Binding characteristics of antibodies to the TSH receptor

**Y Oda¹, J Sanders, S Roberts, M Maruyama¹, R Kato¹, M Perez¹,
V B Petersen, N Wedlock, J Furmaniak¹ and B Rees Smith¹**

FIRS Laboratories, RSR Ltd, Parc Ty Glas, Llanishen, Cardiff, CF4 5DU, UK

¹Department of Medicine, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN, UK

(Requests for offprints should be addressed to J Furmaniak, FIRS Laboratories, RSR Ltd, Parc Ty Glas, Llanishen, Cardiff CF4 5DU, UK)

ABSTRACT

We have used fragments of the TSH receptor (TSHR) expressed in *E. coli* as glutathione S-transferase fusion proteins to produce rabbit polyclonal antibodies and a panel ($n=5$) of monoclonal antibodies to the extracellular fragment of the TSHR. The binding characteristics of the antibodies to linear, conformational, glycosylated and unglycosylated forms of the receptor in different assay systems have been investigated.

The reactivity of these antibodies with the TSHR was assessed by Western blotting with both native and recombinant human TSHR expressed in CHO cells, immunoprecipitation of ³⁵S-labelled full-length TSHR produced in an *in vitro* transcription/translation system, immunoprecipitation of ¹²⁵I-TSH/TSHR complexes, inhibition of ¹²⁵I-TSH binding to the TSHR and fluorescence activated cell sorter (FACS) analysis of binding to CHO-K1 cells expressing the TSHR on their cell surface. Fab fragments of monoclonal antibodies were isolated, labelled with ¹²⁵I and used to determine the affinity constants of these antibodies with receptor, bound and free Fab being separated by polyethylene glycol (PEG) precipitation.

Rabbit polyclonal and mouse monoclonal antibodies reacted with the TSHR in Western blotting and one monoclonal antibody (3C7) was able to inhibit ¹²⁵I-TSH binding to native human TSHR (74% inhibition), recombinant human TSHR (84% inhibition) and porcine TSHR (65% inhibition). Affinity constant values for TSHR monoclonal antibody Fab fragments calculated using Scatchard analysis were about 10^7 M^{-1} . Four out of five monoclonal antibodies reacted in FACS analysis with TSHR expressed on the surface of CHO-K1 cells. The FACS unreactive monoclonal (3C7) bound well to detergent solubilised TSH receptors and this emphasised the importance of using a combination of FACS analysis and radioactively-labelled probes in analysis of the TSH receptor.

The monoclonal antibodies produced in this study were found to be of relatively low affinity but proved useful for detection of the receptor by Western blotting and by FACS analysis.

Journal of Molecular Endocrinology (1998) **20**, 233–244

INTRODUCTION

The thyrotrophin receptor (TSHR) is a major thyroid autoantigen and TSHR autoantibodies (TRAb) are responsible for the hyperthyroidism of Graves' disease (Rees Smith *et al.* 1988, Furmaniak & Rees Smith 1993). Sequence analysis indicates that the TSHR gene codes for a single peptide chain with a computed molecular mass of 84 kDa (Libert *et al.* 1989, Nagayama *et al.* 1989, Frazier *et al.* 1990, Misrahi *et al.* 1990). However the region of the peptide chain linking the receptor's large extracellular domain to its seven membrane spanning domain is readily cleaved giving rise to an A subunit (extracellular domain) linked by a

disulphide bridge(s) to a B subunit (membrane spanning domain) (Buckland & Rees Smith 1984, Kajita *et al.* 1985a,b, Rees Smith *et al.* 1988, Loosfelt *et al.* 1992).

The binding sites for TSH and for TRAb are formed principally by the receptor's A subunit but the A subunit alone does not appear sufficient for high affinity TSH binding (Davies Jones *et al.* 1984, 1985). Similar observations have been reported following studies using recombinant extracellular domain of the TSHR (Harfst *et al.* 1992, Nagayama & Rapoport 1992, Huang *et al.* 1993, Kosugi *et al.* 1997).

Experimentally-produced antibodies to different regions of the TSHR could be helpful in

characterising the receptor and we now describe the preparation of polyclonal and monoclonal antibodies to the extracellular domain of the TSHR.

MATERIALS AND METHODS

Production of an eukaryotic cell line expressing full length TSHR

An ATG start codon in the 5' untranslated region (5'UTR) of the full length hTSHR cDNA (Misrahi *et al.* 1990) was removed by *Ava*I digestion and the cDNA cloned into pRC/CMV (Invitrogen, Leek, The Netherlands). DNA encoding the full length TSHR (pRC/CMV/hTSHR) was transfected into CHO cells (CHO-K1 from ECACC) using the calcium phosphate precipitation method (Chen & Okayama 1987). Clones expressing TSHR were detected using 125 I-TSH binding directly to cells growing on 24-well plates. The clones showing highest TSH binding were expanded and recloned twice by limiting dilution. One stable cell line expressing 4×10^5 TSHRs per cell, as assessed by Scatchard analysis of TSH binding (Scatchard 1949) was selected and grown in continuous culture in DMEM (Gibco-BRL, Life Technologies, Paisley, Strathclyde, UK) containing high glucose and 10% foetal calf serum (FCS), with 1000 µg/ml geneticin sulphate (G-418) (Life Technologies).

Production of GST-TSHR fusion proteins

Two fragments of cDNA coding for the extracellular domain of the TSHR, TSHR800 (amino acids 89–363) and TSHR400 (amino acids 245–380) were cloned into the bacterial expression vector pGEX-2T (Pharmacia, St Albans, UK) to produce in frame fusion proteins with GST (Powell *et al.* 1996). An overnight culture of *E. coli* (strain UT580) transformed with pGEX-2T/TSHR plasmids was diluted 1/5 into 2xYTG medium (16 g tryptone, 10 g yeast extract, 5 g NaCl, 20 g glucose per litre, pH 7.0), incubated for 3 h at 30 °C; isopropyl-β-D-thiogalactopyranoside (IPTG) added to a final concentration of 1 mM to induce protein expression and incubated for a further 3 h. The bacterial pellets were resuspended in PBS (8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄ and 0.24 g KH₂PO₄ per litre, pH 7.4) containing 1% Triton X-100 and sonicated three times for 1 min on ice. The inclusion bodies were pelleted, washed in 4 M urea, solubilised in 8 M urea and separated on 9% acrylamide gels (SDS-polyacrylamide gel electrophoresis; SDS-PAGE) under reducing conditions (Laemmli 1970). The glutathione S-transferase (GST) fusion proteins were electroeluted from

acrylamide gel slices in 0.1 M NaHCO₃ and 0.1% SDS pH 7.8 (Smith & Corcoran 1994) dialysed against 50 mM Tris-HCl pH 8.0 and stored in aliquots at –70 °C.

Production of antibodies

Female New Zealand White rabbits were immunised with 50 µg GST-TSHR fusion proteins in complete Freund's adjuvant and boosted at 4-week intervals with 50 µg GST-TSHR in incomplete Freund's adjuvant until the antibody titre was high (see below for assay method).

Six- to eight-week-old BALB/c mice were immunised with 50 µg GST-TSHR fusion proteins and boosted at 3–4 week intervals until the antibody titre was high (see below for assay method). The fusion of mice spleen cells with X63-Ag8-653 myeloma cells (ECACC) was performed 4 days after the final intraperitoneal boost (De St Groth & Scheidegger 1980). The cells were cultured in 96-well plates in DMEM with high glucose, 20% FCS, 10% Doma Drive (Immune Systems, Paignton, Devon, UK) and selected with HAT (Life Technologies).

Antibody secretion was detected by immunoprecipitation assays (IPA) based on 35 S-labelled full length TSHR produced in the *in vitro* transcription/translation system (TnT) (see below for assay method). Positive wells were recloned two times by limiting dilution (0.5 cells/well), grown up and used to produce ascites.

Immunoprecipitation assay (IPA)

The cDNA encoding full-length TSHR was placed downstream of the T7 promoter in pYES2 (Invitrogen) and used in an *in vitro* TnT system (Promega, Southampton, Hants, UK) to produce TSHR labelled with 35 S-methionine as previously described (Colls *et al.* 1995, Prentice *et al.* 1997).

Briefly, 50 µl 35 S-labelled TSHR (25 000–30 000 c.p.m.) diluted in HSB (150 mM Tris-HCl pH 8.3, 200 mM NaCl and 10 mg/ml bovine serum albumin containing 1% Tween 20) were added to duplicate 50 µl aliquots of diluted test sera or tissue culture supernatant and incubated for 2 h at room temperature. Immune complexes were then precipitated by addition of either protein A Sepharose (Sigma, Poole, Dorset, UK) or anti mouse IgG agarose (Sigma) and counted for 35 S.

Isolation of Fab fragments

TSHR antibody IgGs were purified from ascites fluid using affinity chromatography on Prosep A (Bioprocessing, Consett, UK) according to the manufacturer's instructions.

Purified IgG (10 mg/ml) was dialysed against 70 mM sodium acetate, 50 mM NaCl pH 4.0 and digested for 18 h at 37 °C with 1 mg/ml pepsin. The reaction mixture was dialysed against 1 M glycine/NaOH pH 8.6, 150 mM NaCl and passed through a Prosep A column in the same buffer to remove undigested IgG from the (Fab)₂.

The (Fab)₂ fragments were reduced with 10 mM cysteine in 2 M Tris pH 8.3 for 1 h at 37 °C and the reaction terminated by the addition of 200 mM iodoacetamide in 2 M Tris pH 8.3 to a final concentration of 50 mM. Fab fragments were analysed by 9% SDS-PAGE and 50 µl (20 µg) aliquots of Fab were labelled with ¹²⁵I using the chloramine T method to a specific activity of 500 KBq per µg followed by Sephadex G-25 chromatography (Greenwood *et al.* 1963).

Preparation of detergent solubilised native and recombinant human TSHR

CHO-K1 cells were grown to confluence (4 days) in a 175 cm² flask, the cells were washed with Dulbecco's PBS minus calcium and magnesium ions and scraped into 10 ml ice-cold 50 mM NaCl, 10 mM Tris-HCl pH 7.5, 1 mM phenylmethylsulphonyl fluoride (PMSF; buffer A). The cells were pelleted at 1000 *g* for 5 min at 4 °C, the pellet resuspended in 1 ml buffer A and homogenised with a glass homogeniser on ice. The cell membranes were pelleted at 12 000 *g* for 30 min at 4 °C, resuspended in 1 ml ice-cold buffer A with 1% Triton X-100 and homogenised. The solubilised TSHR preparations were centrifuged at 90 000 *g* for 2 h and the supernatant aliquoted and stored at -70 °C.

Native human TSHR was prepared from human thyroid tissue as previously described (Rickards *et al.* 1981) using 1% Triton X-100.

Precipitation of TSHR/¹²⁵I-TSH complexes

Solubilised porcine TSHR (RSR Ltd, Cardiff, UK) or recombinant human TSHR were incubated for 2 h at room temperature with an equal volume of ¹²⁵I-labelled bovine TSH (24 000 c.p.m.; RSR Ltd) to form TSHR/TSH complexes. Fifty microlitres TSHR/TSH complex were incubated with 50 µl antibody for 1 h at 37 °C. Fifty microlitres protein A (10% suspension; RSR Ltd) were then added and incubated for 1 h at room temperature. The mixture was then diluted with 1 ml assay buffer and centrifuged (1500 *g* for 30 min at 4 °C) and the pellet washed once with 1 ml assay buffer and counted for ¹²⁵I.

Western blot analysis

Twenty five microlitres solubilised CHO-K1 cells, recombinant human TSHR or native human TSHR were mixed with an equal volume of sample buffer (4% SDS, 20% glycerol, 100 mM Tris-HCl pH 6.8, 0.002% bromophenol blue) plus dithiothreitol (10 mM) and heated to 100 °C for 3 min, electrophoresed on 9% SDS-PAGE gels and blotted onto nitrocellulose. Molecular mass markers (6H; Sigma) were run on each gel.

Western blotting analysis was carried out according to the method of Birk and Koepsell (1987). The membranes were blocked using 1 mg/ml polyvinylalcohol (PVA) in PBS and developed using anti-mouse or anti-rabbit horse-radish peroxidase conjugate followed by enhanced chemiluminescence (ECL) reagents (Amersham International plc, Amersham, Bucks, UK). Calculation of relative mobility was carried out using the centre of each protein band as a reference point.

TSH binding inhibition assays

TSH binding inhibition assays were carried out according to the method of Southgate *et al.* (1984) using native human TSHR or recombinant human TSHR (expressed in CHO cells). Briefly, 50 µl detergent solubilised TSHR were preincubated with 50 µl sera, purified IgG or Fab fragment for 15 min at room temperature before the addition of 100 µl ¹²⁵I-TSH (30 000 c.p.m.) and incubation at 37 °C for 1 h. TSHR/¹²⁵I-TSH complexes were precipitated by the addition of 2 ml 16.5% polyethylene glycol (PEG) precipitator (RSR Ltd) and 25 µl healthy blood donor serum, centrifuged at 1500 *g* for 30 min at 4 °C, aspirated and the radioactivity of the pellets counted in a gamma counter.

Scatchard analysis of ¹²⁵I-labelled Fabs binding to detergent solubilised TSHR

The characteristics of ¹²⁵I-labelled Fabs binding to detergent solubilised recombinant human TSHR were assessed by Scatchard analysis (Scatchard 1949). Fifty microlitres solubilised TSHR were incubated with 50 µl unlabelled Fab (concentrations ranging from 0.4 to 40 mg/ml) and 100 µl ¹²⁵I-Fab (15 000 c.p.m.) for 1 h at 37 °C. Fifty microlitres healthy blood donor sera and 2 ml 16.5% PEG (RSR Ltd) were added, mixed, centrifuged at 1500 *g* for 30 min at 4 °C and the radioactivity remaining in the pellet after aspiration counted. The concentrations of bound and free Fab were calculated, and a plot of bound against bound/free was used to calculate the affinities of the Fabs.

Fluorescence activated cell sorter (FACS) analysis

CHO-K1 cells (untransfected or expressing the TSHR) were grown to confluence in 175 cm² flasks in DMEM, 10% FCS at 37 °C and 5% CO₂. Flasks were washed two times with Dulbecco's PBS minus calcium and magnesium ions (PBS(–)) then removed using a cell scraper into 10 ml PBS(–), washed two more times and resuspended using a needle and syringe. A total of 2×10^5 cells were blocked with 100 µl 10% non-immunised rabbit sera in PBS(–) for 20 min at 4 °C, centrifuged at 200 *g* for 5 min at 4 °C and incubated with 100 µl IgG (100 mg/ml) in PBS(–) with 1% BSA for 30 min at 4 °C.

The cells were then washed three times with PBS(–) and incubated with 100 µl second antibody; rabbit anti-mouse Ig F(ab')₂ (Dako), FITC conjugated, diluted 1/40 in PBS(–) with 1% BSA for 30 min at 4 °C. The cells were washed three times with PBS(–) and fixed with 1% paraformaldehyde in PBS(–).

A total of 5000 cells were analysed for each sample using a Becton Dickinson FACScan System and experiments were repeated on three separate occasions.

RESULTS

Reactivity of rabbit and mouse monoclonal antibodies in the IPA

Two rabbits were immunised with each of the TSHR constructs (TSHR400 and TSHR800) and tested every month. Non-immunised rabbit sera and sera from rabbits immunised with a GST/steroid 21-hydroxylase fusion protein (GST/21OH) (Chen *et al.* 1996) used as negative controls bound about 1–2% of ³⁵S-TSHR (full length) at 1/100 dilution (Table 1). In contrast, sera from rabbits immunised with TSHR800 and TSHR400 diluted 1/100 bound 42.5 and 15.1% of ³⁵S-TSHR respectively after 4–12 months of immunisation (Table 1). Five monoclonal antibodies were identified from three fusions (3F3, 3E4, 3C6, 5D8 and 3C7). All five reacted in the IPA, showing binding to ³⁵S-TSHR of between 7 and 46% (Table 2).

Precipitation of TSHR/TSH complexes

Rabbit antisera were used to immunoprecipitate recombinant human and porcine TSHRs complexed with ¹²⁵I-TSH (Table 3). Sera from rabbits immunised with TSHR400 and TSHR800 precipitated both human and porcine TSHR/¹²⁵I-TSH

TABLE 1. Binding of rabbit antibodies to ³⁵S-TSHR (full length). Sera from rabbits immunised with TSHR800 and TSHR400 were tested by IPA based on ³⁵S TSHR (full length) produced in the TnT reaction. Results from the rabbits showing the best responses are shown in the table and are representative of at least five separate experiments carried out in duplicate. GST/21OH = control rabbit sera from rabbits immunised with GST/human steroid 21-hydroxylase fusion protein

	Dilution	% ³⁵ S-TSHR bound
Rabbit serum		
Non-immunised	1/10	2.6
	1/100	1.2
GST/TSHR800	1/10	57.2
	1/100	42.6
GST/TSHR400	1/10	41.2
	1/100	15.1
GST/21OH	1/10	1.0
	1/100	1.2

complexes. Sera from rabbits immunised with TSHR400 cross reacted strongly with the porcine TSHR complexes, 30.4% being bound at 1/10 dilution, compared with sera from rabbits immunised with TSHR800 which precipitated 16.2% at the same dilution and non-immunised rabbit sera which bound 12.7%. Sera from the TSHR800 rabbits reacted more strongly with the human TSHR than sera from the TSHR400 rabbits, 64% of the recombinant human TSHR complex was precipitated at 1/10 dilution with TSHR800 rabbit antiserum compared with 47.5% at the same dilution for TSHR400 rabbit antiserum and 8.3% for non-immunised rabbit sera (Table 3).

Western blot analysis of solubilised human TSHR

The five monoclonal antibodies (3C6, 3C7, 3F3, 3E4 and 5D8) reacted with detergent solubilised recombinant human TSHR on Western blot at 1/100 and 1/1000 dilution. Figure 1 shows the reaction of 3C7 TSHR monoclonal antibody (panels A and B); a TSHR monoclonal antibody directed to the C terminus of the receptor (T3–495; TRANS-BIO, Boulogne, France) (panel C) and a negative control monoclonal antibody to glutamic acid decarboxylase (GAD6; DSHB, Iowa City, IA, USA) (panel D) with solubilised CHO-K1 (untransfected) membranes (lane 1), recombinant human TSHR (lane 2) and native human TSHR (lane 3). Analysis of solubilised CHO cells expressing the full length TSHR with 3C7 shows two bands at 117 ± 4 kDa (mean $\pm$ s.d.; *n*=5) and 99 ± 4 kDa (mean $\pm$ s.d.; *n*=5) and a broad band with the centre at 58 ± 2 kDa (mean $\pm$ s.d.; *n*=5).

TABLE 2. Binding of TSHR monoclonal antibody ascites to ^{35}S -TSHR (full length). The TSHR monoclonals were made to one of two proteins designated TSHR400 and TSHR800. M1/21OH is a negative control monoclonal antibody which was made to a 21-hydroxylase/GST fusion protein produced in *E. coli*. Ascites dilutions were prepared in 150 mM Tris-HCl pH 8.3, 200 mM NaCl and 10 mg/ml BSA containing 1% Tween 20. Results shown are representative of at least five separate experiments carried out in duplicate

Ascites dilution	% ^{35}S -TSHR bound to monoclonal antibody					
	3F3 (TSHR400)	3E4 (TSHR400)	3C6 (TSHR400)	5D8 (TSHR400)	3C7 (TSHR800)	M1/21OH (GST/21OH)
1/100	15.1	18.4	46.4	13.8	25.2	3.9
1/500	8.4	33.6	42.0	8.8	18.8	4.6
1/1000	7.1	29.8	37.1	7.9	14.7	5.8
1/5000	6.2	18.9	22.3	6.9	7.9	5.8
1/10 000	6.0	14.7	14.9	6.5	6.0	5.7

TABLE 3. Immunoprecipitation of TSHR/ ^{125}I -TSH complexes by rabbit antibodies. Sera from rabbits immunised with TSHR400 and TSHR800 tested for their ability to immunoprecipitate both recombinant human (rhTSHR) and porcine TSHR (pTSHR) complexed with ^{125}I -TSH. Results are representative of at least two separate experiments carried out in duplicate

	Dilution	rhTSHR complex (% Binding)	pTSHR complex (% Binding)
Rabbit serum			
	Non-immunised		
	1/10	8.3	12.7
21OH/GST	1/50	6.8	10.1
	1/100	7.0	10.1
	1/10	7.8	12.0
TSHR800/GST	1/50	8.2	11.1
	1/100	7.5	10.1
	1/10	64.0	16.2
TSHR400/GST	1/50	43.5	11.3
	1/100	30.7	10.2
	1/10	47.5	30.4
	1/50	16.5	18.8
	1/100	10.7	14.5

T3-495 antibody recognised bands of about 120 and 100 kDa in the CHO cells expressing the human TSHR. In the case of native TSHR from human tissue, the 3C7 monoclonal antibody reacted with proteins at 93 and 53 kDa whilst T3-495 antibody at a 1/1000 dilution did not show reactivity with native human TSHR. Neither 3C7 nor T3-495 reacted with the untransfected CHO-K1 cell preparations (Fig. 1, lanes 1 in panels A-C).

Figure 2 shows the reaction of the 3C7 TSHR monoclonal antibody (panel B) and 3C6 monoclonal antibody (panel A) with solubilised CHO-K1 (untransfected) membranes (lane 1), recombinant human TSHR (lane 2) and native human TSHR (lane 3). The 3C6 monoclonal showed a similar reactivity to 3C7 (Fig. 1, panels A, B; Fig. 2, panel B) with the two bands at 117 ± 4 kDa (mean $\pm$ s.d.; $n=5$) and 99 ± 1.5 kDa (mean $\pm$ s.d.; $n=5$) but differed from 3C7 in its reactivity to a broad band

with the centre at 52 ± 1 kDa (mean $\pm$ s.d.; $n=5$). 3C6 showed no reaction with the A subunit of the native TSHR extracted from human thyroid tissue.

Antisera from both TSHR400 and TSHR800 immunised rabbits diluted 1/100 and analysed by Western blotting showed a similar pattern of reactivity to recombinant human TSHR as the monoclonal antibodies (3C6 and 3C7). In particular, bands at 119 ± 6 kDa (mean $\pm$ s.d.; $n=5$) and 102 ± 5 kDa (mean $\pm$ s.d.; $n=5$) and a broad band with the centre at 54 ± 1 kDa (mean $\pm$ s.d.; $n=5$) were recognised (data not shown).

Inhibition of TSH binding to TSHR

Only one out of the 5 TSHR monoclonal antibodies (3C7) was able to inhibit ^{125}I -TSH binding to native human TSHR (74% inhibition), recombinant

FIGURE 1. Analysis of reactivity of TSHR monoclonal antibody by Western blotting. Panel A=reaction with TSHR monoclonal antibody (3C7) diluted 1:100. Panel B=reaction with TSHR monoclonal antibody (3C7) diluted 1:1000. Panel C=reaction with TSHR monoclonal antibody directed to the C-terminal part of the receptor (T3-495) diluted 1:1000. Panel D=reaction with monoclonal antibody to GAD6 diluted 1:100. For panels A–D: lane 1=solubilised CHO-K1 (control) membranes; lane 2=solubilised recombinant human TSHR (expressed in CHO-K1 cells); lane 3=solubilised native human TSHR. Calculation of relative mobility was carried out using the centre of each protein band as a reference point.

human TSHR (84% inhibition) and porcine TSHR (65% inhibition) (Fig. 3).

This inhibiting effect was studied in more detail using purified 3C7 IgG (Fig. 4). Concentrations of 3C7 IgG ranging from 0.03 to 2.5 mg/ml showed ^{125}I -TSH binding inhibition ranging from 21 to 92% respectively. Furthermore, 3C7 Fab was able to inhibit TSH binding (41% inhibition at 0.15 mg/ml; data not shown).

Scatchard analysis of anti TSHR Fab fragments

The binding characteristics of two TSHR monoclonal antibody Fabs (3C7 and 3C6) to recombinant human TSHR analysed by a Scatchard plot is shown in Fig. 5. The affinity constant (K_a) was $8.9 \times 10^6 \text{ M}^{-1}$ for 3C7 and $1.3 \times 10^7 \text{ M}^{-1}$ for 3C6.

FACS analysis

Four of the monoclonal antibodies (3C6, 3F3, 3E4 and 5D8) bound to recombinant human TSHR

FIGURE 2. Analysis of reactivity of TSHR monoclonal antibody by Western blotting. Panel A=reaction with TSHR monoclonal antibody (3C6) diluted 1:1000. Panel B=reaction with TSHR monoclonal antibody (3C7) diluted 1:100. For panels A and B: lane 1=solubilised CHO-K1 (control) membranes; lane 2=solubilised recombinant human TSHR (expressed in CHO-K1 cells); lane 3=solubilised native human TSHR. Calculation of relative mobility was carried out using the centre of each protein band as a reference point. Both monoclonals reacted with an unidentified band of 74 kDa in the lane containing solubilised recombinant human TSHR expressed in CHO-K1 cells. This band is most probably a cleavage product of the receptor containing all or part of the extracellular domain.

expressed in CHO-K1 cells in FACS analysis, whereas 3C7 did not appear to recognise the receptor on the cell surface (Fig. 6, Table 4). CHO-K1 cells were used as a negative control cell line and IgG purified from M1/21-OH ascites (RSR Ltd) was used as negative control IgG; Table 4 shows the percentage positive cells detected. The control IgG (M1/21-OH 10 μg per ml) showed similar low reactivity to CHO-K1 cells (untransfected) and CHO cells expressing TSHR (6.8 and 6.9% respectively). The TSHR monoclonal 3C7 IgG (10 μg per ml) also showed similar low reactivity to CHO-K1 cells (6.7%) and CHO cells expressing TSHR (8.6%). However, 3E4, 3C6, 5D8 and 3F3 did not bind to CHO-K1 cells (1.7–5.7% cells positive) but bound well (75.8–83.1% cells positive) to CHO cells expressing the TSHR (Fig. 6; Table 4).

FIGURE 3. Inhibitory effect of antibodies on ¹²⁵I-TSH binding to native human TSHR (a), recombinant human TSHR (b) and native porcine TSHR (c). ■=TRAb in patient serum; ◆=3C7 TSHR monoclonal antibody (ascites) and ●=GAD₆₅ monoclonal antibody (ascites). Results shown in the figure are representative of at least two separate experiments carried out in duplicate.

FIGURE 4. Inhibition of ¹²⁵I-TSH binding to recombinant human TSHR by purified 3C7 TSHR IgG (■), 3C6 TSHR IgG (●) and M1/21OH IgG (◆). Results shown in the figure are representative of at least two separate experiments carried out in duplicate.

DISCUSSION

The TSHR is an important autoantigen in Graves' disease and the experimental production of antibodies to the receptor is critical to our understand-

FIGURE 5. Binding of TSHR antibody Fab fragment to recombinant human TSHR expressed in CHO-K1 cells. 3C6 TSHR Fab (■), 3C7 TSHR Fab (●).

ing of the receptor's structure and function and how it interacts with TSH and TRAb. However, the receptor is present in small amounts on the cell surface and has proved difficult to purify in large enough quantities to immunise experimental animals. Therefore we used two GST-TSHR fusion proteins produced in *E. coli* for immunisation and have produced rabbit polyclonal antibodies and

FIGURE 6. FACS analysis of monoclonal IgG binding to untransfected CHO-K1 cells (CHO-K1) or to CHO-K1 cells expressing the TSHR (CHO-TSHR). (The percentages of positive cells for this experiment are shown in Table 4.) 21-OH IgG: M1/21-OH monoclonal antibody to steroid 21-hydroxylase; 3C6, 3F3, 3E4, 5D8, 3C7: monoclonal antibodies to the TSHR.

a panel of five monoclonal antibodies to the extracellular domain of the human TSHR.

The rabbit polyclonal antibodies raised to both TSHR constructs (TSHR400 and TSHR800) reacted in an immunoprecipitation assay with full length ^{35}S -TSHR; highest titres were produced by sera from the rabbits immunised with the longer (TSHR800) fragment.

Rabbit polyclonal antibodies were able to immunoprecipitate ^{125}I -TSH/TSHR complexes of both human and porcine TSHR. Sera from rabbits immunised with TSHR400 reacted strongly with the human and porcine TSHR/ ^{125}I -TSH complexes compared with sera from rabbits immunised with TSHR800 which reacted only with human TSHR/ ^{125}I -TSH complexes. These differences in crossreactivity with human and porcine receptors suggest that the major epitopes recognised by sera from TSHR800 rabbits are located in regions which are different between human and porcine TSHR sequences. In contrast, sera from rabbits immunised

with TSHR400 recognised some epitopes that are common for both human and porcine TSHR sequences (there is an approximate 70% sequence identity at the amino acid level of the known TSHR extracellular domain sequences between different species) (Furmaniak & Rees Smith 1993).

We were not successful in precipitating either recombinant human or porcine TSHR complexes with any of the monoclonals we produced. The reason for this is unclear but may reflect the relatively low affinities of the monoclonal antibodies. In addition, polyclonal antibodies are generally considered to be more suitable for immunoprecipitation than low affinity monoclonal antibodies (Harlow & Lane 1988).

Western blot analysis of the TSHR with 3C7 showed the recombinant full length receptor to be present as two bands of 117 and 99 kDa and a broad 58 kDa protein band representing the receptor A subunit, whilst analysis with 3C6 showed the two full length bands at 117 and

TABLE 4. FACS analysis of the binding of monoclonal antibody IgGs to intact cells. The percentage positive cells for each IgG with both CHO-K1 cells and CHO-K1 cells expressing the TSHR are shown. Results are means $\pm$ S.D. of three separate experiments

Monoclonal antibody	CHO-K1/TSHR positive cells (%)	CHO-K1 positive cells (%)
M1/210H IgG	6.9 $\pm$ 3.2	6.8 $\pm$ 1.4
3C6 IgG	86.7 $\pm$ 1.5	1.7 $\pm$ 0.9
3F3 IgG	89.1 $\pm$ 1.4	5.4 $\pm$ 1.0
3E4 IgG	75.8 $\pm$ 13.8	2.8 $\pm$ 1.6
5D8 IgG	88.6 $\pm$ 2.0	5.7 $\pm$ 1.7
3C7 IgG	8.6 $\pm$ 1.5	6.7 $\pm$ 1.2

99 kDa but the receptor's A subunit was present as a smaller broad band of 52 kDa. Since both of the antibodies recognise the receptor's extracellular domain these results suggest the presence of two possible cleavage sites in the receptor leading to the variations of the A subunit molecular mass. Similar observations regarding the presence of two cleavage sites in the TSHR have recently been reported by Chazenbalk *et al.* (1997).

Analysis of the sugar residues on the TSHR has suggested that the upper band (117 kDa) of the full length TSHR represents fully glycosylated 'matured' TSHR while the lower band (99 kDa) represents a high mannose precursor (Costagliola *et al.* 1994, Johnstone *et al.* 1994, Chazenbalk *et al.* 1996, Couet *et al.* 1996a,b, Sanders *et al.* 1997). In our studies of native human TSHR, a small amount of the native receptor was present as a single band at 93 kDa whereas most of the receptor was detected by 3C7 at a molecular mass of 53 kDa representing the A subunit (Kajita *et al.* 1985a,b, Rees Smith *et al.* 1988, Loosfelt *et al.* 1992). 3C6 did not react well with the native TSHR extracted from human thyroid tissue in Western blotting analysis and the reason for this is not clear at present. The differences in molecular mass between native and recombinant human TSHRs may be due in part at least to differences in the extent of glycosylation. The T3-495 monoclonal antibody reacted with the 117 and 99 kDa bands of recombinant human TSHR but did not react with bands with molecular weights characteristic of the receptor's A or B subunits (at 1/1000 dilution). Neither 3C7 (diluted 1/1000) nor T3-495 (diluted 1/1000) antibodies reacted with the full length native receptor in Western blotting; this was most probably related to the lower concentration of this form of the receptor in native receptor preparations. 3C7 was produced in response to immunisation with the extracellular region of the TSHR while the antibody T3-495 was

produced using the C-terminus of the receptor (Loosfelt *et al.* 1992). Consequently, in our studies the reactivity of T3-495 with the full length receptor but not the A subunit would be expected. We were unable to detect any reactivity between T3-495 and the receptor B subunit in native or recombinant TSHR preparations but this could be due to a relatively low concentration of the B subunit.

Western blotting analysis showed that the recombinant receptor was present as both full length receptor and the A subunit whereas the native receptor extracted from thyroid tissue was present predominantly as the A subunit (Fig. 1). The differences in the amount of cleaved and uncleaved receptors present in recombinant and native TSHR preparations could be due to the difficulties in preventing rapid protease cleavage of the receptor in native thyroid tissue removed at operation whilst it is relatively simple to add protease inhibitors to cultured cells expressing the receptor as soon as they are harvested. Experimental evidence has suggested that the cleavage of the full length receptor in cultured L cells and thyroid cells may be dependant on a matrix metalloprotease (Couet *et al.* 1996a,b). In addition, recent studies have indicated that the extracellular domain may be cleaved in two positions giving rise to two different A subunit forms (Chazenbalk *et al.* 1997).

We were unable to detect inhibition of TSH binding to either human or porcine TSHRs with the rabbit polyclonal antibodies in contrast to other reports (Dallas *et al.* 1994, Harfst *et al.* 1994). Only one of our five monoclonal antibodies (3C7) was found to inhibit binding of 125 I-TSH to recombinant human, native human and porcine TSHR preparations very effectively in the TRAb assay with a dilution profile similar to that seen for patient sera. The affinity of this antibody (3C7) was similar to that of 3C6 which did not inhibit TSH binding and this suggested that the differences in properties were due to differences in the epitopes recognised. Other laboratories have produced mouse monoclonals which appear to inhibit TSH binding but to a much lesser extent than 3C7 (Johnstone *et al.* 1994, Seetharamaiah *et al.* 1995, Nicholson *et al.* 1996). Consequently, this study and other studies show that to date, most mouse monoclonal antibodies raised to the receptor do not inhibit TSH binding (Johnstone *et al.* 1994, Seetharamaiah *et al.* 1995, Nicholson *et al.* 1996). Furthermore, none of the monoclonal antibodies we have produced had the ability to stimulate cAMP production in CHO-TSHR cells (Nagayama *et al.* 1989) (data not shown). This is consistent with other reports in the literature (Johnstone *et al.* 1994, Seetharamaiah *et al.* 1995, Nicholson *et al.* 1996)

and overall current studies indicate that it is difficult to produce TSHR mouse monoclonal antibodies with thyroid stimulating activity. However, newer approaches using DNA immunisation may be successful (Costagliola 1996, Shimojo *et al.* 1996).

Purification of 3C7 IgG showed that a relatively high concentration (2.5 mg/ml) was needed to obtain over 90% inhibition of TSH binding. These results implied that the affinity of this monoclonal antibody was considerably lower than that of TSH. Consequently, we purified Fab fragments from both 3C7 and 3C6 monoclonal antibodies to investigate their binding to TSHR. The results of Scatchard analysis showed that 3C7 and 3C6 have affinities of 8.9×10^6 and 1.3×10^7 M⁻¹ respectively, approximately 1000 times lower than that of TSH (1×10^{10} M⁻¹); (data not shown). These results are consistent with the high concentrations of 3C7 IgG needed to inhibit TSH binding. However, our data suggest that 3C7 binds at or close to part of the TSH binding site either inhibiting TSH binding directly by binding to the TSH binding site or indirectly by steric hindrance and/or altering the conformation of the receptor preventing TSH binding.

Four of the five antibodies tested in the FACS analysis (3C6, 3F3, 3E4 and 5D8) bound to CHO-K1 cells expressing the human TSHR on their cell surface. Surprisingly 3C7, the antibody which shows inhibition of TSH binding to solubilised TSHR, did not appear to react with receptors on intact cells as judged by FACS analysis. This suggests that the epitope for 3C7 only becomes available after the receptors have been extracted from the cells and/or solubilised with detergent. The reason for this discrepancy is not clear at present but studies with native TSH receptors have shown that TSH binding affinity is increased markedly when the receptor is solubilised in non-ionic detergents (Brennan *et al.* 1980). This effect on TSH binding may be the result of subtle changes in the three dimensional structure of the receptor induced by detergent solubilisation. A similar effect of detergent solubilisation on the region of the receptor involved in 3C7 binding might explain the differences in 3C7 reactivity between cell surface and solubilised TSHR. This observation emphasises the importance of using a combination of FACS analysis and radioactively labelled probes in analysis of the receptor using experimentally produced antibodies.

Overall our studies indicate that the ³⁵S-labelled TSH receptor produced in the TnT system is very useful in screening and characterising experimentally produced antibodies to the TSH receptor. Using this screening technique we have been able to

select the first mouse monoclonal antibody which competes effectively with labelled TSH binding to detergent solubilised TSH receptors.

The monoclonal antibodies produced in this study using fusion proteins expressed in *E. coli* were of relatively low affinity. However, experimentally produced antibodies with the high affinities characteristic of TSH and TRAbs are potentially the most interesting but these have not been described as yet. Successful production of such antibodies is likely to be related to the availability of highly purified, correctly folded TSHR preparations for immunisation or use of techniques such as DNA immunisation (Costagliola 1996).

ACKNOWLEDGEMENTS

Y O, M M, R K and M P were in receipt of RSR fellowships. R K was also supported by a scholarship from the Ministry of Education, Science and Culture, Japan. Kathy Earlam provided excellent secretarial assistance.

REFERENCES

- Birk HW & Koepsell H 1987 Reaction of monoclonal antibodies with plasma membrane proteins after binding on nitrocellulose: renaturation of antigenic sites and reduction of non-specific antibody binding. *Analytical Biochemistry* **164** 12–22.
- Brennan A, Petersen VB, Petersen MM, Rees Smith B & Hall R 1980 Time dependent stabilisation of the TSH–TSH receptor complex. *FEBS Letters* **111** 35–38.
- Buckland PR & Rees Smith B 1984 A structural comparison of guinea pig thyroid and fat TSH receptors by photoaffinity labelling. *FEBS Letters* **116** 109–114.
- Chazenbalk GD, McLachlan SM, Nagayama Y & Rapoport B 1996 Is receptor cleavage into two subunits necessary for thyrotropin action? *Biochemical and Biophysical Research Communications* **225** 479–484.
- Chazenbalk GD, Tanaka K, Nagayama Y, Kakinuma A, Jaume JC, McLachlan SM & Rapoport B 1997 Evidence that the thyrotropin receptor ectodomain contains not one, but two cleavage sites. *Endocrinology* **138** 2893–2899.
- Chen C & Okayama H 1987 High efficiency transformation of mammalian cells by plasmid DNA. *Molecular and Cellular Biology* **7** 2745–2752.
- Chen S, Sawicka J, Betterle C, Powell M, Prentice L, Volpato M, Rees Smith B & Furmaniak J 1996 Autoantibodies to steroidogenic enzymes in autoimmune polyglandular syndrome, Addison's disease and premature ovarian failure. *Journal of Clinical Endocrinology and Metabolism* **81** 1871–1876.
- Colls J, Betterle C, Volpato M, Prentice L, Rees-Smith B & Furmaniak J 1995 Immunoprecipitation assay for autoantibodies to steroid 21-hydroxylase in autoimmune adrenal diseases. *Clinical Chemistry* **41** 375–380.
- Costagliola S 1996 Generation of antibodies recognising the native structure of the TSH receptor by immunisation with cDNA gene constructs. *Journal of Endocrinological Investigation* **19** 1.

- Costagliola S, Alcalde L, Ruf J, Vassart G & Ludgate M 1994 Overexpression of the extracellular domain of the thyrotrophin receptor in bacteria: production of thyrotrophin-binding inhibiting immunoglobulins. *Journal of Molecular Endocrinology* **13** 11–21.
- Couet J, Sar S, Jolivet A, Hai M-TV, Milgrom E & Misrahi M 1996a Shedding of human thyrotropin receptor ectodomain. *Journal of Biological Chemistry* **271** 4545–4552.
- Couet J, Bernard S, Loosfelt H, Saunier B, Milgrom E & Misrahi M 1996b Cell surface protein disulfide isomerase is in the shedding of human thyrotropin receptor ectodomain. *Biochemistry* **35** 14800–14805.
- Dallas JS, Desai RK, Cunningham SJ, Morris JC, Gattadahalli SS, Wagle N, Goldblum RM & Prabhakar BS 1994 Thyrotropin (TSH) interacts with multiple discrete regions of the TSH receptor: polyclonal rabbit antibodies to one or more of these regions can inhibit TSH binding and function. *Endocrinology* **134** 1437–1445.
- Davies Jones E & Rees Smith B 1984 A water-soluble fragment of the thyroid-stimulating hormone receptor which binds both thyroid-stimulating hormone and thyroid-stimulating hormone receptor antibodies. *Journal of Endocrinology* **100** 113–118.
- Davies Jones E, Hashim FA, Kajita Y, Creagh FM, Buckland PR, Petersen VB, Howells RD & Rees-Smith B 1985 Interaction of autoantibodies to thyrotropin receptor with a hydrophilic subunit of the thyrotropin receptor. *Biochemical Journal* **228** 111–117.
- De St Groth SF & Scheidegger D 1980 Production of monoclonal antibodies: strategy and tactics. *Journal of Immunological Methods* **35** 1–21.
- Frazier A, Robbins L, Stork P, Sprengel R, Segaloff D & Cone R 1990 Isolation of TSH and LH/hCG receptor cDNAs from human thyroid: regulation by tissue-specific splicing. *Molecular Endocrinology* **4** 1264–1276.
- Furmaniak J & Rees Smith B 1993 Immunity to the thyroid-stimulating hormone receptor. *Springer Seminars in Immunopathology* **14** 309–321.
- Greenwood FC, Hunter WM & Glover JS 1963 The preparation of ^{131}I -labelled human growth hormone of high specific radioactivity. *Biochemical Journal* **89** 114–123.
- Harfst E, Johnstone AP & Nussey SS 1992 Characterisation of the extracellular region of the human thyrotrophin receptor expressed as a recombinant protein. *Journal of Molecular Endocrinology* **9** 227–236.
- Harfst E, Ross MS, Nussey SS & Johnstone AP 1994 Production of antibodies to the human thyrotropin receptor and their use in characterising eukaryotically expressed functional receptor. *Molecular and Cellular Endocrinology* **102** 77–84.
- Harlow E & Lane D 1988 *Antibodies: A Laboratory Manual*. Cold Spring Harbor: Cold Spring Harbor Laboratory.
- Huang GC, Page MJ, Nicholson LB, Collison KS, McGregor AM & Banga JP 1993 The thyrotrophin hormone receptor of Graves' disease: overexpression of the extracellular domain in insect cells using recombinant baculovirus, immunoaffinity purification and analysis of autoantibody binding. *Journal of Molecular Endocrinology* **10** 127–142.
- Johnstone AP, Cridland JC, Da Costa CR, Harfst E & Shepherd PS 1994 Monoclonal antibodies that recognise the native human thyrotropin receptor. *Molecular and Cellular Endocrinology* **105** 121–129.
- Kajita Y, Rickards CR, Buckland PR, Howells RD & Rees Smith B 1985a Analysis of thyrotropin receptors by photoaffinity labelling: orientation of receptor subunits in the cell membrane. *Biochemical Journal* **227** 413–420.
- Kajita Y, Rickards CR, Buckland PR, Howells RD & Rees Smith B 1985b A structure for the porcine TSH receptor. *FEBS Letters* **181** 218–222.
- Kosugi S, Sugawa H & Mori T 1997 Epitope analysis of the thyrotropin receptor. *Molecular and Cellular Endocrinology* **128** 11–18.
- Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680–684.
- Libert F, Lefort A, Gerard C, Parmentier M, Perret J, Ludgate M, Dumont JE & Vassart G 1989 Cloning, sequencing and expression of the human thyrotropin (TSH) receptor: evidence for binding of autoantibodies. *Biochemical and Biophysical Research Communications* **165** 1250–1255.
- Loosfelt H, Pichon C, Jolivet A, Misrahi M, Caillou B, Jamous M, Vannier B & Milgrom E 1992 Two subunit structure of the human thyrotropin receptor. *Proceedings of the National Academy of Sciences of the USA* **89** 3765–3769.
- Misrahi M, Loosfelt H, Atger M, Sar S, Guiochon-Mantel A & Milgrom E 1990 Cloning, sequencing and expression of the human TSH receptor. *Biochemical and Biophysical Research Communications* **166** 394–403.
- Nagayama Y & Rapoport B 1992 The thyrotropin receptor 25 years after its discovery: new insight after its molecular cloning. *Molecular Endocrinology* **6** 145–156.
- Nagayama Y, Kaufman KD, Seto P & Rapoport B 1989 Molecular cloning, sequence and functional expression of the cDNA for the human thyrotropin receptor. *Biochemical and Biophysical Research Communications* **165** 1184–1190.
- Nicholson LB, Vlase H, Graves P, Nilsson M, Molne J, Huang GC, Morgenthaler NG, Davies TF, McGregor AM & Banga JP 1996 Monoclonal antibodies to the human TSH receptor: epitope mapping and binding to the native receptor on the basolateral plasma membrane of thyroid follicular cells. *Journal of Molecular Endocrinology* **16** 159–170.
- Powell M, Prentice L, Asawa T, Kato R, Sawicka J, Tanaka H, Petersen V, Munkley A, Morgan S, Rees Smith B & Furmaniak J 1996 Glutamic acid decarboxylase autoantibody assay using ^{125}I -labelled recombinant GAD₆₅ produced in yeast. *Clinica Chimica Acta* **256** 175–188.
- Prentice L, Sanders JF, Perez M, Kato R, Sawicka J, Oda Y, Jaskolski D, Furmaniak J & Rees-Smith B 1997 Thyrotropin (TSH) receptor autoantibodies do not appear to bind to the TSH receptor produced in an *in vitro* transcription/translation system. *Journal of Clinical Endocrinology and Metabolism* **82** 1288–1292.
- Rees Smith B, McLachlan SM & Furmaniak J 1988 Autoantibodies to the thyrotrophin receptor. *Endocrine Reviews* **9** 106–121.
- Rickards CR, Buckland PR, Rees Smith B & Hall R 1981 The interaction of Graves' IgG with the thyrotrophin receptor. *FEBS Letters* **127** 17–21.
- Sanders JF, Roberts S, Oda Y, Maruyama M, Furmaniak J & Rees Smith B 1997 Analysis of carbohydrate residues on human recombinant TSH receptor. *Journal of Endocrinological Investigation* **20** 26.
- Scatchard G 1949 The attraction of proteins for small molecules and ions. *Annals of the New York Academy of Sciences* **51** 660–672.
- Seetharamaiah GS, Wagle NM, Morris JC & Prabhakar BS 1995 Generation and characterisation of monoclonal antibodies to the human thyrotropin (TSH) receptor: antibodies can bind to discrete conformational or linear epitopes and block TSH binding. *Endocrinology* **136** 2817–2823.
- Shimojo N, Kohno Y, Yamaguchi KI, Kikuoka SI, Hoshioka A, Niimi H, Hirai A, Tamura Y, Saito Y, Kohn LD & Tahara K 1996 Induction of Graves-like disease in mice by

- immunisation with fibroblasts transfected with the thyrotropin receptor and a class II molecule. *Proceedings of the National Academy of Sciences of the USA* **93** 11074–11079.
- Smith DB & Corcoran LM 1994 Expression and purification of glutathione-S-transferase fusion proteins. *Current Protocols in Molecular Biology* **2** 16·7·1–16·7·7.
- Southgate K, Creagh FM, Teece M, Kingswood C & Rees Smith B 1984 A receptor assay for the measurement of TSH receptor antibodies in unextracted serum. *Clinical Endocrinology* **20** 539–543.

REVISED MANUSCRIPT RECEIVED 29 September 1997