Expression of a human thyrotrophin receptor fragment in *Escherichia coli* and its interaction with the hormone and autoantibodies from patients with Graves' disease

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ABSTRACT

Graves' disease is an autoimmune thyroid disease characterized by the presence of pathogenic autoantibodies to the TSH receptor (TSH-R). By using polymerase chain reaction, the extracellular region of the human TSH-R cDNA has been amplified and used to prepare recombinant TSH-R (extracellular) protein fused with glutathione-S-transferase (GST). Purification of the recombinant TSH-R (extracellular)-GST fusion protein was achieved by preparative gel electrophoresis in SDS or by preparative isoelectric focusing in urea. Following removal of SDS by detergent exchange or urea by dialysis, the purified recombinant receptor preparations were assessed for binding to the hormone or to autoantibodies from Graves' disease patients. The purified recombinant receptor preparations fail to show any binding to the hormone or autoantibodies either by inhibition of binding assays or by immunoblotting. The results imply that the correct folding and/or post-translational modifications of the polypeptide chain which are not achieved in recombinant proteins produced in *Escherichia coli* may be important for the binding of the hormone or Graves' disease autoantibodies to the TSH-R. The recombinant receptor prepared in this manner will be useful for immunological and cellular investigations in patients with Graves' disease.

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INTRODUCTION

Thyroid cell physiology is intimately regulated by the pituitary hormone, thyrotrophin (TSH) (Dumont, 1971). The binding of TSH to its receptor on the thyroid cell leads to thyroid hormone synthesis and thyroid cell growth (Roger, Hotimsky, Moreau & Dumont, 1982). In autoimmune thyroid disease in man, autoantibodies to the TSH receptor (TSH-R) are present which are pathogenic, leading to the hyperthyroidism of Graves' disease (Adams, 1980; Zakaria & McKenzie, 1987; Smith, McLachlan & Furmanik, 1988).

The recent cloning of the human TSH-R cDNA will allow its role in autoimmune thyroid disease to be ascertained. The primary structure of the human TSH-R comprises a single polypeptide of 764 residues which, in common with all other G-binding protein receptors, traverses the membrane seven times (Libert, Lefort, Gerard et al. 1989; Nagayama, Kaufmann, Seto & Rapaport, 1989; Misrahi, Loosfelt, Atger et al. 1990). Following the predicted signal sequence, 395 residues code for the extracellular part of the receptor, which is the likely region that interacts with the bulky glycoprotein hormone. This is also the region to which autoantibodies from autoimmune thyroid disease patients interact, since a proportion of these sera are known to inhibit the binding of TSH to its receptor (Zakaria & McKenzie, 1987). The expression of the TSH-R cDNA as a recombinant protein in eukaryotic cells has shown that the receptor is biologically functional where it maintains reactivity with the hormone (Libert et al. 1989; Nagayama et al. 1989; Misrahi et al. 1990) and Graves' disease autoantibodies (Libert et al. 1989). In this communication, we describe the expression and purification of the extracellular region of the TSH-R as a recombinant fusion protein in *Escherichia coli* (Banga, Barnett, Ewins et al. 1990a). Although the receptor preparation prepared in *E. coli* fails to interact with TSH or receptor autoantibodies, the purified recombinant
protein is suitable for immunological investigations to study its precise role in autoimmune thyroid disease. The work described has been presented in abstract form (Huang, Collison, McGregor & Banga, 1991).

MATERIALS AND METHODS

Amplification by polymerase chain reaction (PCR)

Poly(A)⁺ RNA (5 µg) prepared from Graves’ thyroid tissue, as already described (Huang, Page, Roberts et al. 1990), was used to synthesize first-strand cDNA using oligo d(T) priming with a commercial kit (Pharmacia Biotechnology, Milton Keynes, Bucks, U.K.). The 33 µl reaction volume was diluted to 1 ml in water and used directly for PCR. Oligonucleotides as primers for PCR were designed from the human TSH-R sequence (Nagayama et al. 1989) where the primers flanked the extracellular region of the cDNA coding for the human TSH-R. Additionally, the forward primer was designed to start from amino acid residue 20 following the signal sequence and both the forward and reverse primers incorporated a BamHI and EcoRI restriction site respectively which allowed the amplified cDNA product to be maintained in frame for expression in pGEX-2T (Banga et al. 1990a). The following forward primer and reverse primer oligonucleotides were used 5’-CAGGGGATCCGGCGGAATGGGGTGTTCGTCTCCA-3’ and 5’-TCAGGAATTTCTGTATTATGTC TTCACAGGG-3’ respectively. For PCR, to 2 µl of the diluted cDNA were added 30 pmol of each primer, 1 mΜ MgCl₂ and 2.5 U Taq polymerase in a final volume of 100 µl. PCR was performed in a thermal cycler (Perkin Elmer Cetus, Beaconsfield, Bucks, U.K.) for 40 cycles where each cycle consisted of 92°C for 40 s, 55°C for 1.5 min and 72°C for 3 min. The amplified product (10 µl) was analysed by ethidium bromide staining following agarose gel electrophoresis.

Nucleotide sequencing

The amplified DNA was digested with BamHI and EcoRI and, following electrophoresis in low-melting-point agarose gel, the 1.2 kb DNA fragment was purified (Huang et al. 1990). The cDNA was ligated either into M13mp18 or M13mp19 which had previously been restricted with BamHI and EcoRI. The cDNA inserts were fully sequenced using universal primer and by primer walking using the following three primers (i) 5’-AAAGTGACTCATAGACAA-3’, (ii) 5’-ATCCCCGTGATACATT-3’ and (iii) 5’-GTCTCAAAAGTGT-3’. Sequencing was performed by the chain-termination method (Sanger, Nicklen & Coulson, 1977) using a Sequenase kit (US Biochemicals, Cleveland, OH, U.S.A.) and [α³²P]dATP (Amersham International plc, Amersham, Bucks, U.K.).

Preparation of recombinant TSH-R (extracellular) fusion protein

The cDNA coding for TSH-R (extracellular) which had been fully sequenced in M13 to confirm authenticity was excised from the replicative form by BamHI and EcoRI digestion and purified by gel electrophoresis (Huang et al. 1990). The cDNA was ligated into BamHI/EcoRI-digested pGEX-2T (called pGEX-2T/TSH-R(E)) and used to transform Escherichia coli DH5α cells (Gibco BRL, Paisley, Strathclyde, U.K.). Confirmation of transformation was obtained by subjecting a plasmid preparation from an overnight culture of transformed DH5α bacteria to Southern blotting and hybridization with ³²P-labelled TSH-R(E) cDNA. Expression of recombinant fusion protein was performed as described by Banga et al. (1990a).

Purification of recombinant glutathione-S-transferase-TSH-R (extracellular) fusion protein

The bacterial inclusion bodies were extracted twice with a final concentration of 3 m urea to remove some of the bacterial protein components (Banga, Barnett, Ewins et al. 1990b). Following centrifugation in a bench-top microcentrifuge for 15 min, the pellet containing the insoluble proteins was used for further purification of the recombinant polypeptide. Two different methods were employed for purification as described below.

Electroendosmotic preparative gel electrophoresis

This was performed using an ELFE apparatus (Genofit, Grand-Lancy, Switzerland). Inclusion body pellets (1 ml) which had previously been extracted with 3 m urea were solubilized in 0.125 m Tris–HCl (pH 6.8) containing 2.5% (w/v) SDS and 20% (v/v) glycerol and separated on an 8% (w/v) polyacrylamide gel. Fractions (2 ml) eluting through the capillary tube were collected in a fraction collector and analysed by SDS-polyacrylamide gel electrophoresis. Fractions containing the recombinant TSH-R (extracellular) fusion protein were identified, pooled and used for immunoblotting. For binding experiments with the TSH-receptor antibody (TRAb) assay kit (RSR Ltd, Cardiff, S. Glamorgan, U.K.), lubrol was added to the pooled fractions to a final concentration of 1% (w/v) and dialysed extensively against 10 mM Tris–HCl plus

Journal of Molecular Endocrinology (1992)
50 mM NaCl (pH 7.6) containing 1% (w/v) lubrol (Shrewring & Rees Smith, 1982).

**Preparative isoelectric focusing**
This was performed using a Rotofor apparatus (Bio-Rad, Hemel Hempstead, Herts, U.K.). Inclusion body pellets (1 ml) which had previously been extracted with 3 M urea were solubilized in 8 M urea and the proteins separated by preparative isoelectric focusing using Pharmalyte pH 3–10 (Pharmacia Biotechnology, Milton Keynes, Bucks, U.K.). Twenty individual fractions were collected following isoelectric focusing and analysed by SDS-polyacrylamide gel electrophoresis. The fraction containing the recombinant TSH-R (extracellular) fusion protein was used for binding experiments with the TRAb assay kit by addition of a final 1% lubrol and extensive dialysis against 10 mM Tris–HCl plus 50 mM NaCl (pH 7.6) containing 1% lubrol (Shrewring & Rees Smith, 1982).

**Patients**
A total of 19 sera from newly diagnosed patients with Graves’ disease was used to assess binding to the purified recombinant TSH-R (extracellular) preparations. This panel of characterized sera contained varying levels of cyclic AMP (cAMP) stimulatory activity and/or TSH-binding inhibitory immunoglobulins (TBII). The cAMP stimulatory activity was measured in a bioassay using primary cultures of thyroid cells obtained from thyroid glands from patients with Graves’ disease (Collison, Banga, Barnett & McGregor, 1990; Collison, Banga, Barnett *et al.* 1991) whilst the TBII activity was measured using the TRAb assay kit (Collison *et al.* 1991). Fifteen of these sera from the above panel have been described previously (Collison *et al.* 1990). A pool of normal immunoglobulin from 20 individuals who were negative for cAMP-stimulatory activity was also used.

**Immunoblotting**
Immunoblotting was performed as already described (Banga *et al.* 1990a) using purified preparations of recombinant TSH-R (extracellular) (purified by preparative gel electrophoresis) and glutathione-S-transferase (GST). For immunoblotting, a rabbit antiserum to GST (dilution 1:500) was used to localize the recombinant fusion proteins. Sera from patients (dilution 1:50) or immunoglobulin preparations (0.2 mg/ml) in haemoglobin/phosphate-buffered saline were also used followed by 

Direct binding of 

125I-labelled TSH (approximately 180 000 c.p.m.) (TRAb kit to the receptor preparations was also performed in 10 mM Tris–HCl plus 50 mM NaCl (pH 7.6) (Shrewring & Rees Smith, 1982).

**Binding of 125I-labelled TSH to purified, recombinant preparations from TSH-R (extracellular) fusion protein**
Binding of 

125I-labelled TSH to its receptor was performed using the TRAb assay kit. Briefly, for calibration of the assay, 50 μl porcine thyroid membrane were incubated with various concentrations of unlabelled TSH at room temperature for 15 min. To this were added 100 μl 125I-labelled TSH and the mixture was incubated at 37°C for 60 min. This was followed by addition of assay buffer and precipitation with ice-cold polyethylene glycol (PEG) solution, as specified in the kit. Following centrifugation, the pellets were counted in a gamma counter to ascertain the amount of 125I-labelled TSH present in the precipitate and the results calculated in terms of percentage 

125I-labelled TSH binding.

Recombinant TSH-R (extracellular) fusion proteins, purified by preparative gel electrophoresis, or isoelectric focusing (Rotofor) (50 μl containing 30 pmol recombinant protein) were incubated with 100 μl 125I-labelled TSH at 37°C for 60 min. The mixture was then precipitated with assay buffer and PEG solution and the percentage 125I-labelled TSH bound calculated as described above. To confirm that the recombinant TSH-R (extracellular) receptor preparation was precipitated by the PEG solution of the TRAb assay kit, the precipitated material was subjected to SDS-polyacrylamide gel electrophoresis and immunoblotting using a monoclonal antibody generated to the recombinant protein (G.C. Huang; unpublished observations) and 125I-labelled protein A. In other experiments, various concentrations of the recombinant receptor preparations (30–0.1 pmol/l) were incubated with 100 μl 125I-labelled TSH for 30 min at 37°C followed by the addition of 50 μl porcine thyroid membranes and incubation was continued at 37°C for 60 min. The mixture was precipitated and the percentage of 

125I-labelled TSH bound calculated.

**RESULTS**

**Amplification of the extracellular region of TSH-R**
The extracellular region of the TSH-R encompassing residues 20–414 (coding for 395 amino acids) was amplified from Graves’ thyroid cDNA. A cDNA fragment of approximately 1.2 kb was amplified...
Two different clones were fully sequenced in M13 and were identical to the published sequence (Nagayama et al. 1989) coding for the extracellular region of the receptor (not shown). One of these cDNA clones was excised from M13 and ligated into pGEX-2T. Southern blotting of a plasmid preparation from a colony grown overnight with $^{32}$P-labelled TSH-R(E) cDNA confirmed the transformation with the recombinant plasmid (Fig. 2).

**Preparation and purification of recombinant TSH-R (extracellular) protein**

Colonies from recombinant *E. coli* were grown overnight and tested for expression of recombinant protein following induction with isopropyl thiogalactopyranoside, as already described (Banga et al. 1990a). Bacterial lysates from induced colonies showed the presence of a recombinant protein at approximately 72 kDa in comparison with the non-induced cultures (Fig. 3, lanes 2 and 1 respectively). However, the non-induced lysates also showed the presence of other bacterial proteins which co-migrate with the recombinant TSH-R (extracellular) fusion protein. Inclusion body preparations prepared from induced cultures (Fig. 3, lane 3) were extracted with 3 M urea to remove the bacterial proteins (Banga et al. 1990b) (Fig. 3, lane 4). Under these conditions, the recombinant fusion protein remains insoluble in the pellet (Fig. 3, lane 5). The recombinant protein purified by preparative gel electrophoresis is shown in Fig. 3 (lane 6). Since the purified recombinant protein described above is in the detergent SDS, we also attempted purification in the absence of SDS. This was achieved by solubilizing the pellets extracted with 3 M urea further with 8 M urea which readily solubilizes the recombinant protein (not shown). The solubilized protein was subjected to preparative isoelectric focusing in 8 M urea in the Rotofor followed by collection of the fractions and analysis by SDS-polyacrylamide gel electrophoresis. Two fractions of isoelectric points

![Figure 1](image1.png) **Figure 1.** Agarose gel electrophoresis and ethidium bromide staining to show the 1.2 kb cDNA fragment coding for the extracellular region of TSH-receptor amplified from Graves' disease thyroid cDNA (lane b). The 123 bp (lane a) and 1 kb ladder (lane c) are also shown.

![Figure 2](image2.png) **Figure 2.** Southern blotting on a plasmid preparation derived from an overnight colony to show transformation of *Escherichia coli* with the recombinant plasmid pGEX-2T/TSH-R(E). Plasmid DNA was restricted with BamH1/EcoRI and the blot hybridized with $^{32}$P-labelled TSH-R(E) cDNA and autoradiographed. The arrow indicates the 1.2 kb cDNA band of TSH-R(E) in the plasmid. Southern blotting was performed at 65°C in 5×SSPE (SSPE is 0.18 M NaCl, 10 mM sodium phosphate, pH 7.7, 1 mM EDTA), 5×Denhardt’s solution (Denhardt’s solution is 0.02% (w/v) bovine serum albumin, 0.02% (w/v) Ficoll, 0.02% (w/v) polyvinylpyrollidone), 100 μg single-stranded DNA/ml, 0.5% (w/v) SDS overnight and washed twice in 2×SSPE containing 0.1% (w/v) SDS at room temperature before autoradiography.
immunoblotting with rabbit anti-glutathione-S-transferase to confirm the presence of the recombinant fusion protein following purification. Lanes 1 and 2 are protein-stained gels with preparative gel-purified TSH receptor (extracellular) fusion protein and glutathione-S-transferase (GST) respectively. Lanes 3 and 4 show the immunoblot with GST antiserum binding to the GST recombinant fusion protein and GST respectively. Lane M shows marker proteins (from top to bottom) 205, 116, 97, 66, 45 and 29 kDa.

5.25 and 5.39 containing proteins co-migrating at 72 kDa were visible together with a small number of lower molecular weight components (Fig. 3, lane 7).

Immunoblotting

With anti-GST antibody

The recombinant TSH-R (extracellular) fusion protein and GST, both purified by ELFE, were subjected to immunoblotting with a rabbit antiserum to GST. Binding of the GST antibody to the fusion protein (Fig. 4, lane 3) confirmed that the purified material was a fusion protein with GST. The GST antibody binds GST at 26 kDa (Fig. 4, lane 4).

With sera from patients with Graves' disease

We have described a panel of sera from newly diagnosed patients with Graves' disease which contained high levels of cAMP-stimulatory activity and/or TSH-binding blocking activity (Collison et al. 1990, 1991). Recombinant TSH-R (extracellular) fusion protein, initially purified by preparative gel electrophoresis, was subjected to SDS-polyacrylamide gel electrophoresis for immunoblotting. GST, purified from non-recombinant pGEX-2T transfected E.coli, was also used in immunoblotting. No binding was observed to the purified recombinant receptor preparations by immunoblotting with 19 sera from patients with Graves' disease or the pool of sera from normal individuals (not shown). Additionally, 125I-labelled TSH failed to show any binding by this procedure (not shown).

Binding of 125I-labelled TSH to preparations of purified recombinant receptor fusion protein

125I-Labelled TSH readily binds porcine thyroid membrane which is displaced by various concentrations of unlabelled TSH (5–405 U/l) (Fig. 5). Under these conditions, 125I-labelled TSH shows no binding to recombinant receptor preparations (30 pmol) purified by preparative gel electrophoresis or by isoelectric focusing (not shown) although the recombinant receptor preparations are clearly precipitated by the PEG solution in the TRAb assay kit,
as ascertained by immunoblotting with a monoclonal antibody (see Materials and Methods, not shown). When porcine thyroid membranes were added to various concentrations of recombinant receptor preparations incubated with $^{125}$I-labelled TSH, the percentage binding of $^{125}$I-labelled TSH to the porcine thyroid membranes did not alter significantly (Fig. 5). Thus, the ligand is not adversely affected by the recombinant receptor preparations in the incubation mixture and maintains its capability of binding to the porcine thyroid membranes. The $^{125}$I-labelled TSH was not limiting in this assay under these conditions, since substitution of the recombinant receptor preparation with additional 50 µl porcine thyroid membranes led to a near twofold increase in the $^{125}$I-labelled TSH precipitated in the above assay (not shown).

**DISCUSSION**

In this report we describe the expression of the extracellular region of the TSH-R as a recombinant fusion protein in *E. coli*. High-level expression of the recombinant fusion protein was achieved by this prokaryotic expression system which was enriched in the inclusion body preparations prepared from bacterial lysates. The recombinant protein generated is the TSH-R (extracellular) fusion protein since the cDNA was subjected to DNA sequencing before expression, and the transformation of bacteria with recombinant pGEX-2T/TSH-R(E) construct was confirmed by Southern blotting. Furthermore, immunoblotting with a GST antiserum confirmed the binding of the antibody to the GST moiety of the recombinant fusion protein. Purification of the recombinant receptor by two different biochemical techniques allowed its biological activity to be ascertained. The denaturing detergent, SDS, was removed from the recombinant receptor purified by preparative gel electrophoresis by detergent exchange and extensive dialysis. However, to prepare recombinant receptor preparations that were essentially free of SDS, we also applied
isolectric focusing in urea. The removal of urea by extensive dialysis would also give the unfolded recombinant protein an opportunity to refold during the dialysis procedure. Both preparations of purified recombinant receptor fusion proteins failed to show any binding to radiolabelled TSH in the radioreceptor binding assay. Furthermore, sera from patients with Graves’ disease which were known to contain autoantibodies to the TSH-R, also failed to show any binding to the recombinant receptor preparations.

The use of purified, denatured E.coli recombinant fusion protein may be responsible for the lack of binding of TSH or Graves’ disease autoantibodies. This may also explain the failure to clone TSH-R by screening human thyroid λgt11 cDNA expression libraries with immunoglobulin preparations from patients with Graves’ disease (Chan, Lerman, Prabhakar et al. 1989). However, it is also possible that the failure of the recombinant receptor preparations to interact with the hormone or autoantibodies may be due to the use of a truncated receptor molecule. The full recombinant receptor produced in a eukaryotic expression system binds radiolabelled TSH (Libert et al. 1989; Nagayama et al. 1989; Misrahi et al. 1990), although at present there are no reports on whether a truncated, extracellular region of the TSH-R expressed in eukaryotic systems is capable of interacting with the hormone. It is, however, interesting that other receptors in the glycoprotein hormone receptor family, such as the luteinizing hormone/human chorionic gonadotrophin receptor, when expressed as extracellular regions with various lengths of truncations in Chinese hamster ovary cells, continue to show high-affinity binding to the ligand hormone (Xie, Wang & Segaloff, 1990; Braun, Schofield & Sprengel, 1991; Ji & Ji, 1991).

Although the purified recombinant TSH-R (extracellular) fusion protein produced in E.coli does not show any reactivity with its ligand or Graves’ disease autoantibodies, it is suitable as a source of purified antigen for cellular investigations to study the role of T cells from patients with Graves’ disease (Nicholson, Ewins, Huang et al. 1991) and production of monoclonal antibodies (G.C. Huang; unpublished observation). The ability to use denatured recombinant protein to produce monoclonal antibodies which then show reactivity with native protein has been demonstrated previously (Man, Cartwright, Morris et al. 1990). The availability of monoclonal antibodies to TSH-R will allow the purification of the recombinant receptor produced in eukaryotic systems (Page, 1988) by antibody affinity chromatography, as well as the analysis of the autoantigenic epitopes recognized by Graves’ disease.

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