₀ values) of the native TSH β -subunit for MAB 279 and 29% for MAB 299 (Table 1) following trypsin digestion. SDS/PAGE (under both reducing and non-reducing conditions) revealed that the subunit was completely digested (Figure 2); therefore, the observed immunoreactivity could not be attributed to residual, undigested bTSH β -subunit. Under non-reducing conditions, the trypsin-digested β -subunit migrated as a single band with an apparent molecular mass of 19000 Da on SDS/PAGE (Figure 2, lane 2), compared with the native β -subunit which migrated with an apparent molecular mass of 24500 Da (Figure 2, lane 1). This decrease in molecular mass indicates that either a portion(s) of the subunit was cleaved following digestion and/or that significant changes in the hydrodynamic shape of the digested protein had occurred. The reduced, digested TSH β -subunit migrated in SDS/PAGE with the dye-front (Figure 2, lane 5), indicating that fragments of less than 11700 Da were formed and further emphasizing that complete digestion occurred.

Characterization of the trypsin-digested bTSH β -subunit

The cleavage sites of the trypsin-digested bTSH β -subunit were characterized by N-terminal sequencing of the unseparated cleavage products. These results are summarized in Tables 3 and 4. The yields of the N-terminal sequence (Phe¹-Cys²-Ile³) were used as a guide to determine the amount of each fragment sequenced at each automated Edman cycle. Comparison of these sequencing results with the known primary structure and tryptic cleavage sites of the reduced and alkylated bTSH (Figure 3)

enabled the identification of these sites in the present study. Using this approach, the following preferred trypsin cleavage sites of the native bTSH β -subunit were determined: namely between the residues β 34–35, β 39–40, β 44–45 and β 69–70. The peptides β 35–39 and β 40–44 were completely excised from the β -subunit following trypsin digestion, as both of these peptides lack half-cystine residues.

DISCUSSION

Although previous studies have extensively characterized the epitopes recognized by MAbs raised against hCG, LH and FSH, there are very few reports of the characterization of epitopes recognized by anti-TSH MAbs. In this present investigation, the structures of the epitopes on the TSH β -subunit recognized by two murine anti-TSH MAbs, designated MAb 279 and MAb 299, have been partially characterized. The affinities of these MAbs for the various intact GPHs and free subunits (Table 1) indicate a number of similarities as well as differences in their epitope specificities. Both antibodies were highly specific for TSH, with neither MAb cross-reacting with any of the other GPHs tested. This result is consistent with the β -subunits for each of the GPHs possessing unique amino acid sequences. However, it has previously been demonstrated that MAbs specific for two (out of a total of six) distinct epitopes on hTSH β epitopes can also recognize other GPHs [4]. The inability of MAb 279 and MAb 299 to bind to the other GPHs indicates that the epitopes for these MAbs are unique to TSH and hence potentially involve amino acid sequence regions which confer TSH hormonal specificity and which are of low sequence similarity with the other GPH β -subunits. The results also indicate that both epitopes are conformational (as opposed to contiguous) in nature, as they are dependent on the disulphide bridges being intact to maintain their structures. Other studies have also previously demonstrated that the hTSH epitopes are dependent on the disulphide bond structure [5].

Various studies have revealed that the carbohydrate moieties can play a role in the immunogenicity of the GPHs [13,14,24,25]. In particular, studies with enzymically deglycosylated hTSH [13,14] have indicated that the majority of the glycosylation-dependent epitopes are located on the β -subunit. In the present study, removal of the carbohydrate moieties from both subunits of bTSH by chemical deglycosylation resulted in an approximate 2-fold decrease in the affinity of both MAb 279 and MAb 299 compared with the fully glycosylated bTSH (Table 1). This result, however, most probably reflects an overall alteration in the hormone conformation rather than direct involvement of the carbohydrates in the epitopes as suggested by Schwarz et al. [26] for hCG. The specificities of MAb 279 and MAb 299 are therefore likely to be different to those investigated by Papandreou et al. [13]. Other studies with both enzymically deglycosylated hTSH [5] and hCG [26] have similarly determined that removal of the carbohydrates results in only a relatively small change in the affinity of the MAbs for the deglycosylated hormone. Since the carbohydrate moiety on bTSH β is attached to Asn²³, it is also likely that both the epitopes recognized by MAb 279 and MAb 299 do not involve any amino acid residues directly adjacent to this site.

The affinity of MAb 279 for the free bovine TSH β -subunit was higher than that observed for intact hTSH or bTSH, even though the MAb was raised against the intact hormone. It has been recently shown that the free hCG β -subunit is processed by antigen-presenting cells in the same manner as when the β -subunit is complexed with hCG α -subunit [27]. A similar ex-

planation can be applied to MAb 299, which also has a higher affinity for the bTSH β -subunit compared with the intact bTSH. However, in the case of MAb 299, the affinity for intact human TSH is greater than that of intact bovine TSH (and free bTSH β -subunit), while MAb 279 binds both the intact bovine and human hormones with the same affinity. This result indicates that the epitope for MAb 279 is located either within an amino acid sequence region that is completely structurally conserved between these two species, or alternatively, the three-dimensional presentation of the epitope is very similar. The structure of the epitope in the bovine β -subunit compared with the human β -subunit that is recognized by MAb 299, however, is only partially conserved. There are just three non-conservative substitutions in bTSH β compared with hTSH β : namely Thr⁸ $\rightarrow$ Met, Leu⁶⁹ $\rightarrow$ Arg and Ala⁷² $\rightarrow$ Thr [28]. Hence the MAb 299 epitope may involve one, two or conceivably all three of these amino acid residues.

Both MAb 279 and MAb 299 displayed significantly lower affinity for pTSH than either the human or bovine hormones. There are a number of non-conservative substitutions in the pTSH β -subunit compared with hTSH β -subunit, namely Ile³⁶ $\rightarrow$ Phe, Asn³⁷ $\rightarrow$ Asp, Leu⁶⁹ $\rightarrow$ His, Asn⁸⁹ $\rightarrow$ Asp and Gln¹⁰⁹ $\rightarrow$ Glu. Any one, or possibly a combination of these amino acid residues, may therefore be involved in the two epitopes. It is noteworthy that Leu⁶⁹ is substituted in both the bovine and porcine TSH β -subunits (compared with hTSH β), and therefore this substitution may contribute to the observed reduced affinity of both of these hormones for MAb 299.

The distinct epitope specificities of MAb 279 and MAb 299 were further emphasized by IRMA results which demonstrated that MAb 279 could simultaneously bind to hTSH when MAb 299 was employed as the capture antibody (Table 2). When the MAbs were used in the reverse order, MAb 299 was unable to bind. False negative results have previously been described with this type of experiment and explained by the detection antibody being at a critically low concentration, of low affinity or damaged by iodination [29]. However, in the experiments reported in this present investigation the MAb 299 was clearly able to bind when used in combination with capture MAbs other than MAb 279 (e.g. MAb 435). It appears therefore that steric factors may be involved, with the epitopes recognized by MAb 279 and MAb 299 closely related in terms of spatial arrangement and may even partially overlap. Alternatively, the binding of MAb 279 to its epitope may possibly induce a conformational change in the epitope recognized by MAb 299 such that MAb 299 is then unable to bind. The ability of MAbs to induce conformational changes upon binding to GPHs has been suggested in studies with MAbs to hCG [30] and equine CG [31]. Whichever explanation is the case, the differential binding of MAb 279 and MAb 299 to hTSH when other MAbs were employed in the IRMA as capture MAbs, further emphasizes their distinct epitope specificities (Table 2).

MAbs 279 and 299 were both able to inhibit the interaction of radio-iodinated bTSH with its receptor in a dose-dependent manner (Figure 1). Similar observations have been made in other studies in which MAbs to TSH [10,11] and other GPHs (e.g. [31–33]) have been found to inhibit both receptor binding and stimulation of adenylate cyclase activity. These effects result from the binding of the MAb to the GPH at a site directly involved in, or proximal to, the receptor binding domain. In the latter case, the antibody would sterically hinder the hormone–receptor interaction. In the case of MAb 279 and MAb 299 this latter possibility cannot be ruled out when the relative sizes of the TSH β -subunit and the MAbs are compared. However, some antibodies to hTSH have previously been shown to be incapable

of inhibiting receptor binding when prebound to the intact hormone [11]. In addition, MABs can bind to two (out of a total of seven) of the distinct epitopes on hCG β when it is complexed with its receptor [34]. Hence, interference of the hormone-receptor interaction will not always occur despite the relative sizes of the GPH and the antibody.

The effects of various modifications to bTSH were examined to identify key structural features of the epitopes recognized by MAb 279 and MAb 299. Citraconylation of bTSH was employed to determine the contribution of the positive charge groups associated with the side chains of lysine residues. There are nine lysine residues in bTSH β (and eight in hTSH β) at the sequence positions β 14, 39, 44, 60, 84, 87, 101, 107 and 110. Because most of the lysine residues will be chemically modified [35] it was not surprising that this modification of bTSH resulted in a significant reduction in the binding of both MAb 279 and MAb 299. The immunoreactivity was partially recovered following removal of the citraconyl group. This result indicates a possible requirement for positively charged groups in the interaction of TSH with MAb 279 and MAb 299, although the influence of the introduced negative charge interfering in the binding cannot be totally discounted.

Trypsin digestion of the native bTSH β -subunit and characterization of the products were performed to enable elucidation of the potential lysine and arginine residues which may be involved in the epitopes recognized by MAb 279 and MAb 299. Excision of the sequence region between bTSH β 35-44 was evident following N-terminal sequencing of the trypsin-digested bTSH β -subunit (Tables 3 and 4). A proteolytic clip was also introduced into the hormone between β Arg⁶⁹-His⁷⁰. These results represent to our knowledge the first report on the fragmentation sites leading to the tryptic-core of TSH, and are comparable with other data obtained for the tryptic-core fragment of hCG [36] (Figure 3). The cleavage observed at bTSH β 34-35 (i.e. at the sequence position analogous to hCG β 41-42 upon alignment of the sequences of the hCG β and TSH β subunits) is very close to the hCG β 43-44 trypsin cleavage site. Proteolytic cleavage at Arg⁴³ in hCG β by endoprotease-Arg C has also been observed previously [37]. Similarly, the bTSH β 69-70 cleavage site determined in the present investigation is directly analogous to the hCG β 74-75 trypsin cleavage site. Due to the presence of the six disulphide bonds, no peptides can be released from hCG following trypsin digestion (except the C-terminal extension peptide hCG β 114-145 which is not present in TSH), while with TSH β the region between Asp³⁵-Lys⁴⁴ can clearly be excised. This difference is readily explained by the fact that there is not an additional trypsin cleavage site in the analogous region of hCG β (Figure 3). In summary, these results support the conclusion that the regions between β 31-52 in TSH and β 38-57 in hCG are surface exposed, consistent with the proposal that this region constitutes a receptor-binding domain for the GPHs [8,38,39]. The X-ray crystallographic structure of hCG [2,3] indicates that this region forms a large, outwardly projecting loop structure with the cleavage sites analogous to TSH β 34-35 and β 39-40 buried at the α/β -subunit interface in the intact hormone, while the β 44-45 cleavage site is exposed to the solvent. In the free β -subunit, however, all of these cleavage sites are likely to be exposed to the solvent as the above results demonstrate.

The TSH β 35-44 region cannot be involved in the epitopes recognized by either MAb 279 or MAb 299 as significant binding to both MABs was retained by bTSH β following the complete excision of this region by trypsin digestion. The observed decrease in antibody binding after trypsin digestion is likely to be due to an overall conformational change in TSH β following the excision

of the bTSH β 35-44 region and the introduction of the proteolytic nick at bTSH β 69-70. The excision of a significant portion of the β 31-52 putative receptor-binding domain indicates that the other proposed sites for TSH receptor binding, i.e. β 1-15, β 71-85 and β 101-112 [8,9], are probably more relevant candidates as sequence regions contributing to the epitopes recognized by MAb 279 and MAb 299. Studies using anti-peptide antibodies raised to the C-terminus of hTSH β [6,7] have indicated that the C-terminal region (i.e. β 95-112) of hTSH is antibody-accessible in both the intact hormone and the free subunit, while conflicting results have been obtained for antibodies to peptides from the N-terminal region (i.e. β 1-15) [6,7]. Anti-peptide antibodies raised against a peptide encompassing the sequence β 53-76 have also been shown to be capable of binding to the TSH β -subunit and the native intact hormone [7].

In summary, the epitopes recognized by MAb 279 and MAb 299 are distinct but are closely related in spatial terms, are dependent on the integrity of the disulphide bonds of TSH β and may involve lysine residues. The sequence region between TSH β 35-44 and the carbohydrate moieties are not involved in this immunorecognition. In addition, it is likely that both epitopes coincide with the putative receptor binding sites TSH β 1-15, β 71-85 or β 101-112.

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REFERENCES

- Ryan, R. J., Charlesworth, M. C., McCormick, D. J., Milius, R. P. and Keutmann, H. T. (1988) *FASEB J.* **2**, 2661-2669
- Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J. and Isaacs, N. W. (1994) *Nature (London)* **369**, 455-461
- Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E. and Hendrickson, W. A. (1994) *Structure* **2**, 545-558
- Benkirane, M. M., Bon, D., Costagliola, S., Paolucci, F., Darboret, B., Prince, P. and Carayon, P. (1987) *Endocrinology* **121**, 1171-1177
- Endo, Y., Miyai, K., Iijima, Y., Nakajima, T., Eda, Y., Fujita, H. and Unoki, M. (1989) *Acta Endocrinol.* **120**, 201-209
- Bidart, J.-M., Troalen, F., Ghillani, P., Puisieux, A., Buhon, C. and Bellet, D. (1991) *J. Biol. Chem.* **266**, 19238-19244
- Labbe-Jullie, C., Sergi, I., Canonne, C., Darbon, H. and Ronin, C. (1992) *Endocrinology* **131**, 1999-2009
- Morris, J. C., McCormick, D. J. and Ryan, R. J. (1989) *J. Biol. Chem.* **265**, 1881-1884
- Leinung, M. C., Bergert, E. R., McCormick, D. J. and Morris, J. C. (1992) *Biochemistry* **31**, 10094-10098
- Hill, B. L., Lustbader, J. W. and Erlanger, B. F. (1987) *Endocrinology* **120**, 574-581
- Costagliola, S., Madec, A.-M., Benkirane, M. M., Orgiazzi, J. and Carayon, P. (1988) *Mol. Endocrinol.* **2**, 613-618
- Endo, Y., Tetsumoto, T., Nagasaki, H., Kashiwai, T., Tamaki, H., Amino, N. and Miyai, K. (1990) *Endocrinology* **127**, 149-154
- Papandreou, M.-J., Sergi, I., Benkirane, M. and Ronin, C. (1990) *Mol. Cell. Endocrinol.* **73**, 15-26
- Papandreou, M.-J., Sergi, I., Medri, G., Labbe-Jullie, C., Braun, J. M., Canonne, C. and Ronin, C. (1991) *Mol. Cell. Endocrinol.* **78**, 137-150
- Stanton, P. G. and Hearn, M. T. W. (1987) *J. Chromatogr.* **397**, 379-387
- Cohen, S. A. and Strydom, D. J. (1988) *Anal. Biochem.* **174**, 1-16
- Stanton, P. G., Robertson, D. M., Burgon, P. G., Schmauk-White, B. and Hearn, M. T. (1992) *Endocrinology* **130**, 2820-2832
- Lissitzky, S., Fayet, G. and Verrier, B. (1976) in *Methods in Receptor Research*, part II (Blecher M., ed.), pp. 641-655, Marcel-Dekker, New York
- Stanton, P. G. and Hearn, M. T. W. (1986) *Endocrinology* **118**, 14-22
- Manjunath, P. and Sairam, M. R. (1985) *Methods Enzymol.* **109**, 725-735
- Atassi, M. Z. and Habeeb, A. F. S. A. (1972) *Methods Enzymol.* **25**, 546-553
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680-685
- Wray, W., Boulikis, T., Wray, V. P. and Hancock, R. (1981) *Anal. Biochem.* **118**, 197-203
- Sairam, M. R., Lingenn, J. and Bhargavi, G. N. (1988) *Biosci. Rep.* **8**, 271-278
- Lamarre, L. and Sairam, M. R. (1989) *Mol. Cell. Endocrinol.* **66**, 181-187

- 26 Schwarz, S., Krude, H., Klieber, R., Dirnhofer, S., Lottersberger, C., Merz, W. E., Wick, D. and Berger, P. (1991) *Mol. Cell. Endocrinol.* **80**, 33–40
- 27 Rouas, N., Christophe, S., Housseau, F., Bellet, D., Guillet, J.-G. and Bidart, J.-M. (1993) *J. Immunol.* **150**, 782–792
- 28 Ward, D. N., Bousfield, G. R. and Mar, A. O. (1989) in *Structure-Function Relationship of Gonadotropins* (Bellet, D., ed.), pp. 1–21, Serono Symposia Publications, Raven Press, New York
- 29 Schwarz, S., Berger, P. and Wick, G. (1986) *Endocrinology* **118**, 189–197
- 30 Hattori, M., Hachisu, T., Shimohigashi, Y. and Wakabayashi, K. (1988) *Mol. Cell. Endocrinol.* **57**, 17–23
- 31 Chopineau, M., Maurel, M.-C., Combarnous, Y. and Durand, P. (1993) *Mol. Cell. Endocrinol.* **92**, 229–239
- 32 Schwarz, S., Berger, P., Nelboeck, E., Khashabi, D., Panmoung, W., Klieber, R. and Wick, G. (1988) *J. Recept. Res.* **8**, 437–453
- 33 Alonso-Whipple, C., Couet, M. L., Doss, R., Koziarz, J., Ogunro, E. A. and Crowley, J. R. W. F. (1988) *Endocrinology* **123**, 1854–1860
- 34 Schwarz, S., Krude, H., Wick, G. and Berger, P. (1991) *Mol. Cell. Endocrinol.* **82**, 71–79
- 35 Liu, W.-K., Yang, K.-P., Nakagawa, Y. and Ward, D. N. (1974) *J. Biol. Chem.* **249**, 5544–5550
- 36 Birken, S., Gawinowicz Kolks, M. A., Amr, S., Nisula, B. and Puett, D. (1987) *Endocrinology* **121**, 657–666
- 37 Bousfield, G. R. and Ward, D. N. (1988) *J. Biol. Chem.* **263**, 12602–12607
- 38 Keutmann, H. T., Charlesworth, M. C., Mason, K. A., Ostrea, T., Johnson, L. and Ryan, R. J. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 2038–2042
- 39 Santa-Coloma, T. A., Dattatreymurty, B. and Reichert, L. E. (1990) *Biochemistry* **29**, 1194–1200
- 40 Liao, T.-H. and Pierce, J. G. (1971) *J. Biol. Chem.* **246**, 850–865