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Full Papers

Structure of the porcine thyrotropin receptor: a 200 kilodalton glycoprotein heterocomplex

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Summary. We have determined that the porcine thyroidal TSH receptor is a glycoprotein heterotetramer composed of two $M_r \sim 35{,}000$ (ε) covalently linked subunits which interact noncovalently with two copies of δ (M_r 66,000) chains.
Key words. Thyrotropin receptor; glycoprotein; δ and ε subunits; electrophoresis.

Introduction

Thyrotropin (TSH) is a glycoprotein hormone composed of two noncovalently associated subunits[31,32]. Interest in the structure of the TSH receptor is to a large measure related to the fact that the hyperthyroidism of Graves' disease, which has a prevalence of 1%, is associated with spontaneous antibodies against this receptor[14,26,37].

Many attempts have been made to elucidate the receptor structure[4–6,10,11,17,22,34,38,41]; none have yielded unequivocal evidence as to its molecular mass nor its organization. Estimates of molecular mass varied from 15,000 to 500,000 daltons[14]. One group[4] suggested that two $M_r \sim 50{,}000$ subunits linked by disulphide bonds made up the receptor, whereas Koizumi et al.[22] suggested that affinity purified bovine TSH receptor included M_r 38,000 and 66,000 subunits. The present study took advantage of the knowledge that the receptor was an acidic glycoprotein[6].

We report here that the porcine thyroidal TSH receptor is a glycoprotein heterotetramer. It is composed of two $M_r \sim 35{,}000$ (ε) covalently linked subunits which interact noncovalently with two copies of δ (M_r 66,000) chains.

Methods

Partial purification of TSH receptor

Partially purified plasma membranes were prepared from porcine thyroids as previously described[19]. Membrane pellets thus obtained were resuspended in 50 mM Tris-HCl pH 7.5, protein concentration adjusted to 3 mg/ml and homogenized with an equal volume of 0.2 M lithium-diiodosalicylate (LIS) in 50 mM Tris-HCl, pH 7.5. The homogenate was stirred for 1 h at 4 °C and the insoluble material was removed by centrifugation at 113,000 × g for 45 min at 4 °C. The supernatant was dialyzed against three changes of 20 mM Tris-HCl pH 8.0 at 4 °C and then centrifuged at 131,000 × g for 45 min. 20 g of thyroid tissue usually yielded 0.7–1.3 mg of solubilized membrane proteins. All steps of thyroid plasma membrane isolation were carried out in the presence of 2 mM phenylmethylsulfonylfluoride. 20–25 ml of membrane lysate were applied to a DEAE-Sephacel column (1.5 × 20 cm), bed volume 20 ml equilibrated with 20 mM Tris-HCl, pH 8.0. After washing the column with three bed volumes of equilibrating buffer, it was eluted with 30 mM Tris-HCl, 1 M NaCl, pH 8.0 until all the protein material, as determined by absorbance at 280 nm was removed. Fractions containing protein were pooled and desalted by filtration through Sephadex-G-25M (4 × 40 cm) column. Material from two such columns was applied to a new DEAE-Sephacel column which was then eluted with a 0.0–1.0 M linear gradient of NaCl in 20 mM Tris-HCl, pH 8.0 at a flow rate of 17 ml/h. Fractions collected at 10-min intervals were appropriately pooled and concentrated using cx-10 immersible millipore filter (Millipore Corp. Bedford, MA, USA). Pooled fractions were designated I–VIII (fig. 1a) and tested for their capacity to bind ^{125}I-bTSH.

In order to further resolve the fraction which contained this activity (peak VIII), 400 µg–3 mg of fraction VIII in 200 µl of 20 mM Tris-HCl pH 8.0 were applied to a

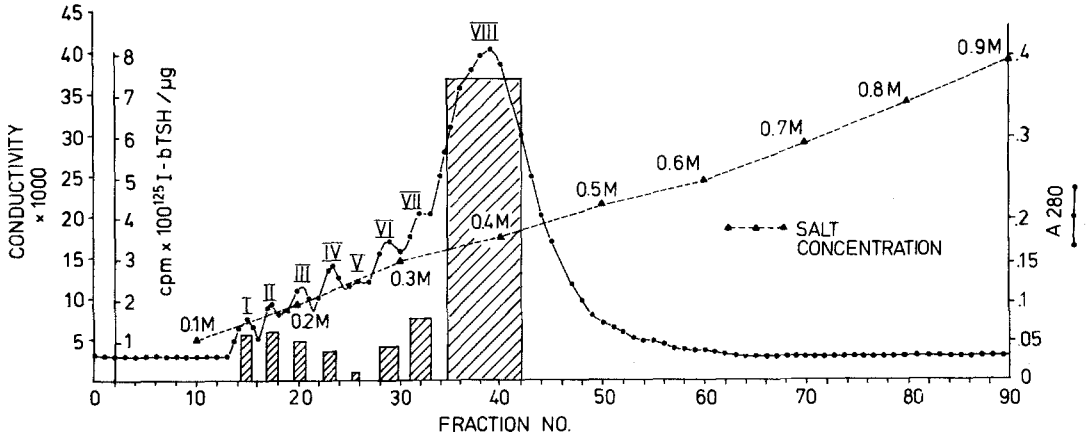

Figure 1a. Elution profile of thyroid membrane lysates from DEAE-Sephacel by NaCl gradient. The hatched bars depict ^{125}I-bTSH specifically bound per µg of protein as assessed in a radiometric assay. Peak VIII was the only peak with ^{125}I bTSH binding activity (742 cpm/µg of protein).

1.6 × 40 cm Sephadex G-200 column (bed volume 72 ml). The column was equilibrated with 20 mM Tris-HCl pH 8.0 and calibrated with MW standard proteins. The column was eluted with equilibration buffer at a rate of 6 ml/h. Two fractions were eluted (FR I and II). The high molecular weight fraction (FR I) eluted ahead of blue dextran; FR II eluted with Kav of 0.72 compared to 0.78 for ^{125}I-bTSH.

Affinity chromatography

The cross-linking of bTSH to Affigel-10 (Bio-rad, Richmond, CA) was previously described[19]. The TSH-Affigel conjugate was washed with 3 M NaCl and equilibrated with 40 mM Tris-HCl pH 7.4. 5 ml of the conjugate were packed into 0.9 × 15 cm column and incubated with 1.5 mg of peak VIII proteins for 16 h at 4 °C. The column was then washed with 40 mM Tris-HCl, pH 7.4 until absorbance (at 280 nm) dropped to baseline; proteins bound to the column were eluted with the same buffer containing 3 M NaCl. The eluates were desalted by dialysis against 50 mM Tris-HCl 2 M sucrose, pH 7.4 and sucrose concentration subsequently reduced to 0.25 M.

Polyacrylamide gel electrophoresis

100 µg of eluted proteins were boiled for 5 min in the presence of 2% sodium dodecylsulphate (SDS) with or without 2% β-mercaptoethanol (ME) and resolved on a 7.5–15% SDS slab linear gradient gel using Laemmli's discontinuous buffer system[23]. Following electrophoresis, proteins were visualized by staining by a sensitive silver staining method[42] or with Coomassie brilliant blue. Staining for glycoprotein bands was undertaken by three different methods which rely upon periodic oxidation of the vicinal hydroxy groups of glycoprotein carbohydrate moieties[12,13,15]. The following standards were used to estimate molecular weights: rabbit muscle myosin (M_r 205,000), phosphorylase b (M_r 94,000), bovine serum albumin (M_r 67,000), ovalbumin (M_r 43,000), carbonic anhydrase (M_r 30,000), soybean trypsin inhibitor (M_r 20,000) and α-lactalbumin (M_r 14,000).

Protein blotting

Proteins resolved on 7.5–15% SDS-PAGE in absence of reductant were electrotransferred onto nitrocellulose paper[3,40] for 14 h at 200 mA with a Hoffer Scientific instrument TE 42 Transphor-electrophoretic transfer unit. Individual lanes were cut and incubated with bTSH for 4 h at 4 °C, and the unreacted sites blocked by a mixture of 10% horse serum and 3% bovine serum albumin (BSA) in 50 mM Tris-HCl, 200 mM NaCl, pH 7.4 (TBS). After washing, the strips were incubated with rabbit anti-TSH antibody (U.B.S. Bioproducts, Brussels, Belgium) for 14 h at 4 °C. At the end of the incubation the nitrocellulose paper strips were washed several times and peroxidase conjugated species-specific anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) was added and incubated at room temperature for 4 h. After several washes in TBS the paper strips were developed with 4-chloro-1-napthol (Sigma, St. Louis, MO) and hydrogen peroxide for 15 min (fig. 3)[21].

Two-dimensional electrophoresis

Proteins of peak VIII were subjected to two-dimensional gel electrophoresis as by O'Farrell et al.[28] as well as in the absence of reductant or boiling. In the latter case, 20–30 µg of peak VIII proteins suspended in 50 µl Tris-HCl buffer containing 2% Ampholines, 15% sucrose and 3 M urea was applied to 2.5% polyacrylamide gel rods (0.28 × 13 cm) containing 2% Nonidet P-40 and 3 M urea. The gels containing 3.5–10.0 Ampholines were prefocussed for 1 h at 250 V and focussed at 1000 V for 4 h. The anode electrode buffer was 0.01 glutamic acid and the cathode buffer 0.01 M histidine. The focussed proteins were then resolved on 5–15% SDS polyacrylamide gels in the presence of 1% SDS in the absence of reductant. Receptor peptides could not be focussed in the absence of NP-40 or urea.

Peak VIII proteins were also dissolved in nonequilibrium pH gradient electrophoresis (NEPHAGE) sample buffer (9.2 M urea containing 2% Nonidet P-40 and 5% Ampholines) and focussed in NEPHAGE tube gels 0.28 × 13 cm containing pH 3.5–10 Ampholines[28]. An essentially linear pH gradient extending from 3.5 to 7.0 was obtained. After equilibrating the NEPHAGE gel with SDS-PAGE sample buffer, the polypeptides were resolved in the presence or absence of reductant as described above.

Radiostaining of receptor-rich fractions

10–20 µg of peak VIII fraction resolved on Sephadex G-200 were resuspended in 100 µl of 0.1 $NaHCO_3$, pH 8.3, and incubated with 0.25 mCi ^{125}I-Bolton Hunter reagent for 2 h at room temperature[2]. The reaction was stopped by adding 5 µl of 1 M glycine. Labeled proteins were further processed as described by Shing and Ruoho[36] except that 10% rather than 7.5% gels were used for SDS-PAGE. Dried gels were exposed to X-o mat (Kodak) at 4 °C for 24–48 h.

Radiometric assay for the receptor

Hormone binding activity of solubilized membranes and polypeptides resolved by column chromatography were assayed by a solid phase radiometric assay. Highly purified bTSH (30 mU/mg, supplied by Dr John Pierce) was iodinated by the lactoperoxidase method[39]. Briefly, 5–15 µg protein samples were dotted on 6 × 6 cm nitrocellulose paper grids. After blocking the unreacted sites with 3% BSA 0.05 pmoles ^{125}I-bTSH (2 × 10^5 cpm) were added in the absence or presence of increasing concentrations of native bTSH, insulin and human chorionic gonadotropin (to test for ligand specificity). Unbound ^{125}I-bTSH was removed by repeated washing with TBS. Counts bound in the presence of 500 mU of bTSH were taken as nonspecific and were ≤ 8% of bound counts. Characteristics of ^{125}I-bTSH binding to plasma membrane and peptide fraction were determined by the method of Scatchard[35]. Human chorionic gonadotropin and insulin cross-reacted to < 0.005% in the assay.

Results and discussion

The thyrotropin receptor was isolated from porcine thyroid plasma membranes. Throughout the purification procedure the ability of the material to bind ^{125}I-bTSH was determined by a solid-phase radiometric assay. 3.25 mg of receptor-rich glycoprotein was recovered from each 100-g batch of starting porcine thyroid tissue (fig. 1). TSH receptor or its breakdown products constituted > 65% of the final preparation.

The native form of TSH receptor is an $M_r \sim 200{,}000$ glycoprotein heterocomplex (fig. 2). Several lines of evidence lead to this conclusion 1) native TSH bound specifically to this polypeptide on protein blots of thyroid plasma membranes (fig. 3)[21]; a similar result was obtained with receptor-rich glycoprotein, although at high concentrations an additional $M_r \sim 65{,}000$–$71{,}000$ was also visualized, 2) polyclonal antibodies[19] known to interact with the TSH binding site and to trigger receptor-mediated adenylate cyclase activation bound exclusively to this band, 3) this polypeptide was one of several covalently cross-linked with ^{125}I-bTSH and was quantitativly displaced by native TSH but not by other related hormones[1], 4) when a crude glycoprotein extract of thyroid membranes was incubated in a TSH-affinity column, the $M_r \sim 200{,}000$ polypeptide was the predominant glycoprotein eluted (fig. 2). The fact that receptor

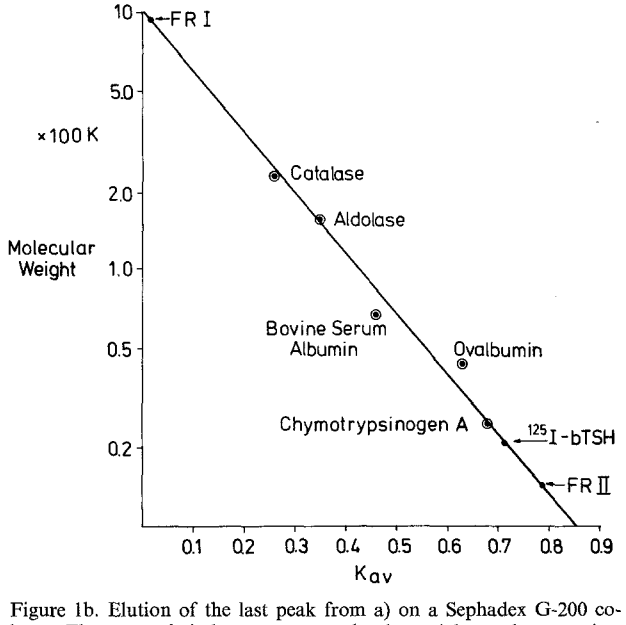

Figure 1b. Elution of the last peak from a) on a Sephadex G-200 column. The targeted circles represent molecular weight marker proteins. FR = fraction.

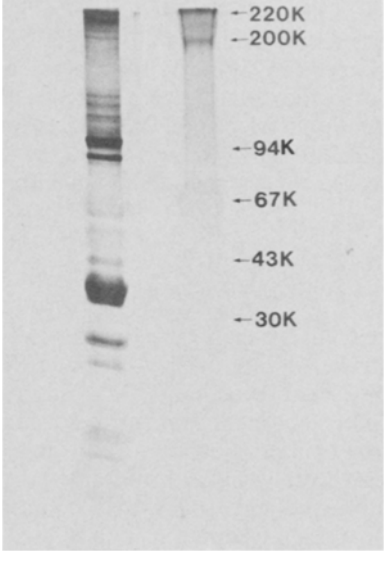

Figure 2. Analysis of material eluted off TSH affinity column The lane of the left shows the starting material applied to the column, whereas the peptides eluted off the affinity column is shown on the right, ^{125}I-bTSH binding activity increasing 4.2-fold as a result. Note the predominant M_r 197,000 peptide eluted. The peptides under study were resolved on a 7.5–15% SDS gradient gel in the absence of reductant. Protein bands were identified by the silver staining method described in Wray et al.[42].

activity determined by ^{125}I bTSH was only increased 390-fold in this preparation compared to crude membrane starting material reflects the extensive receptor inactivation during isolation steps[22]; by contrast, 0.084% protein content of starting material was recovered. In contrast to thyroid plasma membranes where two classes of ^{125}I-bTSH binding sites were found[35] one of high affinity ($K_D = 1.3 \times 10^{-10}$ M)[1, 19, 30, 33] and the other of a low affinity ($K_D = 2.5 \times 10^{-8}$ M), purified receptor preparation exhibited one high affinity binding site with $K_D = 3.4 \times 10^{-11}$ M (table). Although a number of explanations may be invoked to account for the low affinity site[11], recent data indicate that it is related to the high-affinity site by aggregation[27]. Moreover, we found that upon treatment with SDS, the high molecular weight fraction of the Sephadex G-200 eluate yield similar polypeptide bands to the low molecular weight fraction.

After the completion of this work, Iida et al.[18] estimated, by gel filtration, the size of ^{125}I-bTSH-human TSH receptor complex to be ~ 180,000. Receptor was purified by DEAE-Sephadex, concanavalin A and TSH-affinity chromatography in sequence to a degree comparable to that reported here.

Characteristics of ^{125}I-bTSH binding sites

	Solubilized membranes	Purified receptor
^{125}I-bTSH specifically bound cpm/μg protein	22	860
K_d (M^{-1})*	1.3×10^{-10}	3.4×10^{-11}
ID_{50} (mU)**	3	0.75
Binding capacity (pM/mg protein)	~ 1.0	4

*Value for high affinity binding site quoted. **The dose of bTSH in milliunits which cause 50% inhibition of ^{125}I-bTSH bound in absence of bTSH.

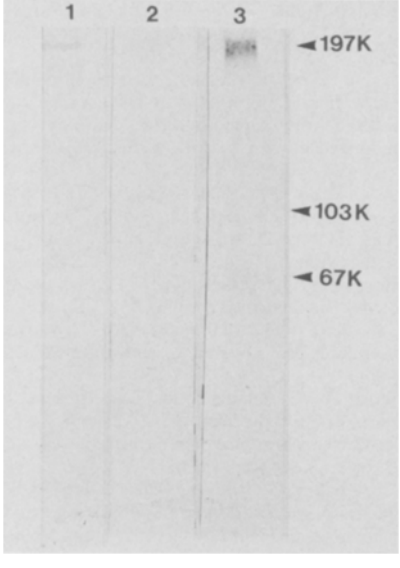

Figure 3. The thyrotropin holoreceptor (M_r ~ 197,000) band in 1) lithium diiodosalicylate-membrane lysates, 2) lysates dialyzed against 20 mm Tris-HCl, pH 8.0, 3) peak VIII of the DEAE-Sephacel eluate. The material was resolved on SDS-PAGE in the absence of reductant, subjected to protein blotting and receptor peptides identified by an enzyme linked immunoassay (see text). In presence of high concentration of peak VIII (100 μg), faint bands of lower molecular weight are identified.

The TSH holoreceptor is made up of two copies of the $M_r = 66{,}000$ (δ) heavy chain which interact noncovalently with two light (ε) chains of M_r ~ 35,000. The two ε chains are covalently linked. Two-dimensional gel electrophoresis in the presence of 3 M urea and 1% SDS, without boiling, results in two spots of M_r ~ 65,000–70,000 (fig. 4a). We also noted that receptor purified by TSH-affinity column is more likely to dissociate into its constituent parts in hypotonic compared to isotonic buffer systems. Analysis of radiostained receptor prepared in 20 mM Tris-HCl, pH 8.0 on a 7.5% SDS-PAGE nonreducing gel yields two bands of $M_r = 66{,}000$ and 70,000, respectively. Stochiometric ratios were based on the relative degree of radiostaining of the two receptor components and assumes similar abundance of free amino groups on both chains (fig. 4b). Under reducing conditions the M_r 70,000 chain is broken down to two similar (if not identical) subunits of M_r ~ 33,000–37,000. Two-dimensional electrophoresis by the technique of O'Farrell[28] indicated that δ has a pI of 6.5 and ε subunit a pI of 5.0 (fig. 4c). In contrast to 2:1 stochiometry of δ:(2ε) chains, the former stained less in the PAS-Schiff reaction[13] or by silver nitrate following periodic oxidation of the vicinal hydroxy[12] groups; both chains showed poor florescence with dansyl hydrazine[15]. The glycoprotein nature of the receptor is further confirmed by its retention on concanavalin A-Sepharose column and its elution with α-methyl D-mannoside[11]. Glycolipids were not detected in either of the two receptor chains, although the presence of minor glycolipids cannot be excluded. Both receptor chains, are exposed on thyroid follicular cell surface, in as much as they were both surface-labeled with ^{125}I-bTSH on viable thyrocytes[20].

The dissociation of noncovalently-linked chains, conformational changes consequent upon disulphide bond reduction and possibly endogenous proteolysis contribute to the additional electrophoretic bands ($M_r = 135{,}000$, $M_r = 103{,}000$, $M_r < 30{,}000$) in TSH receptor preparations (fig. 2). The M_r ~ 200,000, 135,000 and 103,000 bands resolved on SDS-PAGE in the absence of reductant were cut and re-electrophoresed after reduction in 5% ME. As a consequence only M_r ~ 66,000 and 35,000 bands were visualized.

TSH binding to thyroid plasma membrane is diminished by −S−S− bond reducing reagents[16, 29] and the occupancy of the receptor by TSH protects against the disruptive influence of sulfhydryl group reductants[1, 9]. These observations coupled with photoaffinity crosslinking of the receptor components by radiolabeled TSH[1] suggest that the locus of TSH binding is between the two ε subunits held by one or more disulphide bonds. Whether the δ chains function as affinity modulators or signal transducers[14], they generally become dissociated upon reduction of the inter-ε chain disulphide bond(s). If the human thyrotropin receptor is organized similar to the porcine receptor, spontaneously arising anti-receptor antibodies may exhibit different biological activities depending upon the receptor determinant with which they interact or on the predomi-

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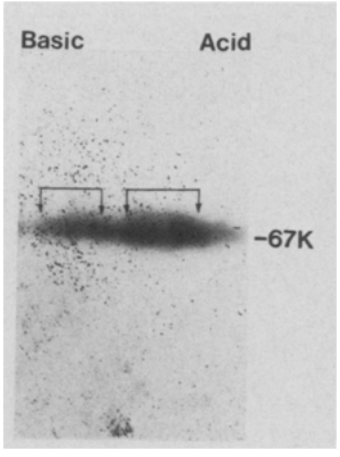
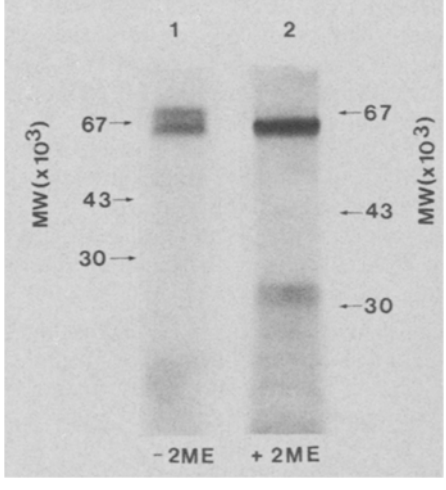
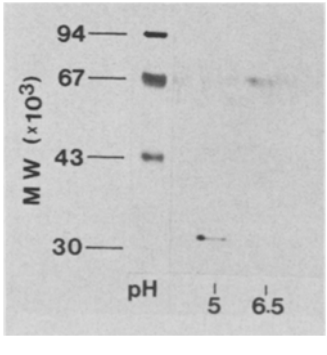
Figure 4. Characterization of the TSH receptor structure.



a) Two-dimensional gel electrophoresis in the absence of reductant or sample boiling. The gel (5–15%) was stained with Coommassie blue. Two confluent spots are visualized, the more basic of which has an $M_r \sim 66{,}000$ whereas the acidic spot has an $M_r \sim 70{,}000$ (arrows). The range of M.W. markers noted in the text were used to estimate M_r of the peptides; we show here only the position of bovine serum albumin (M_r 67,000). The bracketed protein migrate in O'Farrell's gel[28] as 35,000 and 66,000 peptides (see 4c).



b) Radiostaining of Sephadex G-200 FR II resolved on a 10% gel. In the absence of reductant, two bands ($M_r = 70{,}000$ and 66,000, respectively) were visualized. In the presence of 2-mercaptoethanol, the 66,000 band was again seen; the $M_r = 70{,}000$ was, however, reduced to $M_r = 33{,}000$–$36{,}000$ polypeptide.



c) Two-dimensional gel electrophoresis of peak VIII by the technique of O'Farrell et al.[28]. The protein spots were visualized by silver nitrate. The $M_r = 66{,}000$ spot has a PI of 6.5 and $M_r = 35{,}000$ spot a PI of 5.0.

nance of one over the other determinant-specific antibody[14].

Until recently, no attempts were made to study the organization of partially purified TSH receptor preparations. Notwithstanding possible species variation, our results are compatible with the observations that the bovine TSH receptor involves two glycoproteins of $\sim 70{,}000$[6] and that it includes M_r 38,000 and M_r 66,000 subunits[22]. Using photoaffinity cross-linking techniques two M_r 50,000 covalently linked subunits[4] as well as a complex involving M_r 60,000 and M_r 45,000 subunits were proposed for receptor structure[27]. Caution must, however, be exercised in deducing the structure of a multimeric glycoprotein receptor for TSH both of whose glycoprotein subunits are radiolabeled[1].

TSH is one of a family of glycoprotein hormones which share a common α subunit and mediate, at least some of their biological actions, through the activation of adenylate cyclase[31, 32]. Although minimal cross-reactions of these hormones with each other's receptor suggested evolutionary divergence of receptors[8], recent structural evidence may argue otherwise. The LH-HCG receptor was reported to be composed of two identical units of M_r 120,000–140,000 each of which contains subunits of $M_r = 85{,}000$ and 38,000 apparently linked by disulphide bonds[7]. Variation in presence, number and position of disulphide bonds upon a parent holoreceptor have been described for the receptor for insulin and insulin-like growth factors[24, 25].

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