A structure for the porcine TSH receptor

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Affinity purified, detergent-solubilised porcine TSH receptors have been crosslinked to a ^{125}I-labelled photoactive derivative of TSH and analysed by gel electrophoresis, gel filtration and sucrose density gradient centrifugation. Our studies suggest that the porcine TSH receptor is made up of a hydrophilic A subunit with an M_r of about 45000 linked to an amphiphilic B subunit (M_r approx. 25000) by a disulphide bridge(s). The A subunit forms the binding site for TSH on the outside of the cell membrane. The B subunit appears to penetrate the membrane and form the site for interaction with adenylate cyclase either in the lipid bilayer or close to the cytoplasmic surface of the membrane.

Thyrotropin *Receptor* *Thyroid* *Graves' disease* *Affinity labelling* *Crosslinking*

1. INTRODUCTION

Recent studies have shown that the TSH receptor can be covalently crosslinked to ^{125}I-labelled TSH and the crosslinked complex used to characterise the receptor [1,2]. We now describe an analysis of the porcine TSH receptor using this approach in combination with polyacrylamide gel electrophoresis in SDS (SDS–PAGE), sucrose density gradient centrifugation and gel filtration. Our studies suggest that the porcine TSH receptor is made up of a hydrophilic A-subunit with M_r of about 45000 linked to an amphiphilic B-subunit (M_r approx. 25000) by a disulphide bridge(s). The A-subunit forms the binding site for TSH on the outside of the cell membrane. The B-subunit appears to penetrate the membrane and form the site for interaction with adenylate cyclase either in the lipid bilayer or close to the cytoplasmic surface of the membrane.

2. MATERIALS AND METHODS

2.1. *Preparation and SDS–PAGE analysis of crosslinked TSH–TSH receptor complexes*

Highly purified bovine TSH (70 units per mg) was coupled to the photoactive crosslinking reagent *N*-hydroxysuccinimidyl-4-azidobenzoate (HSAB) and labelled with ^{125}I [1,2]. This material contained similar amounts of ^{125}I and HSAB on each subunit of TSH [1,2].

Affinity purified Lubrol solubilised [3] porcine TSH receptors (5 ml) were incubated in the dark for 3 h at room temperature with 5 ml (200 μCi) of ^{125}I-labelled HSAB-TSH, photolysed (254 nm; 20 min) and run at 2–4°C on a 250-ml column of Sephacryl S-300 in 50 mM NaCl; 10 mM Tris–HCl, pH 7.5 (Tris–NaCl) containing 0.1% Lubrol 12A9 (ICI Organics Division) to remove free TSH and small amounts of aggregate [7]. Formation of the complex was completely inhibited by an excess (100 mU) of unlabelled TSH.

The partially purified crosslinked TSH–TSH receptor complex was stored at 0°C for up to 4 weeks. Most of the ^{125}I-HSAB-TSH was crosslinked to the receptor as shown by the relatively small amount of free ^{125}I-HSAB-TSH observed on SDS–PAGE (fig.1). Furthermore, when the crosslinked complex was rerun on the S-300 column in the presence of 100 mU of unlabelled TSH only 10% of the eluted radioactivity corresponded to free ^{125}I-HSAB-TSH.

SDS–PAGE and autoradiography were carried out as in [1,2] using 8–12% gradient gels and a

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water-cooled Biorad apparatus. In some cases 10 mM DTT (final concentration) was used to reduce the crosslinked complexes prior to electrophoresis. Warming of samples in SDS prior to electrophoresis caused dissociation of TSH subunits and a more complex pattern [1,2] on the gels. Consequently, care was taken to apply samples to the gel immediately after preparation. This resulted in more precise M_r determinations than reported previously [1].

2.2. *Analysis of crosslinked TSH–TSH receptor complexes by sucrose density gradient centrifugation*

Crosslinked complexes (1-ml aliquots) were run (150 min; 300000 × *g*; 4°C) on linear sucrose gradients (5–20%, w/v; 17 ml) using a vertical rotor. Marker proteins were analysed under the same conditions in the same run. After each run, tubes were emptied from the bottom and fractions of about 0.5 ml were collected into tubes of known weight. After monitoring for ^{125}I the fractions were weighed, absorbance at 280 nm and refractive index determined and fraction volume calculated.

2.3. *Release of the TSH receptor A-subunit crosslinked to labelled TSH from thyroid membranes by treatment with DTT*

A crude membrane preparation was obtained from 5 g porcine thyroid tissue by differential centrifugation [3,7] and suspended in 5 ml of Tris–NaCl. Aliquots (500 μl) were incubated with unlabelled TSH (10 μl; 0–0.5 units) for 15 min at 20°C followed by ^{125}I-labelled HSAB-TSH (500 μl) for 1 h at 37°C. After photolysis (254 nm for 20 min) and centrifugation (5 min; 14000 × *g*; 4°C) the membranes were washed 3 times with Tris–NaCl (1 ml). The final pellets were resuspended in 100 μl of Tris–NaCl or Tris–NaCl containing 10 mM DTT. After incubation for 1 h at room temperature, the suspensions were centrifuged (100000 × *g*; 1 h; 4°C) and the supernatants analysed by SDS–PAGE or gel filtration on Sephacryl S-300 (250 ml) in Tris–NaCl without Lubrol.

3. RESULTS AND DISCUSSION

Analysis by SDS–PAGE showed that under non-reducing conditions, the crosslinked TSH–TSH receptor complex had an M_r of about 100000 and after subtraction of 28000 for the M_r of TSH [4] this suggested that the receptor itself had an M_r of about 70000. After mild reduction however, the M_r of the crosslinked complex was about 73000 giving an M_r for the reduced receptor of about 45000. Consequently, mild reduction resulted in the loss from the receptor of a subunit of M_r approx. 25000 which was not crosslinked to TSH. These results suggested that the TSH receptor contained 2 subunits linked by a disulphide bridge(s). One subunit (defined as the A-subunit) had an M_r of about 45000 and was crosslinked to TSH, the other (defined as the B-subunit) had an M_r of about 25000 and was not crosslinked to TSH.

Gels run under non-reducing conditions showed the presence of some aggregate material (which did not penetrate the gradient gel) and a diffuse background at higher M_r (fig.1). These effects were markedly less in reduced samples but there was some evidence of a small amount of incompletely reduced crosslinked complex at about 100 K and TSH subunits at low M_r. The diffuse background may have been due to ^{125}I-HSAB-TSH crosslinked non-specifically to lipid or detergent.

Other subunits of the receptor, not crosslinked to TSH, may have been associated non-covalently with the A- or B-subunits but these were not evident when additional crosslinking was attempted by adding disuccinimidyl suberate (DSS; 0.5 and 1 mM final concentration) to affinity purified porcine TSH receptors crosslinked to ^{125}I-HSAB-TSH (not shown). However, it was possible that any additional subunits were not accessible to crosslinking with DSS.

When the partially purified crosslinked complexes were analysed in 0.1% Lubrol by sucrose density gradient centrifugation (fig.2) the crosslinked material sedimented at a rate slightly slower than catalase (11 S; M_r 240000) suggesting a structure considerably larger than the M_r of about 100000 observed on SDS gels (fig.1). However, the association of a large Lubrol micelle (M_r approx. 100000) [5] with the receptor during centrifugation would account for the apparent discrepancy.

Treatment of crosslinked complexes with 10 mM dithiothreitol (DTT) prior to centrifuga-

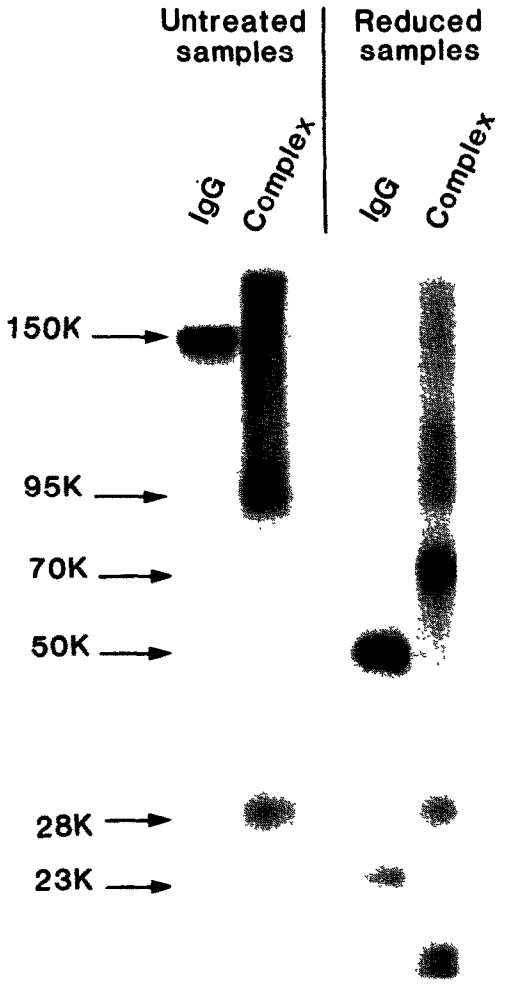

Fig.1. Analysis of crosslinked TSH–TSH receptor complex by SDS–PAGE (see text for experimental details).

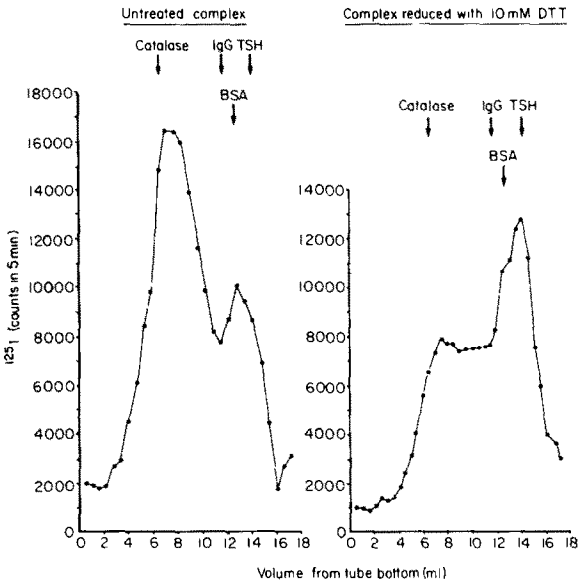

Fig.2. Analysis of crosslinked TSH–TSH receptor complex by sucrose density gradient centrifugation (see text for experimental data).

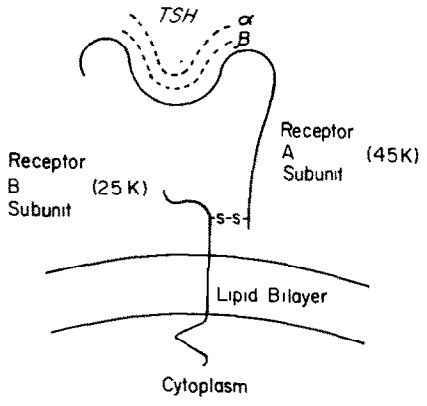

Fig.3. Proposed structure of the porcine TSH receptor. Currently, our studies do not provide an indication as to whether the B-subunit merely penetrates the lipid bilayer or spans the membrane to form a functionally important site at its cytoplasmic surface. The exact role of the 2 subunits of TSH in forming the hormone's receptor binding site is not known at present except that confirmational and/or structural contributions are required from both subunits [13]. Previous studies have suggested that the disulphide bridge(s) joining the A- and B-subunits is close to one end of the A-subunit [2].

tion, caused a marked change in sedimentation characteristics with most of the radioactivity running between TSH and BSA (fig.2). This suggested that after mild reduction, labelled TSH was principally associated with a much smaller structure; possibly due to the loss of the B-subunit from the receptor together with the Lubrol micelle.

To investigate this possibility further, analyses were carried out using a calibrated column (250 ml) of Sephacryl S-300 run in 0.1% Lubrol. Non-reduced complexes were eluted as a symmetrical peak (K_{av} = 0.14) [6,7] together with a small amount of free TSH (K_{av} = 0.50). Complexes

reduced in 10 mM DTT prior to chromatography, however, showed a different elution profile with the appearance of a major peak (K_{av} = 0.32) eluting in a position between the non-reduced complex and TSH. Analysis of this K_{av} = 0.32 peak on SDS–PAGE showed it to be the A-subunit of the receptor crosslinked to intact TSH (M_r = 73000). Conversion of the A-subunit crosslinked to ^{125}I-HSAB-TSH from the K_{av} = 0.14 form to K_{av} = 0.32 by mild reduction appeared to be almost complete as SDS–PAGE of the residual high-M_r radioactive material eluting from the column only showed the presence of small amounts of ^{125}I-HSAB-TSH and a diffuse background at higher M_r (not shown). Similarly, most of the crosslinked complex was converted to slowly sedimenting material on sucrose density gradients after reduction (fig.2).

To determine whether the A-subunit of the receptor crosslinked to TSH (K_{av} = 0.32 peak) was associated with Lubrol micelles or not, Lubrol micelles were labelled with [^{3}H]oleic acid ([^{3}H]oleic acid is spontaneously incorporated into Lubrol micelles) and run on the S-300 column. The

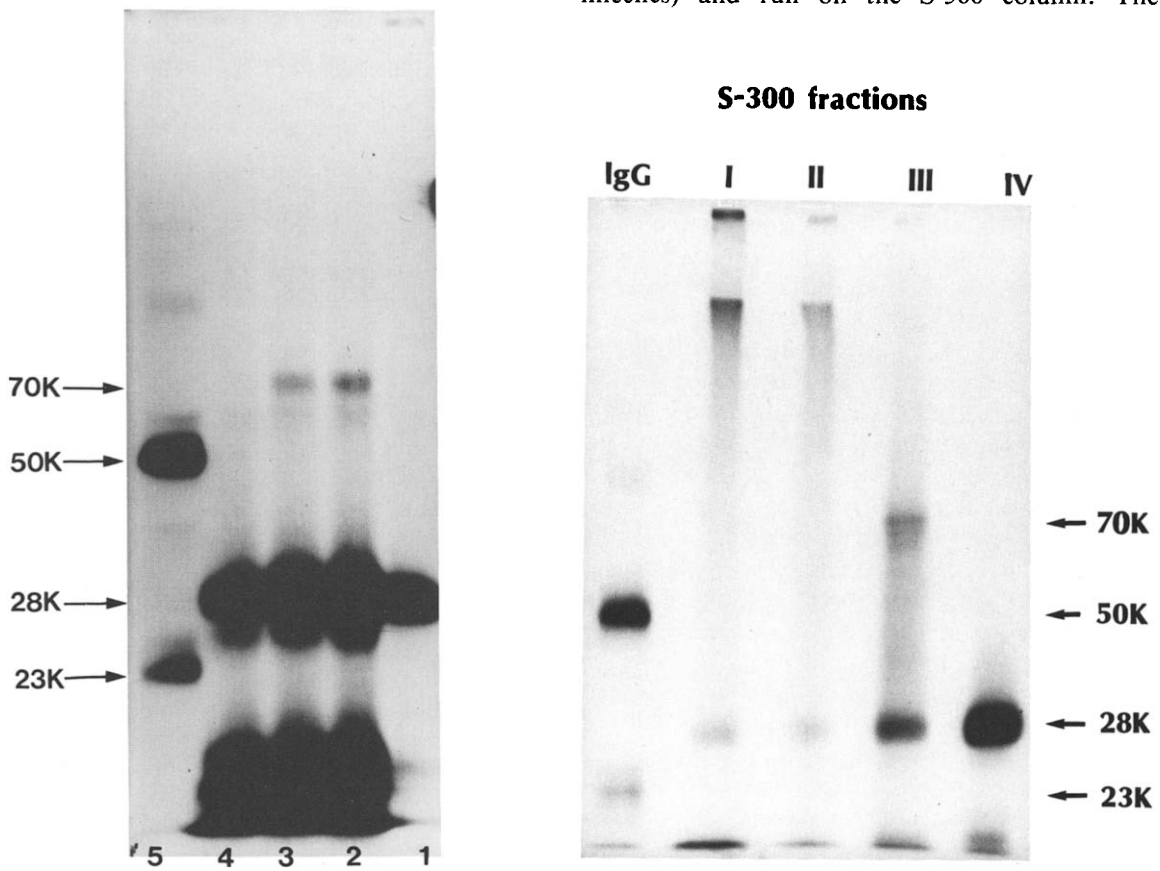

Fig.4. Release of the TSH receptor A-subunit crosslinked to labelled TSH from thyroid membranes by treatment with DTT (see text for experimental details). (a) SDS–PAGE. Lane 1, membranes crosslinked to labelled TSH and treated with Tris–NaCl. Lane 2, membranes crosslinked to labelled TSH and treated with Tris–NaCl containing 10 mM DTT. Lane 3, membranes preincubated with 1 mU unlabelled TSH, crosslinked to labelled TSH and treated with Tris–NaCl containing 10 mM DTT. Lane 4, membranes preincubated with 10 mU unlabelled TSH, crosslinked to labelled TSH and treated with Tris–NaCl containing 10 mM DTT. Lane 5, reduced ^{125}I-labelled IgG. The crosslinked labelled TSH–TSH receptor A-subunit complex is the band with M_r = 73000. The material in each of lanes 1–4 was derived from the same amount of thyroid membranes (500 μl) and ^{125}I-labelled HSAB-TSH (500 μl). (b) Chromatography on Sephacryl S-300 in Tris–NaCl (i.e., in the absence of detergent) of material prepared as for lane 2 in panel a followed by SDS–PAGE. I, Void volume peak. II, Material eluting between void volume peak and peak III. III, Peak with K_{av} = 0.35. IV, Peak with K_{av} = 0.55.

micelles eluted from the column in a single symmetrical peak with $K_{av} = 0.275$ indicating that the micelles had a considerably larger Stokes radius than the TSH–TSH receptor A-subunit complex and consequently, the TSH–TSH receptor A-subunit complex could not have been associated with a Lubrol micelle during chromatography. This suggested that the A-subunit of the receptor was a hydrophilic peptide. As the intact receptor was associated with a Lubrol micelle these observations suggest that the micelle was associated with the B-subunit of the receptor.

If this suggestion is correct the region of the B-subunit which bound to the Lubrol micelle would be expected to penetrate the cell membrane prior to detergent solubilisation and the data suggest a possible structure for the receptor corresponding to that shown in fig.3. If this model is correct it should be possible to release the TSH receptor A-subunit crosslinked to TSH from thyroid membrane preparations merely by addition of reducing agent. This effect could be demonstrated as shown in fig.4a. The proportion of TSH receptor A-subunit crosslinked to ^{125}I-HSAB-TSH released from the membranes by DTT treatment was difficult to estimate principally because of the need to use an excess of ^{125}I-HSAB-TSH in the binding studies to provide sufficient counts in the crosslinked complex for visualisation on the gels. However, previous studies have shown that incubation of membrane-bound or detergent-solubilised TSH receptors with 5 mM DTT completely inactivated the receptor in terms of TSH binding [8].

The material released by DTT treatment of thyroid membranes crosslinked to ^{125}I-HSAB-TSH was also run on the Sephacryl S-300 column after the column had been equilibrated with aqueous buffer (Tris–NaCl). Under these conditions, the A-subunit crosslinked to ^{125}I-HSAB-TSH eluted with a K_{av} of 0.35 (fig.4b) confirming the hydrophilic nature of the A-subunit and providing further evidence for the structure proposed in fig.3.

Several comments concerning the proposed structure are appropriate: (i) There is a resemblance to the structure of the major histocompatibility antigens [9]; (ii) preliminary studies with TSH receptor preparations from different species suggest that their structures are similar with small differences in subunit M_r values between species [1,2]; (iii) freezing and thawing of human thyroid homogenates releases a water soluble component of the TSH receptor which contains binding sites for both TSH and TSH receptor antibodies [10,11]. This component is probably closely related to the A-subunit of the receptor; (iv) the ability of the A- and B-subunits to dissociate readily after reduction of their interchain disulphide bridges indicates that interchain non-covalent interactions in the crosslinked complex are of relatively low affinity; (v) the association of the B-subunit with the cell membrane suggests that it is this subunit which is involved in interaction with the regulatory subunits of adenylate cyclase [12]. These interactions may occur inside the lipid bilayer or at the cytoplasmic surface of the membrane.

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