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A mouse monoclonal antibody to the TSHR

Hanna Stankowiak-Kulpa^{1, 2, 3ABCDEF}, Jane Sanders^{1ABCDEF},
Hilde Depraetere^{1ABCDE}, Jennifer Jeffreys^{1ABCDEF}, Michele Evans^{1BE},
Tonya Richards^{1BCDE}, Jadwiga Furmaniak^{1, 2ABCDEF}
and Bernard Rees Smith^{1, 2ADEG}

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

- ¹ FIRS Laboratories, RSR Ltd., Llanishen, Cardiff, UK
- ² Department of Medicine University of Wales College of Medicine, Cardiff, UK
- ³ Department of Medicine, Poznań University of Medical Sciences, Poznań, Poland

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Summary

- Introduction:** Mouse monoclonal antibodies (mAbs) with the ability to inhibit thyrotropin (TSH) binding to the TSH receptor (TSHR) are useful tools to study TSH-TSHR interaction. The 3C3 mAb we produced was found to inhibit binding of TSH to human (h)TSHR but not to porcine (p)TSHR.
- Materials and Methods:** Purified 3C3 immunoglobulin G (IgG) and its antibody-binding fragment were prepared using standard methods and their ability to inhibit TSH binding to hTSHR or pTSHR was analyzed using a coated tube assay. The TSHR epitope reactive with 3C3 IgG was determined using Western blotting, ELISA based on peptides corresponding to the TSHR sequence, and the SPOT synthesis technique. RNA was isolated from 3C3 hybridoma cells and the mAb variable (V) region genes were sequenced and analyzed.
- Results:** 3C3 mAb had a 1×10^8 l/mol binding affinity to the hTSHR as assessed by Scatchard analysis. 3C3 reacted with the hTSHR region between amino acids (aa) 212-230, and two aa differences were found between the corresponding regions in the hTSHR and pTSHR. The light chain (LC) genes of 3C3 were derived from the Vk21 germ-line (97.6% homology) and Jk2 genes. The heavy chain (HC) genes were from the V130 germ-line (94.6% homology) combined with a D gene (not identified) and JH3 gene. The replacement/silent mutation ratios of 6.0 and 6.5 for the LC and the HC V regions, respectively, indicated that 3C3 underwent antigen-driven maturation.
- Conclusion:** Mouse mAbs of this type should be useful in studying the interactions between the TSHR, TSH, and mAbs in more detail.
- Key words:** thyrotropin receptor • monoclonal antibodies • thyroid • Graves's disease

Abbreviations: aa – amino acid(s), cDNA – complementary deoxyribonucleic acid, cpm – counts per minute, ELISA – enzyme-linked immunosorbent assay, Fab – antibody-binding fragment, TSH – thyrotropin, hTSH – human TSH, pTSH – porcine TSH, TSHR – GST/hTSHR – TSH receptor, glutathione sulfur transferase/hTSHR, TRAb – TSHR autoantibody, HC – heavy chain, IgG – immunoglobulin G, LC – light chain, mAb – monoclonal antibody, RNA – ribonucleic acid, mRNA – messenger RNA, PBS – phosphate-buffered saline, RT-PCR – reverse transcriptase polymerase chain reaction, Tg – thyroglobulin, V region – variable region.

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Author's address: Hanna Stankowiak-Kulpa, Department of Medicine University of Wales College of Medicine, 39 Jenkins Way, St. Mellons, Cardiff CF3 0RN, UK, tel.: +44 7792 995 549, e-mail: hania_kulpa@hotmail.com

INTRODUCTION

The thyrotropin receptor (TSHR) is one of the major thyroid autoantigens and as such has an important role in the pathogenesis of autoimmune thyroid disease (AITD)^{8, 23, 24}. Autoantibodies to the TSHR (TRAb) with TSH agonist activity, known as stimulating autoantibodies, are responsible for the development of Graves's disease^{8, 23, 24}. TRAb with TSH antagonist activity (blocking antibodies) play a role in the development of hypothyroidism^{8, 23, 24}. Studying the interaction between TRAbs and the TSHR is likely to lead to a better understanding of the pathogenesis of AITD^{8, 23, 24}.

Mouse monoclonal antibodies (mAbs) to the TSHR are useful tools for studying the receptor's structure^{15, 20, 21, 23, 32, 34}. Furthermore, mAbs that have the ability to inhibit binding of TSH to the TSHR allow studying the interaction between TSH and the receptor in more detail^{12, 15, 20, 25, 27, 28, 32, 34}. Here we describe an analysis of the interaction of 3C3 mAb, which can bind and inhibit TSH binding to the human TSHR (hTSHR) but not to the porcine TSHR (pTSHR). Studies with 3C3 provided a better insight into the binding site for TSH on the TSHR molecule and should be helpful in further understanding the interactions between TSH and the TSHR as well as mAbs and the TSHR.

MATERIALS AND METHODS

Production of GST/hTSHR fusion proteins

The full-length extracellular domain (Ex1 aa 1-418) and the TSHR extracellular domain fragments (Ex2 aa 1-367, Ex3 aa 1-164, Ex4 aa 1-260, TSHR 800 aa 91-363, TSHR 400 aa 246-380) were cloned into the bacterial expression vector pGEX-2T (Pharmacia, St. Albans, UK) to produce in-frame fusion proteins as described before^{20, 21}. The glutathione sulfur transferase (GST)/hTSHR fusion proteins were produced in *E. coli* (strain TG1) and were solubilized in 8 mol/l urea^{20, 21}.

Production of TSHR mouse mAbs

Six- to eight-week-old BALB/c mice were immunized with 50 µg of GST/hTSHR fusion proteins in complete Freund's adjuvant and boosted at 3–4 week intervals using incomplete Freund's adjuvant until the antibody titer was high, as described in detail before^{7, 21}. The mouse spleen cells were fused with X63-Ag8.653 myeloma cells (European Collection of Animal Cell Cultures; Porton Down, UK) and positive clones were selected and cloned by limiting dilution as described previously²¹. The hybridoma cells were cultured in 96-

-well plates in DMEM with high glucose, 20% fetal calf serum, and 10% Doma Drive (Immune System, Paignton, Devon, UK) using standard methods²¹.

Purification of 3C3 mouse mAb and Fab preparation

Immunoglobulin G (IgG) preparations were isolated from 3C3 hybridoma culture supernatants using a Prosep-A High Capacity column (Millipore UK Ltd., Watford, UK) according to the manufacturer's instructions. 3C3 purified IgG was incubated with a mixture of L-cystein/papain (both from Sigma Aldrich Co., Ltd., Gillingham, UK) at an enzyme-to-protein ratio of 1:50 and passed through a Prosep-A High Capacity column to separate intact IgG or Fc fragments from antibody-binding fragment (Fab) preparations²². Fab preparations were dialyzed against phosphate-buffered saline (PBS; 8 mmol/l Na₂HPO₄, 14 mmol/l KH₂PO₄, 26 mmol/l KCl, 136 mmol/l NaCl, containing 3 mmol/l of NaN₃, pH 7.4) overnight at 4°C and analyzed on 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE)¹⁶.

Inhibition of ¹²⁵I-TSH binding to the TSHR

TSH binding inhibition assays were carried out using tubes coated with solubilized hTSHR or pTSHR as described before^{26, 33} using reagents from RSR Ltd., Cardiff, UK. Briefly, 100 µl of purified IgG or Fab fragment were incubated in the TSHR-coated tubes at room temperature for 2 h with gentle shaking. After aspiration, the tubes were washed twice with 1 ml of assay buffer (50 mmol/l NaCl, 10 mmol/l Tris-HCl, pH 7.5, 0.1% Triton X-100) before addition of 100 µl of ¹²⁵I-TSH (80,000 counts per minute – cpm) and incubation at room temperature for 20 min with shaking. The tubes were then washed twice with 1 ml of assay buffer, aspirated, and counted in a gamma counter. Inhibition of TSH binding was expressed using the formula:

$$\text{binding inhibition} = [1 - B/Bo] \times 100,$$

where B – binding (in cpm) in the presence of test IgG or Fab, and Bo – binding (in cpm) in the presence of negative control IgG. 2B4 mAb (TSHR specific, binds to the C-terminus of the TSHR extracellular domain) was used as a positive control²⁰, while 2G2 (Tg specific RSR Ltd.), 14C4, and 3C6 mAbs (TSHR specific, interact with the epitopes within the extracellular domain of the TSHR)²⁰ were used as negative controls.

Scatchard analysis of ¹²⁵I-3C3 Fab binding to hTSHR

3C3 Fab was labeled with ¹²⁵I using the chloramine T method as described before²⁵. The characteristics of

^{125}I -3C3 Fab binding to detergent-solubilized recombinant hTSHR were assessed by Scatchard analysis³¹. Fifty μl of solubilized hTSHR was incubated with 50 μl of unlabeled 3C3 Fab (concentrations ranging from 0.1 $\mu\text{g/ml}$ to 200 $\mu\text{g/ml}$) and 100 μl ^{125}I -3C3 Fab (approx. 25,000 cpm per tube) for 2 h at room temperature (18–22°C). Fifty μl of normal pool serum and 2 ml of 16.5% PEG (RSR Ltd.) were added, mixed, and centrifuged at 1500 $\times$ g for 30 min at 4°C. The radioactivity was counted in the pellet remaining after aspiration. The concentrations of bound and free 3C3 Fab were calculated, and a plot of bound vs. bound/free 3C3 Fab was used to calculate the affinity of the 3C3 Fab binding to the hTSHR³¹.

Western blotting

Samples containing GST/hTSHR fusion proteins (Ex1: full-length TSHR, Ex2, Ex3, Ex4, TSHR 800, TSHR 400) were run on 9% SDS-PAGE, blotted onto nitrocellulose and Western blotting carried out according to the method of Birk and Koepsell¹. Supernatant from *E. coli* not expressing GST/hTSHR fusion proteins was used as a negative control. 3C3 IgG was used in a concentration of 10 $\mu\text{g/ml}$. The Western blotting reactions were developed using enhanced chemoluminescence (ECL) reagents (Perbio Science UK Ltd., Chester, UK).

TSHR peptide ELISA

Twenty-six peptides, each 20 aa long, corresponding to an extracellular domain of the hTSHR sequence (aa 22–416) were kindly provided by Dr. J. Morris (Mayo Clinic, Rochester, MN, USA)¹⁸. The peptides (10 $\mu\text{g/ml}$ in 100 mmol/l NaHCO_3 , pH 9.5) were coated 100 μl per well onto an enzyme-linked immunosorbent assay (ELISA) plate (Nunc, Maxisorp, Roskilde, Denmark). The coated plates were incubated overnight at 4°C, washed with 0.05% Tween-20 in PBS (4 times), then blocked with 250 μl of 1% bovine serum albumin in PBS (1 h at room temperature on an orbital shaker), and washed 4 times with 0.05% Tween-20 in PBS. Binding of the 3C3 IgG to the peptides was assessed by addition of 100 μl of purified 3C3 IgG (1 $\mu\text{g/ml}$ in sterile water) to the plate wells and incubation at room temperature on an orbital shaker. After 2 h, the plate wells were washed 6 times with 0.05% Tween-20 in PBS. Bound 3C3 mouse IgG was quantified using anti-mouse IgG conjugated to HRP (Perbio, Science UK Ltd.) followed by the peroxidase substrate tetramethylbenzidine (Enhanced K- blue[®] TMB Substrate, Neogen Corp., Lexington, UK). Absorbances were read using an ELISA plate reader (EL $\times$ 800 Biotek Insbument) at 450 nm².

The human steroid 21-hydroxylase peptide (C1, SSS-RVPYKDRARLPL), which binds to M21-OH5 mAb (RSR Ltd.), was used as a positive control, and a peptide reactive with 8B7 mAb, which binds to the C-terminal domain of the hTSHR (RSR Ltd.), and PBS were used as negative controls.

Epitope mapping with synthetic peptides prepared by SPOT synthesis

A SPOT membrane (prepared by Jerini AG, Berlin, Germany) with N-terminally acetylated overlapping peptides (n=8) covering the hTSHR aa 202–236 corresponding to the 3C3 epitope determined by peptide ELISA were used. Before use, the membrane was taken out of the freezer, allowed to warm to room temperature, and washed with methanol and TBS buffer (50 mmol/l Tris, 137 mmol/l NaCl, 2.7 mmol/l KCl, pH 8.0). Then the membrane was blocked by using blocking buffer (Western Blocking Reagent, supplied by Roche Diagnostics, Lewes, UK, diluted 1:10 in TBS) for 1 h at room temperature and washed with T-TBS (TBS pH 8.0 containing 0.05% Tween 20). 3C3 IgG was diluted to 1 $\mu\text{g/ml}$ in blocking buffer (diluted 1:20 in T-TBS) and added to the membrane. After 2 h incubation with 3C3 IgG the membrane was washed with TBS (3 times, 10 min each time). HRP-conjugated anti-mouse antibody (Sigma, UK; diluted 1:12,000 in blocking buffer 1:20 in TBS) was added to the membrane and incubated for 1 h at room temperature on an orbital shaker. Then the membrane was washed with T-TBS (3 times, 10 min each time) and placed in equal volumes of both ECL reagents for 5 min. Finally, the membrane was exposed to X-ray film and the film was developed using the standard procedure. Dry membrane was stored at –20°C¹⁰.

Comparison of mammalian TSHR sequences

Nucleotide sequences of hTSHR and pTSHR were taken from PUBMED (www.ncbi.nlm.nih.gov). The open reading frames within each of these sequences were found and translated to protein sequences using the EditSeq program (Dnastar Inc., Madison, Wisconsin, USA). MegAlign program (Dnastar Inc., Madison, Wisconsin, USA) was used to compare the TSHR sequences. In particular, three fragments of the TSHR found to be important for 3C3 mAb binding in Western blotting analysis (aa 165–245), in the peptide ELISA (aa 202–236), and as assessed by the SPOT synthesis technique (aa 212–230) were compared.

Variable region gene analysis

Ribonucleic acid (RNA) was isolated from 1.3×10^7 hybridoma cells secreting mouse mAb 3C3 by the

guanidinium thiocyanate extraction/isopropanol precipitation method³ and mRNA was prepared using the Dynabeads[®] mRNA Purification Kit (DynaL Ltd., UK) according to the manufacturer's instructions. Reverse transcriptase polymerase chain reaction (RT-PCR) was performed using SuperScript[™] One-Step RT-PCR with RT-Platinum[®] Taq mixture from Invitrogen (Paisley, UK).

3' and 5' PCR primers were synthesized according to Kettleborough et al.¹⁴ (Invitrogen, Paisley, UK). RT-PCR was performed according to the kit manufacturer's instructions; 40 cycles of the reactions: complementary DNA synthesis at 45°C for 30 min, denaturation at 94°C for 1 min, annealing at 72°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 10 min were carried out.

RT-PCR products of the heavy chain (HC) genes were cloned into pUC 18 using AatII and Sal I restriction sites and of the light chain (LC) genes into pNH 14 using XhoI and NcoI sites and sequenced by the Sanger-Coulson method^{29, 30} using the Sequenase version II kit from Amersham, UK.

RESULTS AND DISCUSSION

The 3C3 mouse mAb to the TSHR was derived from a BALB/c mouse immunized with GST/hTSHR fusion protein and was of the IgG1 subclass combined with the kappa LC. To date, TSHR mAbs raised in different strain of mice, including BALB/c mice^{5, 9, 12, 20, 21, 23, 34}, NMRI mice^{4, 12}, and 5C7/ BL6 mice¹⁹, have been reported. Also, different TSHR preparations were used to immunize mice and these included the GST/hTSHR fusion proteins as used in this study^{19, 21}, recombinant hTSHR ectodomain of full-length purified TSHR^{9, 12, 19, 20, 32}, recombinant murine TSHR ectodomain^{6, 34}, and complementary deoxyribonucleic acid (cDNA) coding for the hTSHR^{4, 5, 12}. TSHR mouse mAbs reported from other laboratories were predominantly of the IgG1 subclass^{6, 9, 12, 19, 32}; however, the mouse mAbs obtained from cDNA immunization reported previously were of the IgG2a subclass^{5, 12}.

3C3 mAb inhibited binding of ¹²⁵I-TSH to the tubes coated with hTSHR but not with pTSHR. Both 3C3 IgG and 3C3 Fab showed inhibition of ¹²⁵I-TSH binding to the hTSHR. In the case of 3C3 IgG, the inhibition was 33.3% at 200 µg/ml, and 22.8% inhibition was observed with 3C3 Fab at 200 µg/ml (Fig. 1). 3C3 IgG and 3C3 Fab at lower concentrations (0.2–20 µg/ml) did not have an effect on ¹²⁵I-TSH binding to the hTSHR (Fig. 1), 14C4 and 3C6 (negative control TSHR antibodies) did not inhibit ¹²⁵I-TSH binding to

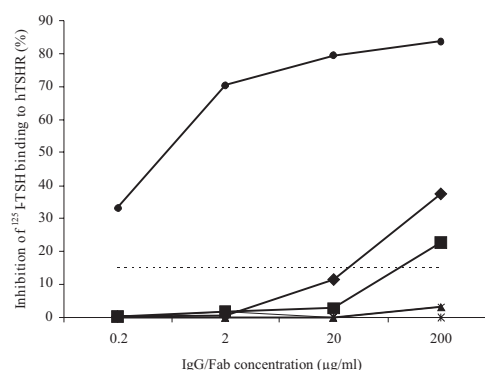


Figure 1. Effect of 3C3 mAb and 3C3 Fab on ¹²⁵I-TSH binding to the hTSHR. Effect on ¹²⁵I-TSH binding to the hTSHR by 3C3 IgG, 3C3 Fab and control mAbs: 3C3 IgG (◆), 3C3 Fab (■), 2G2 (▲), 14C4 (×), 3C6 (*), 2B4 (●). 2G2 mAb to thyroglobulin and TSHR specific mAbs (14C4, 3C6) that bind to the epitopes within the C-terminus of the TSHR were used as negative controls. 2B4 TSHR specific mAb that binds to the C-terminus of the TSHR extracellular domain was used as a positive control.

the hTSHR, while 2B4 (positive control TSHR mAb) showed dose-dependent inhibition of ¹²⁵I-TSH binding to the hTSHR (83.7% at 200 µg/ml, 79.7% at 20 µg/ml, 70.5% at 2 µg/ml, 33.3% at 0.2 µg/ml; Fig. 1). Neither 3C3 IgG nor 3C3 Fab showed inhibition of ¹²⁵I-TSH binding to the pTSHR (Fig. 2). 2B4 IgG (positive control antibody) showed dose-dependent inhibition of ¹²⁵I-TSH binding to the pTSHR (83.3% at 200 µg/ml, 79.3% at 20 µg/ml, 59.3% at 2 µg/ml, and 29.7% at 0.2 µg/ml), while 2G2 (negative control Tg mAb) and 3C6 and 14C4 (negative control TSHR mAbs) did not show an effect on ¹²⁵I-TSH binding to the pTSHR (Fig. 2). The differences in the effect on TSH binding to the TSHR observed between 3C3 and 2B4 are most likely related to differences in the

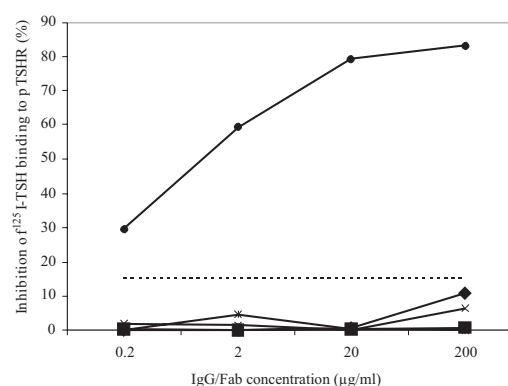


Figure 2. Effect of 3C3 mAb and 3C3 Fab on ¹²⁵I-TSH binding to the pTSHR. Effect on ¹²⁵I-TSH binding to the pTSHR by 3C3 IgG, 3C3 Fab and control mAbs: 3C3 IgG (◆), 3C3 Fab (■), 2G2 (▲), 14C4 (×), 3C6 (*), 2B4 (●). 2G2 mAb to thyroglobulin and TSHR specific mAbs (14C4, 3C6) that bind to the epitopes within the TSHR C-terminus were used as negative controls. 2B4 TSHR specific mAb that binds to the C-terminus of the extracellular domain was used as a positive control.

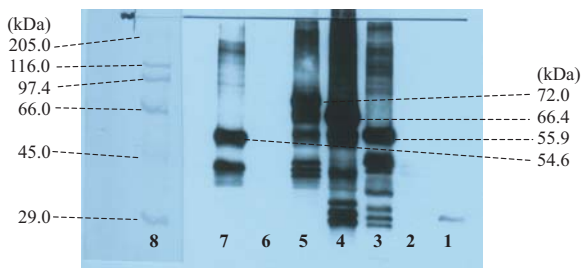


Figure 3. Western blotting – analysis of 3C3 IgG interaction with GST/hTSHR fusion proteins corresponding to the extracellular domain of the hTSHR. Control and TSHR preparations in lanes 1–8: lane 1 – S/N (supernatant from *E. coli* not expressing GST/hTSHR fusion proteins), lane 2 – 400 (aa 246–380), expressed molecular weight 40.7 kDa, lane 3 – 800 (aa 91–363), expressed molecular weight 55.9 kDa, lane 4 – Ex 2 (aa 1–367), expressed molecular weight 66.4 kDa, lane 5 – Ex 1 (aa 1–418), expressed molecular weight 72.0 kDa, lane 6 – Ex 3 (aa 1–164), expressed molecular weight 44.0 kDa, lane 7 – Ex 4 (aa 1–260), expressed molecular weight 54.6 kDa, lane 8 – high molecular weight marker.

affinities of these two mAbs (2B4 binding affinity for TSHR is 1×10^{10} l/mol¹²; for 3C3 affinity, see below).

These experiments demonstrated that 3C3 IgG and 3C3 Fab inhibit ¹²⁵I-TSH binding to the hTSHR and do not inhibit ¹²⁵I-TSH binding to the pTSHR (Fig. 2). Furthermore, ¹²⁵I-labeled 3C3 did not bind solubilized preparations of pTSHR, but showed dose-dependent binding to solubilized hTSHR (data not shown, see below).

Mouse mAbs to the TSHR that have the ability to inhibit TSH binding to the TSHR have been reported previously. Some of the previously reported mouse mAbs showed similar TSH binding inhibition activity to 3C3 mAb^{15, 32}; however some of the mAbs were more powerful inhibitors (50–90% inhibition)^{6, 20, 21}.

Table 1. Analysis of 3C3 IgG binding to the hTSHR peptides in ELISA (absorbance at 450 nm)

Controls/peptides	Absorbance at 450 nm (duplicates)	
	reading 1	reading 2
Blank	+0.002	–0.002
M21-OH	0.115	0.118
8B7	0.056	0.054
PBS	0.028	0.030
aa 202-221	0.937	0.823
aa 217-236	>4	>4

Table 1 contains absorbance values at 450 nm for 3C3 IgG, which reacted with 2 out of 26 hTSHR peptides (aa 202–221 and aa 217–236). In addition, absorbance values at 450 nm are shown for the blank, the PBS negative control, a positive control (21OH mAb, M21-OH5, binding to a 21-OH peptide), and a negative control (8B7 mAb that binds to the C-terminal part of the TSHR). Absorbance at 450 nm obtained with 3C3 mAb reacting with the remaining hTSHR peptides tested ranged 0.006–0.156.

3C3 Fab binding affinity for hTSHR as assessed by Scatchard analysis was $1.0 (\pm 0.1) \times 10^8$ l/mol. 3C3 Fab affinity was higher than the affinities of the mAbs derived from the BALB/c mice immunized with the GST/hTSHR fusion proteins reported previously³². Mouse mAbs of higher affinity (10^{10} – 10^{11} l/mol) were isolated from NMRI or BALB/c mice immunized with cDNA coding for the hTSHR^{4, 25}.

In order to identify the specific epitope on the hTSHR recognized by 3C3 mAb, several experiments were carried out. First, 3C3 reactivity with different fragments of TSHR's extracellular domain expressed as GST/hTSHR fusion proteins was analyzed by Western blotting. These analyses indicated that 3C3 recognized a TSHR epitope between aa 165 and 245 (Fig. 3). This observation was confirmed and the epitope was determined in more detail by peptide ELISA. 3C3 IgG was able to bind to peptides 13 and 14 covering 202–236 amino acids of the hTSHR's extracellular domain, but not to the peptides corresponding to the hTSHR sequences between aa 22–201 and aa 237–416 (Table 1). The aa 202–236 epitope was then analyzed further by SPOT synthesis analysis. As shown in Fig. 4, 3C3 IgG reacted with the peptides corresponding to the amino acids of the extracellular domain of the hTSHR between aa 212 and 230. This result, compared with the previous result of the peptide ELISA (aa 202–236), suggests the hTSHR aa from 212 to 230 are important for 3C3 binding to the hTSHR.

The TSHR epitope reactive with 3C3 IgG (aa 212–230) is in close proximity to the aa involved in forming the TSH binding pocket on the TSHR described previously¹², particularly the aa sequence 246–260^{12, 19, 20}. Furthermore, Vlase et al.³⁴ reported mouse mAbs reactive with aa 217–236 (overlapping with the 3C3 epitope) which had the ability to inhibit TSH binding to the TSHR.

We then compared the hTSHR sequence reactive with 3C3 hTSHR (aa 212–230) with the correspond-



Figure 4. Small SPOT membrane (8 overlapping peptides). Sequences of the peptides attached to the membrane are shown below (peptides reacting with 3C3 IgG are marked in bold): 1 – LDAVYLNKKNKYLT (aa 202–215), 2 – YLNKKNKYLTVIDK (aa 206–218), 3 – KNKYLTVIDKDAF (aa 209–221), 4 – **YLTVIDKDAFGGV** (aa 212–224), 5 – **VIDKDAFGGVYSG** (aa 215–227), 6 – **KDAFGGVYSGPSL** (aa 218–230), 7 – FGGVYSGPSLLDV (aa 221–233), 8 – VYSGPSLLDVST (aa 224–236). The membrane was reacted with 3C3 IgG at 1 µg/ml (see text for details).

hTSHR	(165) TSIPVNAFQGLCNETLTCLKLYNNGFTSVQGYAFNGT
pTSHR	(165) TSIPANAFQGLCNETLTCLKLYNNGFTSVQGHAFNGT
hTSHR	(201) K LD A VYLN K NKY L <u>TVIDKDAFGGVYSGPSL</u> L DV S Q T SV T AL P SK G (245)
pTSHR	(201) K LD A VYLN K NKY L <u>TVIDKDAFGGVYSGPTL</u> L DV S Y T SV T AL P PK G (245)

Figure 5. Comparison of hTSHR and pTSHR aa 165-245 sequences detected in Western blotting analysis. The sequence of the hTSHR corresponding to 3C3 mAb epitope determined in peptide ELISA (aa 202-236) is marked in bold and the 3C3 mAb epitope detected in SPOT synthesis (aa 212-230) is underlined. The aa differences between hTSHR and pTSHR are marked with o above the letter. Single letter code for amino acids is used. Explanations: Y – tyrosine, F – phenylalanine, S – serine, T – threonine.

ing sequence of pTSHR (Fig. 5). Three base pair changes: adenine to thymine at 672 bp, adenine to thymine at 674 bp, and guanine to cytosine at 686 bp, were detected within the corresponding sequences of the hTSHR and the pTSHR, resulting in two amino acid differences. Tyrosine at position 225 in the hTSHR amino-acid sequence corresponds to phenylalanine in the pTSHR amino acid sequence and serine at aa 229 to threonine in the pTSHR. It is likely that these two amino acid differences within the aa 212-230 fragment of the pTSHR and the hTSHR contribute to the observed differences in the interaction of 3C3 with the hTSHR and the pTSHR.

Variable (V) region genes of 3C3 IgG were sequenced and analyzed. The degree of complementarity of the 3C3 LC to its germ-line counterpart (Vk21)¹¹ was 97.6%. There were 3 oligonucleotide mutations (2 replacement and 1 silent) within FWR I (95.5% homology), 2 replacement mutations within FWR II (95.6% homology), 1 replacement mutation within CDR II (95.2% homology), and 1 replacement mutation within the CDR III (93.3% homology). Overall, the replacement to silent (R/S) mutation ratio for the LC V region was 6.0.

The degree of complementarity of the 3C3 HC to its germ-line gene V130¹⁷ was 94.6% due to 3 replacement type mutations within FWR III (96.5% homology), 1 replacement mutation and 1 silent mutation

within FWR II (95.2% homology), 1 silent mutation within FWR I (98.8% homology), 6 replacement mutations within CDR II (88.2% homology), and 3 replacement mutations within CDR I (80% homology). In total, 15 mutations were found within the 3C3 HC. There were 2 silent mutations and 13 replacement mutations. The R/S ratio for the whole 3C3 HC V region was 6.5. The high R/S ratios for the V region of the LC and the HC of 3C3 suggest that this mAb underwent antigen-driven maturation¹³.

Overall, a high affinity mouse mAb to the TSHR (3C3) having the ability to inhibit TSH binding to the hTSHR but not to the pTSHR has been produced and characterized. Detailed analysis of the 3C3 binding site on the TSHR has allowed the identification of two amino acid differences between the corresponding sequences of the hTSHR and the pTSHR that are likely to be important in the specificity of 3C3 to the hTSHR. The availability of mouse mAbs of this type may be very helpful in further studies of the interaction of TSH with the TSHR of different species and detailed studies of the binding site for TSH on the TSHR.

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