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Photoaffinity labelling of the TSH receptor on $FRTL_5$ cells

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An investigation of the properties of TSH receptors on $FRTL_5$ cells using affinity labelling with a ^{125}I-labelled photoactive derivative of TSH is described. Our studies suggest that $FRTL_5$ cells contain 2 principal types of cell surface TSH receptors. One form, probably a precursor, consists of a single polypeptide chain (M_r 120000) with an intrachain loop of amino acids formed by a disulphide bridge. The other type of receptor consists of a water-soluble A chain (M_r 55000) linked to an amphiphilic B chain (M_r 35000) by a disulphide bridge. The 2 chain structure is probably derived from the single chain 120000 protein by enzymatic cleavage of peptide sequences within the loop of amino acids formed by the intrachain disulphide bridge.

Thyroid; Thyrotropin; TSH receptor; ($FRTL_5$ cell)

1. INTRODUCTION

The continuous line of functioning rat thyroid cells ($FRTL_5$) developed by Ambesi-Impiombato and his colleagues [1] have proved to be a most useful model for investigating the properties of thyroid cells. In particular the cells retain most of the features of the thyroid gland in vivo, including responsiveness to TSH and TSH receptor autoantibodies [2]. We now describe an investigation of the properties of TSH receptors on $FRTL_5$ cells using affinity labelling with a ^{125}I-labelled photoactive derivative of TSH. Our studies suggest that $FRTL_5$ cells contain 2 principal types of cell surface TSH receptors. One form, probably a precursor, consists of a single polypeptide chain M_r 120000, with an intrachain loop of amino acids, formed by a disulphide bridge. The other type of receptor consists of a water soluble A chain (M_r 55000) linked to an amphiphilic B chain (M_r 35000) by a disulphide bridge. The 2 chain structure is probably derived from the single chain 120000 protein by enzymatic cleavage of peptide sequences within the loop of amino acids formed by the intrachain disulphide bridge.

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2. MATERIALS AND METHODS

2.1. *Culture of $FRTL_5$ cells*

$FRTL_5$ cells were grown in Coon's modified Hank's F12 medium (Imperial Laboratories) containing 5% newborn calf serum (Gibco) as well as a mixture of TSH, insulin, hydrocortisone, transferrin, glycyl-L-lysine acetate and somatostatin (defined as 6 hormone medium, 6H) for 14 days under 5% CO_2 in air in a water-saturated incubator [1,2]. After 14 days growth in 6H medium, the cells were either (i) detached from their plastic supports using a mixture of trypsin, collagenase and chicken serum [1], washed, resuspended in 6H medium and recultured or (ii) 6H medium was replaced with 5H medium (6H medium without TSH) and culture continued for 4 days.

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In the case of cultures in 6H medium followed by 5H medium, the cells were washed twice in Earle's balanced salt solution (EBSS) without phenol red (Gibco) and then detached from the flasks using 3 mM EGTA in 20 mM Hepes, pH 7.3. The cell suspensions were then washed once again with EBSS before further analysis. Cell viability was assessed by the method of Parks et al. [3] and found to be approx. 80%.

In one series of experiments involving several passages, a slow growing line of cells with different morphology developed. Membranes prepared from these cells did not bind ^{125}I-labelled TSH (see below) but they were used as an additional control in photoaffinity labelling studies.

2.2. *TSH preparations*

TSH was prepared from bovine pituitaries using described procedures [4,5]. The purified material contained 70–80 units (MRC bovine TSH standard) per mg of protein as judged by TSH receptor assay [5] and bioassay [6].

2.3. *Preparation of ^{125}I-labelled TSH for receptor binding studies*

Highly purified bovine TSH (70–80 MRC units per mg of protein) was labelled with ^{125}I to a specific activity of 50 μCi per μg by the Iodogen method [5,7]. After receptor purification, the material was stored for up to 2 weeks at −70°C.

2.4. *Receptor binding with ^{125}I-labelled TSH*

FRTL$_5$ cells were grown in 6H medium for 14 days and then in 5H medium for 4 days before being detached from the culture flasks with 3 mM EGTA. After suspension (20×10^6 per ml) in EBSS, aliquots of the cells were treated with trypsin (0–0.1%) for 15 min at room temperature followed by a 2-fold excess (w/w) of trypsin inhibitor (10 min at room temperature). After washing in EBSS, a crude membrane fraction was prepared from homogenates of the cells (see section 2.7) and suspended in 50 mM NaCl; 10 mM Tris-HCl, pH 7.5; 1 mg^{-1} bovine serum albumin (membranes from 20×10^6 cells per ml of suspension buffer). ^{125}I-labelled TSH binding was then carried out by incubating triplicate 100 μl aliquots of the membrane suspension (membranes from 2×10^6 cells per 100 μl) with 100 μl highly purified unlabelled TSH (0–0.5 μg) and 100 μl of receptor purified ^{125}I-labelled TSH (5000 cpm) for 1 h at 37°C. Bound and free labelled TSH were then separated by addition of 500 μl of ice-cold suspension buffer and centrifugation at 15000 × g for 20 min.

2.5. *Preparation of ^{125}I-HSAB-TSH for crosslinking studies*

Highly purified bovine TSH was coupled to the photoactive hetero-bifunctional reagent *N*-hydroxysuccinimidyl-4-azidobenzoate (HSAB) as described [8]. After labelling with ^{125}I to a specific activity of 50 μCi per μg by the Iodogen method [5,7], the material (^{125}I-HSAB-TSH) was purified by gel filtration [5] and stored at −70°C for up to 2 weeks.

2.6. *Crosslinking of ^{125}I-HSAB-TSH to TSH receptors on intact FRTL$_5$ cells*

After washing in EBSS, the cells (20×10^6) were suspended in 1 ml of EBSS without phenol red and incubated in the dark at 37°C, with an equal volume of ^{125}I-HSAB-TSH (20 μCi) for 20 min. In some experiments, unlabelled bovine TSH (5 μg; 350 mU) was added to the cell suspension 5 min prior to addition of ^{125}I-HSAB-TSH. The material was then photolysed for 15 min, 14 cm from a 250 W UV lamp to effect crosslinking and the cells washed twice in 20 ml of EBSS.

2.7. *Preparation of FRTL$_5$ cell membranes*

The affinity labelled cells (or in some cases untreated cells) were first homogenized in 10 mM Tris-HCl, pH 7.5, containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF) and 15 mM iodoacetamide. A crude membrane fraction was then sedimented by centrifugation at 14000 × g for 10 min at 4°C and used for further analysis. In some experiments, a crude membrane fraction was also prepared from homogenates of normal Fisher rat thyroid tissue.

2.8. *Solubilization of crosslinked TSH-TSH receptor complexes with sodium deoxycholate*

Membranes containing TSH receptors crosslinked to ^{125}I-HSAB-TSH were homogenized in 1% sodium deoxycholate (DOC) in 50 mM NaCl; 10 mM Tris-HCl, pH 8.3, containing 0.1 mM PMSF (50 μl of 1% DOC per membrane pellet obtained from 20×10^6 cells) and incubated at room

temperature for 30 min. After centrifugation (100000 × g, 1 h, 4°C) the supernatants were collected and analysed by SDS-PAGE and autoradiography as described below.

2.9. *Reduction of membrane bound TSH-TSH receptor complexes with dithiothreitol*

Cell membranes containing TSH receptors crosslinked to ^{125}I-HSAB-TSH were homogenized in 50 mM NaCl; 10 mM Tris-HCl, pH 8.3, containing 0.1 mM PMSF and 10 mM dithiothreitol (DTT) (50 μl per membrane pellet obtained from 20×10^6 cells) and incubated for 30 min at room temperature [9,10]. Parallel experiments were carried out in which the membrane preparations were homogenized in buffer without DTT. The homogenates were then centrifuged at 100000 × g for 1 h at 4°C and the supernatants treated with a 5-fold molar excess of iodoacetamide prior to analysis by SDS-PAGE and autoradiography.

2.10. *Crosslinking to membrane preparations*

In some experiments, crude membrane fractions were prepared from $FRTL_5$ cells (20×10^6) or normal Fisher rat thyroid tissue (250 mg) and then affinity labelled with ^{125}I-HSAB-TSH (20 μCi). After washing with suspension buffer (2 × 1 ml), containing 0.1 mM PMSF, the final pellets were treated with DTT or DOC in the same way as membrane pellets from affinity labelling of intact $FRTL_5$ cells (see above).

2.11. *Gel electrophoresis and autoradiography*

Samples for analysis by SDS-PAGE were diluted in an equal volume of electrophoresis sample buffer (4% SDS in Tris-HCl buffer, pH 6.8, containing 20% glycerol) with or without 10 mM DTT and analysed on 9% polyacrylamide gels [8–11]. After electrophoresis gels were stained, fixed and set for autoradiography using Kodak X-OMAT film and Cronex Lighting Plus enhancing screens. The following M_r markers were run on each gel: myosin (M_r 205000), β-galactosidase (116000), phosphorylase *b* (97000), BSA (67000), immunoglobulin G and its component heavy and light chains (150000, 50000 and 23000, respectively) and ovalbumin (44000). Analysis under reducing and non-reducing conditions used reduced and non-reduced standard proteins, respectively.

3. RESULTS

Trypsin treatment of $FRTL_5$ cell suspensions did not affect their viability as judged by ethidium bromide exclusion. However, specific ^{125}I-TSH binding to the membranes prepared from trypsin-treated cells was markedly diminished, from 12% of counts added in the case of membranes from untreated cells to 3.8% with 0.01% trypsin and 2.5% with 0.1% trypsin. Scatchard analysis [12] of TSH binding to membranes prepared from non-trypsin-treated $FRTL_5$ cells was carried out and showed linear plots (fig.1). Cells grown in 6H medium followed by 5H medium gave membrane preparations with an association constant of 3.7×10^9 M^{-1} and binding capacity of 11×10^3 sites per cell. Cells grown in 6H medium only gave membrane preparations with a similar association con-

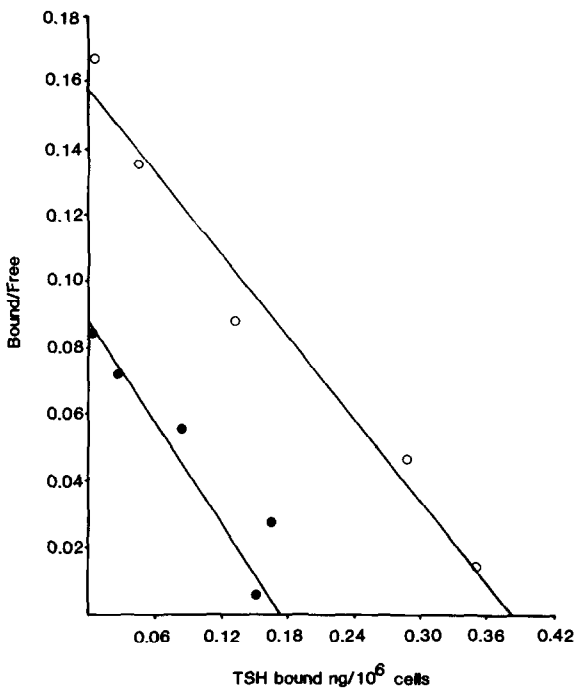

Fig.1. Scatchard analysis of TSH binding to membranes prepared from $FRTL_5$ cells grown in 6H medium only (●) or 6H medium followed by 5H medium (○). See text for experimental details. Data for each point were obtained using membranes from 2×10^6 cells (in duplicate). Non-specific binding was determined using 0.15 μg (10 mU) of unlabelled TSH. The data shown are typical of 3 separate experiments.

stant (4×10^9 M^{-1}) but markedly lower binding capacity (4×10^3 sites per cell).

When TSH receptors on $FRTL_5$ cells were affinity labelled with ^{125}I-HSAB-TSH and the membranes from these cells treated with DTT, analysis by SDS-PAGE and autoradiography showed the presence of a major labelled band at 80 kDa (fig.2). Additional labelled bands at 28 and 14 kDa corresponding to intact TSH and TSH subunits respectively were also present. The 80 kDa band was not observed when $FRTL_5$ cells were incubated with 5 μg (350 mU) of unlabelled TSH prior to addition of ^{125}I-labelled HSAB-TSH (fig.2a) or when membranes from affinity labelled cells (in the absence of unlabelled TSH) were homogenized in buffer rather than DTT (fig.2b).

SDS-PAGE analysis of DOC solubilized membranes from affinity labelled $FRTL_5$ cells under non-reducing conditions is shown in fig.3a. In addition to TSH, major bands at 120 kDa and about 150 kDa were observed and a weak band at 80 kDa. Under reducing conditions, the 120 kDa band was not seen, but clear labelled bands were present at 140 and 85 kDa (fig.3b). The formation of the non-TSH bands was not observed in DOC solubilized membranes prepared from cells incubated with 5 μg/350 mU of unlabelled TSH prior to ^{125}I-labelled HSAB-TSH (not shown). Furthermore, no bands additional to TSH were observed in crosslinking experiments with mutant

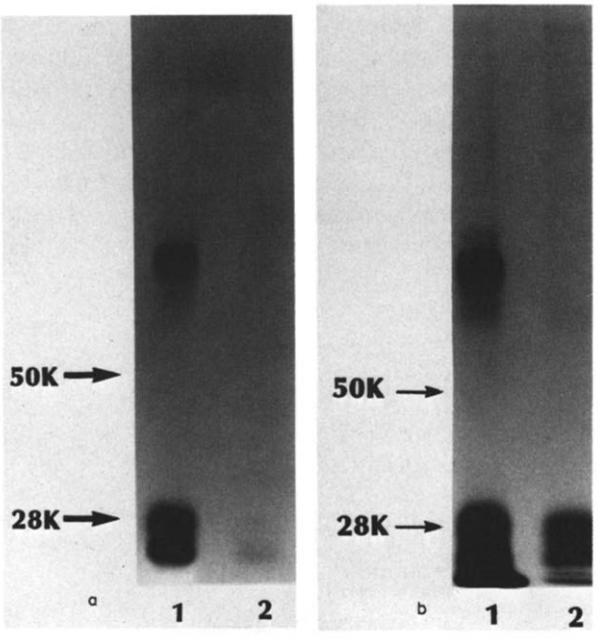

Fig.2. Effect of DTT on membranes prepared from affinity labelled $FRTL_5$ cells. (a) Lanes: 1, membranes treated with DTT, centrifuged (100000 × *g*) and supernatant analysed by SDS-PAGE and autoradiography; 2, as lane 1 except cells incubated with an excess of unlabelled TSH prior to addition of ^{125}I-HSAB-TSH and photolysis. (b) Lane 1: as lane 1 in a. Lane 2: as lane 1 in a except DTT omitted from buffer used to homogenize membranes. The material applied to each lane was derived from 10×10^6 cells.

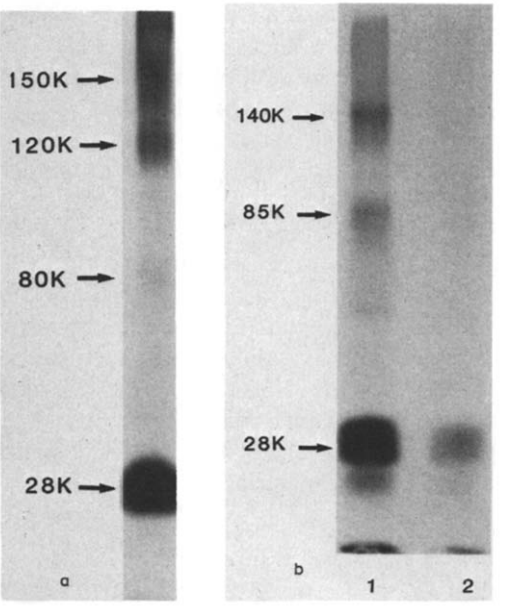

Fig.3. Analysis of DOC solubilized membranes prepared from affinity labelled $FRTL_5$ cells. (a) SDS-PAGE under non-reducing conditions followed by autoradiography. The bands at 150, 120 and 80 kDa were not detected in experiments in which cells were incubated with an excess of unlabelled TSH prior to addition of ^{125}I-HSAB-TSH and photolysis. (b) SDS-PAGE under reducing conditions followed by autoradiography. Lanes: 1, DOC solubilized membranes from affinity labelled $FRTL_5$ cells; 2, DOC solubilized membranes from affinity labelled mutant $FRTL_5$ cells which did not have detectable TSH receptors (see text for details). The 140 and 85 kDa bands shown in lane 1 were not detected in experiments in which cells were incubated with an excess of unlabelled TSH prior to addition of ^{125}I-HSAB-TSH and photolysis; the results being very similar to that shown in lane 2. The material applied to each lane was derived from 10×10^6 cells.

$FRTL_5$ cells which did not have detectable TSH receptors.

A summary of the data obtained by SDS-PAGE and autoradiography is given in table 1.

In an additional series of experiments membranes were isolated from $FRTL_5$ cells and then affinity labelled with ^{125}I-HSAB-TSH. Analysis by SDS-PAGE and autoradiography of the supernatants obtained after treatment with DTT or DOC and centrifugation gave similar results to those obtained when affinity labelling was carried out using intact cells (table 1). However, the bands were less intense. Studies with membranes prepared from normal Fisher rat thyroid tissue gave even weaker bands but in one experiment release of 80 kDa material by DTT could be seen clearly (not shown).

4. DISCUSSION

Trypsin treatment of $FRTL_5$ cells did not influence cell viability but markedly diminished the ability of membranes prepared from the cells to bind labelled TSH. This indicated that the cell's TSH receptors were principally present on the cell surface. Scatchard analysis [12] of membranes prepared from $FRTL_5$ cells suggested the presence of a single population of low capacity high-affinity TSH binding sites. The number of binding sites was markedly increased in membranes prepared from cells cultured for 4 days in the absence of TSH as reported [13]. Consequently, our results support the conclusion of this report that TSH down-regulates its own receptor in $FRTL_5$ cells.

When TSH receptors on $FRTL_5$ cells were affinity labelled with ^{125}I-labelled HSAB-TSH and treated with the reducing agent DTT in the absence of detergent, a water-soluble component (M_r 80000) of the TSH receptor crosslinked to TSH was released. Similar results have been observed after DTT treatment of affinity labelled TSH receptors in membranes prepared from human, porcine and guinea pig thyroid tissue and from guinea pig fat [8–10,14,15]. Consequently, our data indicate that the $FRTL_5$ cell TSH receptor contains a water-soluble subunit (defined as the A subunit) with M_r 50000–55000 (after subtraction of M_r 28000 for TSH from M_r 80000 for the A subunit TSH complex). Our studies also indicated that TSH receptors in normal Fisher rat thyroid membranes contained a water-soluble A subunit with M_r 50000–55000.

The results obtained with DOC solubilized, affinity labelled, $FRTL_5$ cell TSH receptors however showed some differences from those observed with porcine or human thyroidal TSH receptors

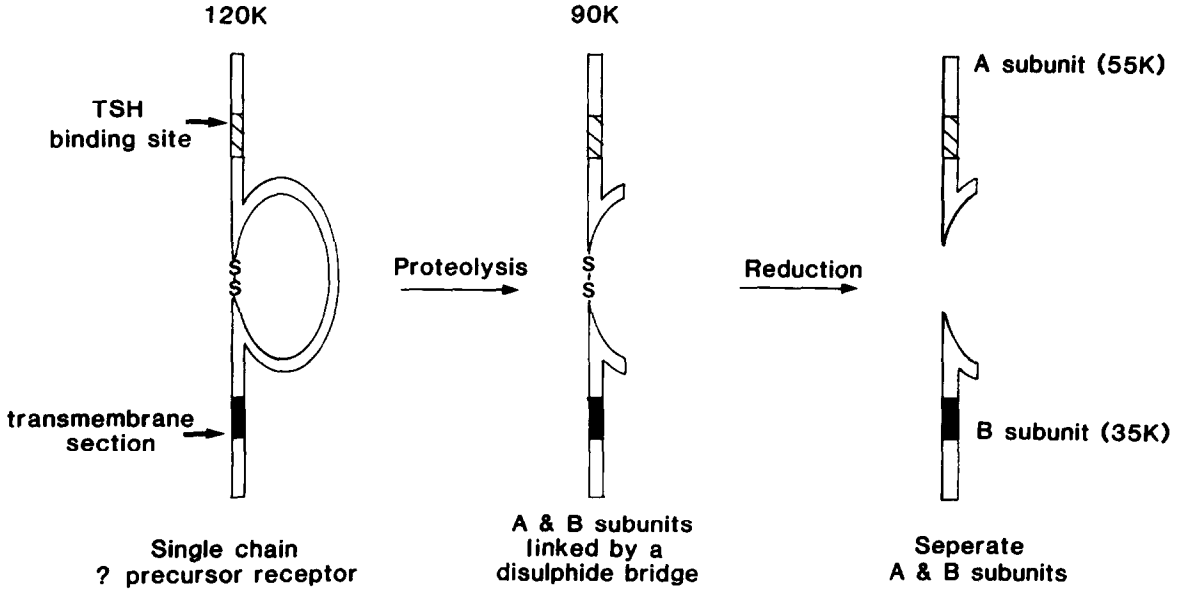

Fig.4. Proposed relationship between different forms of the $FRTL_5$ TSH receptor.

[8–10,14,15]; in particuar the presence of a major broad band at M_r 150000 in addition to a major band at M_r 120000 and minor band at M_r 80000 under non-reducing conditions (fig.3). The band of M_r 150000 showed only a minor change in M_r value (to 140000) on reduction indicating that it did not contain any major subunits linked by disulphide bridges. The band of M_r 120000 showed a marked change in M_r value (to 85000) on reduction and the material ran on the gels essentially as the A subunit of the receptor crosslinked to TSH (M_r 80000). The slightly higher M_r of A subunit-TSH complex derived from DOC solubilized membranes (86000 ± 4000) compared to complex prepared by treatment of membranes by DTT in the absence of DOC (80000 ± 4000) was consistent. This difference could have been due to continued association of some DOC micelles with the A subunit-TSH complex during SDS-PAGE in the case of studies with DOC solubilized preparations. The intensity of the minor band at 80 kDa under non-reducing conditions was markedly increased if iodoacetamide was omitted from the homogenization buffers, suggesting that this 80 kDa band could possibly be formed by the actions of an endogenous reducing system on 120 kDa material.

Overall, our observations suggest that the 120 kDa band shown in fig.3a consists of:

(i) TSH (M_r 28000) crosslinked to the A subunit of the TSH receptor; and
(ii) an amphiphilic B subunit (M_r 35000) which does not bind TSH, but is linked to the A subunit by a disulphide bridge. The B subunit must also contain the trans-membrane section of the 120 kDa band.

It seems likely that the TSH receptor component of the 150 kDa band is a precursor of the A and B subunit form present in the 120 kDa band as shown in fig.4. We can propose that the precursor form of the receptor consists of a single peptide chain (M_r 120000) with a loop of amino acids formed by a disulphide bridge (fig.4). Proteolytic cleavage of peptide bonds within the loop of amino acids then gives rise to a form of the receptor in which 2 subunits (A and B) are linked by the disulphide bridge which originally formed the loop (fig.4). Reduction of the disulphide bridge then allows release of the water-soluble A subunit. This type of processing is well established in the cases of several proteins including insulin and influenza virus hemagglutinin [16]. Any differences in affinity between the 'precursor' and 'processed' forms of the receptor were not evident from Scatchard analysis of TSH binding data (fig.1).

The single chain precursor form of the receptor was not detected in previous studies of crosslinked

Table 1

Summary of results obtained by SDS-PAGE analysis of affinity labelled TSH receptors

Method used to treat affinity labelled preparations	Major labelled bands observed on SDS-PAGE additional to TSH (mean M_r ± SD)					
	Bands of M_r 150000		Bands of M_r 120000		Bands of M_r 80000	
	Crosslinking to intact cells	Crosslinking to membrane preparations	Crosslinking to intact cells	Crosslinking to membrane preparations	Crosslinking to intact cells	Crosslinking to membrane preparations
DTT in the absence of detergent	ND	ND	ND	ND	80000 ± 4000 (n = 5)	80000 (n = 1)
DOC in the absence of DTT	154000 ± 3000 (n = 4)	150000 (n = 1)	119000 ± 2000 (n = 4)	119000 (n = 1)	minor band at M_r 85000 (n = 4)	minor band at M_r 86000 (n = 1)
DOC followed by DTT	139000 ± 10000 (n = 6)	137000 (n = 1)	ND	ND	86000 ± 4000 (n = 6)	86000 (n = 1)

ND, not detected; n, number of separate experiments

TSH-TSH receptor complexes prepared from human, porcine and guinea pig thyroid or guinea pig fat cell membranes [8–10,14,15]. However, it is likely that the 2-chain structure observed in these tissues is synthesised initially as a single polypeptide chain precursor molecule of the type shown in fig.4 [16].

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