Endocrine Journal 1998, **45** (4), 467–473

Effect of Site Directed Mutagenesis in the CMGCC Region of the α-Subunit on Immunoreactive Human Thyrotropin

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Abstract. The cDNA of the common α-subunit of human glycoprotein hormone was mutated by site directed mutagenesis in the CMGCC region composed of cysteine-methionine-glycine-cysteine-cysteine (position 28–32). The cDNA of wild-type human thyrotropin (hTSH) β-subunit and that of wild-type or mutant common α-subunits were co-transfected into COS-I cells. The concentration of hTSH determined by two immunoradiometric assay systems was detectable in culture media of COS-I cells transfected with wild-type (CMGCC) and a mutant (CRGCC) α-subunits but not four other mutants (YMGCC) (CMRCC) (CMACC) (CMDCC). The present data with the other studies on wild-type or mutant glycoprotein hormones support our hypothesis that an amino acid motif of "C-X-G-X-C" in the common α-(CMGCC in human) and β-(CAGYC in human) subunits play an important role in biosynthesis of glycoprotein hormones in all species.

Key words: CMGCC and CAGYC regions, C-X-G-X-C motif, α Subunit of glycoprotein hormones, Site directed mutagenesis, Immunoreactive TSH

(*Endocrine Journal* **45**: 467–473, 1998)

THE glycoprotein hormones, TSH and gonadotropin, are composed of two non-covalently linked α- and β-subunits. The amino acid sequences of α-subunits (common α-subunit) are identical in all glycoprotein hormones and those of β-subunits are specific for each hormone. The structural relationships of glycoprotein hormones with their biological and immunological activities have been investigated by chemical and immunological techniques [1–5]. The recent introduction of selective site-directed mutagenesis has made it possible to more precisely identify amino acid residues or sequences of glycoprotein hormones relating to the activity [6, 7].

Our previous studies on patients with congenital isolated TSH deficiency as well as site directed mutagenesis of β-subunits of hTSH [8–10] and human chorionic gonadotropin (hCG) [11] indicated that the CAGYC region composed of cysteine-alanine-glycine-tyrosine-cysteine was related to their biological and immunological activities. Subsequently, the CMGCC region composed of cysteine-methionine-glycine-cysteine-cysteine in the human common α-subunit was found to be important for the biological activity of hCG [12]. These data led us to postulate the hypothesis that a motif of amino acid sequence C-X-G-X-C may play an important role in the biosynthesis of

Received: December 3, 1997
Accepted: March 16, 1998
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glycoprotein hormones [10].

We therefore studied the effect of site directed mutagenesis in the CMGCC region of the α-subunit of hTSH on the immunological activity that has not yet been studied.

Materials and Methods

Preparation of recombinant hTSH

Plasmid construction: The cDNA of the common α-subunit of human glycoprotein hormones was kindly provided by Dr. J. C. Fiddes and that of hTSH β-subunit by Dr. Y. Hayashizaki. The cDNA of α-subunit was mutated by site-directed mutagenesis as described previously [12].

The wild-type and mutant cDNAs of α-subunit were subcloned into pSVK3 vector (Pharmacia LKB Biotechnology, Sweden), which is constructed as an expression vector in SV40 infected mammalian cells, at Kpn I and Pst I sites. The DNA and amino acid sequences of the CMGCC region of wild-type and mutant α-subunits are shown in Fig. 1. The cDNA of wild-type hTSH β-subunit was also subcloned into pSVK3 vector at Sma I and Sal I sites.

Preparation of cells for transfection: The COS-1 cell line which was derived from monkey kidney was maintained in Dubecco's Modified Eagle Medium (DMEM) (Gibco BRL Products, USA) supplemented with 10% fetal calf serum (FCS) (JRH Biosciences, USA). COS-1 cells were harvested from sub-confluent cultures by trypsinization, then collected by centrifugation, washed twice with phosphate buffered saline (PBS) and resuspended in K-PBS buffer (30.8 mM NaCl, 120.7 mM KCl, 8.1 mM Na_2HPO_4, 1.46 mM KH_2PO_4, 10 mM $MgCl_2$) at 5×10^6 cells/0.6 m*l*.

Electroporation: Prepared COS-1 cells were incubated for 15 min on ice with 25 μg of α-subunit cDNA/pSVK3 and 50 μg of hTSH β-subunit cDNA/pSVK3. Electroporation was performed by Gene Pulser, as suggested by the manufacturer (Bio-Rad Laboratories, USA) at 220 V and 960 mFD on 5×10^6 COS-1 cells in KPBS buffer. After incubation in KPBS buffer on ice for 5 min, the cells were cultured at 37 °C in DMEM/Ham F12 medium (Gibco BRL Products, USA) (1:1) supplemented with 10% FCS. The medium was changed to serum free medium after 24 h and the cells were further incubated for 46 h. Supernatants were harvested and stored at −80 °C until measurement of the concentration of hormone produced by transfected COS-1 cells.

		28	29	30	31	32
Wild type		Cys (C) T G C	Met (M) A T G	Gly (G) G G C	Cys (C) T G C	Cys (C) T G C
Mutant 1	C 28 Y	Tyr (Y) T A C	Met (M) A T G	Gly (G) G G C	Cys (C) T G C	Cys (C) T G C
Mutant 2	M 29 R	Cys (C) T G C	Arg (R) A G G	Gly (G) G G C	Cys (C) T G C	Cys (C) T G C
Mutant 3	G 30 R	Cys (C) T G C	Met (M) A T G	Arg (R) C G C	Cys (C) T G C	Cys (C) T G C
Mutant 4	G 30 A	Cys (C) T G C	Met (M) A T G	Ala (A) G C C	Cys (C) T G C	Cys (C) T G C
Mutant 5	G 30 D	Cys (C) T G C	Met (M) A T G	Asp (D) G A C	Cys (C) T G C	Cys (C) T G C

Fig. 1. DNA and amino acid sequences in the CMGCC region of wild-type and mutant common α-subunits. Mutations were indicated by boxes.

Immunoassay for hormones

hTSH: The concentration of hTSH was determined by a two site immunoradiometric assay (IRMA) with two different commercial kits, SPAC-S TSH kit® (Daiichi Radioisotope Laboratories, Japan) and TSH·RIABEAD II® (Dainabot, Japan). Briefly, samples or standard were incubated with a mouse anti hTSH monoclonal antibody coated on tubes (SPAC-S TSH kit®) or beads (TSH·RIABEAD II®). The tubes or beads were washed and incubated with I^{125} labeled anti hTSH monoclonal antibody. Radioactivity was determined in the washed tubes or beads. The minimum detectable doses were 0.1 mU/*l* (SPAC-S TSH kit®) and 0.05 mU/*l* (TSH·RIABEAD II®) respectively.

Free subunits: Concentrations of free hTSH α- and β-subunits were determined by a double-antibody radioimmunoassay. Reagents used for each assay were as follows, hTSHα-(kindly provided by NIH, USA) and hTSHβ-(Hoechst, Japan) subunits; rabbit antibodies to hTSHα- and β- subunits (NIH) (first antibodies); goat antibodies to rabbit immunogloblin G (Eiken Chemical Co Ltd Japan) (second antibody). Each subunit was labeled with ^{125}I by the glucose oxidase method. Briefly samples or standards were incubated with the first

antibody and ^{125}I labeled subunit. After the second antibody was added the mixture was centrifuged. The radioactivity in precipitates was determined. The minimum detectable dose of each assay was 0.3 $\mu g/l$.

Calibration for cross-reactivity: The cross-reactivities in each assay were very small but the concentration of each hormone was calculated with a calibration coefficient as follows;

$$X = \frac{(1-Bz\cdot Cy)X' + (Az\cdot Cy-Ay)Y' + (Ay\cdot Bz-Az)Z'}{1-Ay\cdot Bx-Az\cdot Cx-Bz\cdot Cy + Ay\cdot Bz\cdot Cx + Az\cdot Bx\cdot Cy}$$

$$Y = \frac{(Bz\cdot Cx-Bx)X' + (1-Az\cdot Cx)Y' + (Az\cdot Bx-Bz)Z'}{1-Ay\cdot Bx-Az\cdot Cx-Bz\cdot Cy + Ay\cdot Bz\cdot Cx + Az\cdot Bx\cdot Cy}$$

$$Z = \frac{(Bx\cdot Cy-Cx)X' + (Ay\cdot Cx-Cy)Y' + (1-Ay\cdot Bx)Z'}{1-Ay\cdot Bx-Az\cdot Cx-Bz\cdot Cy + Ay\cdot Bz\cdot Cx + Az\cdot Bx\cdot Cy}$$

where

X is the true concentration of hTSH ,
Y is that of free hTSHα-subunit,
Z is that of free hTSHβ-subunit;
X′ is the observed hTSH value according to the hTSH assay,
Y′ is that of hTSHα-subunit according to the hTSHα assay,
Z′ is that of hTSHβ-subunit according to the hTSHβ assay ,
Ay is the cross-reactivity of free hTSHα-subunit in the hTSH assay ,
Az is that of free hTSHβ-subunit in the hTSH assay,
Bx is that of hTSH in the hTSHα assay ,
Bz is that of hTSHβ-subunit in the hTSHα assay,
Cx is that of hTSH in the hTSHβ assay ,
Cy is that of hTSHα-subunit in the hTSHβ assay.

Results

As shown in Fig. 2, the concentration of hTSH determined by the two methods (SPAC-S TSH kit® and TSH·RIABEAD II®) was detectable in the culture media of COS-1 cells transfected with DNAs of wild type common α[CMGCC]- plus wild-type hTSHβ-subunits (Wt) and that of mutant 2 common α[CRGCC] plus wild-type hTSHβ-subunit (Mt-2). No hTSH was detectable in the other mutants (Mt-1[YMGCC], Mt-3[CMRCC], Mt-4[CMACC] and Mt-5[CMDCC]) or the control.

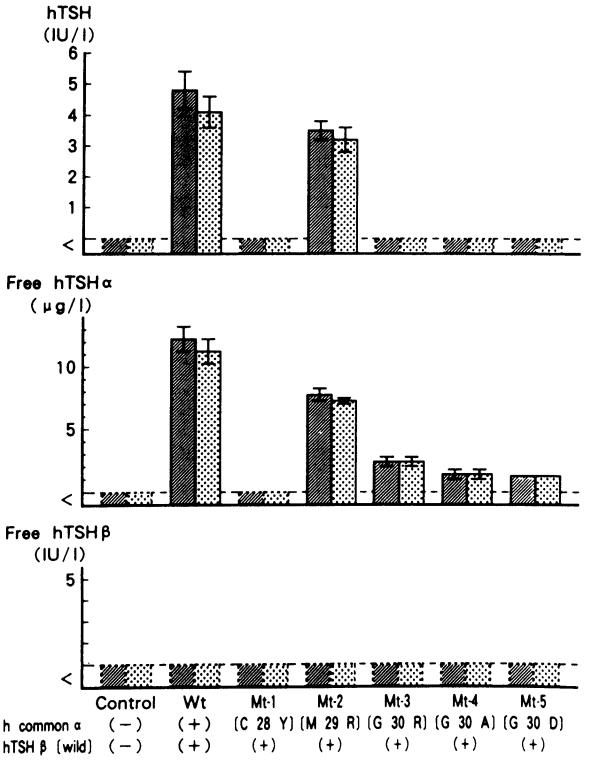

Fig. 2. Concentrations of immunoreactive hTSH, free hTSHα- and hTSHβ-subunits in culture media of COS-1 cells transfected with cDNAs of wild-type hTSH β-subunit and wild-type or mutant common α-subunits. The DNA and amino acid sequences of the common α-subunit are shown in Fig. 1. Immunoreactive hTSH was determined by two methods, SPAC-TSH kit® (hatched column) and TSH·RIABEAD II® (stippled column). Bars indicate mean and SE. Columns shown with dotted line and (<) indicate below minimum detectable level. All immunoreactive values were calibrated for respective cross reactivities as described in Materials and Methods.

Free TSHα-subunit was detectable in wild-type (Wt) and all mutants except Mt-1. No free TSHβ-subunit was detectable in any medium.

Discussion

Immunoreactive hTSH determined by two methods had a similar pattern to that of hTSH detected in Mt-2 but not in Mt-1, Mt-3, Mt-4 or Mt-5. Bioassay and receptor assays have not yet been done because of their low sensitivity in detecting hTSH in small samples. Since, however, our study

on the biological activity of hCG with the same site directed mutagenesis of α-subunit [12] demonstrated similar patterns of hCG to those determined by time resolved fluoroimmunoassay (unpublished data), it seems to be reasonable to assume that the biological activity of hTSH is similar to that of the immunoreactive hTSH in the present experiment.

At least three mechanisms are suggested to account for the loss of immunoreactivity of hTSH caused by the site directed mutagenesis. The first possibility—decrease in the expression or secretion of common α-subunit by transfection with mutant cDNAs (Mt-3, -4, -5)— is unlikely because immunoreactive free common α-subunit was detectable in these mutants. The concentration of hTSH and its subunits in cells was not determined although cell viability was found to be intact. The wild-type common α-subunit is found to be critical for the secretion and stability of the hTSHβ-subunit in an expression experiment [13], so that it cannot be denied that the production or secretion of hTSH β-subunit was decreased with the mutant common α-subunits or small amounts of cDNAs of hTSH β-subunits used in this experiment. The second possibility is increased degradation of the mutant hTSH produced. The third possibility is that the epitope(s) of mutant hTSHs, which are responsible for immunometric assay were changed. Epitope mappings have demonstrated that different monoclonal antibodies recognized different epitopes of wild-type hTSH [14], but the epitope mappings of mutant hTSHs have not yet been done.

Table 1 summarizes amino acid sequences of the CMGCC region of common α-subunits and the CAGYC region of β-subunits relating to immunological and/or biological activities of mutant glycoprotein hormones. Data on site directed mutagenesis of CMGCC and CAGYC regions of LH and FSH, C C, in CMGC C and Y C in CAGY C regions and biological activity of mutant TSH (CMGCC) and CG (CAGYC) have not been available, so that information on wild type glycoprotein hormones with biological activity is also listed in Table 1. Amino acid sequences of the wild-type hormones are highly conserved beyond species. Immunological and/or biological activities of glycoprotein hormones were maintained with various changes in M in the CMGCC region ([M to K, V, S or T] in wild-type and [M to R] in mutagenesis), those of A in the CAGYC region([A to S, M, E, G, T or S] in wild type and [A to D] in mutagenesis) and those of Y in the CAGYC region ([Y to F, L,Q or H] in wild-type). A substitution of [C to Y] in the CMGCC region in mutagenesis resulted in a complete loss of immunoreactivity of hTSH and hCG suggesting a gross alteration of the structure. Alterations of G in the CMGCC region ([G to R, A or D] in mutagenesis) and those in the CAGYC region ([G to R or D] in mutagenesis and [G to R] in patients with isolated TSH deficiency) resulted in loss of immunological and/or biological activities of hTSH or hCG, indicating an important role of this amino acid.

Since the amino acids replaced are acidic (D), basic (R) and neutral (A), changes in the charge seem to be unlikly to cause loss of activities. It is interesting that replacement with even a non bulky amino acid (A) causes a loss of activity of the mutant hormones. The CMGCC (amino acid number in human common α-subunit 28–32) and the CAGYC (hTSHβ 27-31 ; hCGβ, hLHβ 34–38; hFSH 28–32) regions are not directly related to amino acid residues which are responsible for glycosylation (human common α 52, 78 (Asn); hTSHβ 23 (Asn); hCGβ 13, 30 (Asn), 120, 127, 132, 138 (Ser); hLHβ 30 (Asn); hFSH 7, 24 (Asn)) [1].

These regions are also different from a domain of hCGα 37–40 required for assembly with the hCGβ-subunit [6] and that of hCGα 88–92 relating to receptor binding and biological activities of hCG [7].

Our previous prediction analysis of the secondary structure by Robson's and Chou-Fasman's methods indicated that the CMGCC and CAGYC regions may form a tight β-turn structure [10, 12], but a recent study on the crystal structure of hCG by x-ray analysis revealed that these regions form a β sheet structure [15]. They possess a unique disulphide bridge, a "cystine-knot", which is found in hCGα 28 (CMGCC)-82 and β 34 (CAGYC)-88. The hCG α 32 (CMGCC) -84 and β 38 (CAGYC)-90 form usual disulphide bridges. The CMGCC region of the common α-subunit and the CAGYC region of the β-subunit are located near each other in the heterodimer. Substitutions of amino acid residues in these regions may result in considerable changes in the three-dimensional structure to form a non covalent assembly of the two subunits responsible for immunological and biological activities.

Table 1. Relationship between amino acid sequence of C-X-G-X-C motif (italic letters) and the activity of wild type and mutant glycoprotein hormones. Replacement of amino acid indicated by parentheses results in loss of immunological and/or biological activities

	Glycoprotein hormones	Amino acid sequences				
COMMON α-SUBUNIT						
wild type	Common α : h b c g m o p pg r	*C*	M	*G*	C	*C*
	: e	–	K	–	–	–
	: ee tu	–	V	–	–	–
	: f	–	S	–	–	–
	: ch q s(chu,masu) t	–	T	–	–	–
mutagenesis	TSH-α : h	–	R	–	–	–
	: h	*(Y)*	–	–	–	–
	: h	–	–	*(R)*	–	–
	: h	–	–	*(A)*	–	–
	: h	–	–	*(D)*	–	–
	CG-α : h	–	R	–	–	–
	: h	*(Y)*	–	–	–	–
	: h	–	–	*(R)*	–	–
	: h	–	–	*(A)*	–	–
	: h	–	–	*(D)*	–	–
β-SUBUNIT						
wild type	TSH-β : h b m p r	*C*	A	*G*	Y	*C*
	: f	–	S	–	–	–
	: q	–	–	–	F	–
	: ee tr	–	M	–	F	–
	FSH-β : h b e o p	–	–	–	–	–
	: r	–	E	–	–	–
	LH-β : h b e l o p r w	–	–	–	–	–
	: ch q	–	G	–	–	–
	: f	–	T	–	–	–
	CG-β : h e	–	–	–	–	–
	GTH-β : g(I) s(chu,masu)	–	–	–	L	–
	: bo(I) st(I) tu(I)	–	E	–	Q	–
	: k	–	S	–	–	–
	: bo(II) c ee g(II) s(chi) st(II) tu(II)	–	S	–	H	–
mutagenesis	TSH β : h	–	–	*(R)*	–	–
	CG β : h	–	D	–	–	–
	: h	–	–	*(R)*	–	–
	: h	–	–	*(D)*	–	–
patient	TSH β : h	–	–	*(R)*	–	–

h, human; b, bovine; bo, bonito; c, carp; ch, chicken; e, equine; ee, eel; f, frog; g, goldfish; k, killfish; l, lagomorph; m, mouse; o, ovine; p, porcine; pg, porgy; q, quail; r, rat; s, salmon (chi: chinook, chu: chum); st, striped bass; t, turkey; tr, trout; tu, tuna; w, whale; GTH, gonadotropin. The sequences were taken from the following sources: wild-type common α-[1, 3, 19–21], TSH β-[1, 22, 23], FSH β-[1, 22, 24], LH β-[1, 19, 22, 24], CG β-[1] and GTH β-subunit [19, 24, 25]; mutagenesis of TSH α-(the present study), CG α-[12], TSH β-[8] and CG β-subunit [11]; patients with mutation in TSH β-subunit gene [8, 9, 10].

These findings support our hypothesis presented previously that an amino acid motif of "C-X-G-X-C" in common α- and β-subunits plays an important role in the biosynthesis of glycoprotein hormones [10, 12]. The "C-X-G-X-C" motif has been found in various other biologically active proteins in humans such as low density lipoprotein receptor (amino acid 4253–4257; CAGYC) [16], C-

erb-B-2 (220–224; CAGGC) [17], and transforming growth factor (44–48: CAGAC) [18], etc. Further studies are required to elucidate the significance and mechanism of this motif.

Acknowledgements

We are grateful to Dr. J. S. Fiddes and Dr. Y. Hayasizaki for the gifts of the cDNAs of common α- and TSHβ-subunits, and to NIH for hTSHα-subunit and antibodies to hTSHα- and β-subunits respectively. Our thanks go to Mr M. Kondo and Mr Y. Izumiguchi (Osaka Kessei Research Laboratories Inc) and members of SRL for their technical help with the TSH assay.

We thank Mr T. Mashimo and Mr H. Goto (Nisho Corporation Research and Development Laboratory) and Dr. K. Ishibashi (Eiken Chemical Co. Ltd) for their helpful suggestions and encouragement, and Miss M. Kawaguchi and Miss M. Kondo for their secretarial assistance.

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