

**Interactions of human autoantibodies with
the TSH receptor at the molecular level;
new perspectives for the management of
Graves' disease**

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ABBREVIATIONS

aa	amino acid
AITD	autoimmune thyroid disease
AMP	adenosine monophosphate
ATD	antithyroid drugs
CD	cleavage domain
cDNA	complementary deoxyribonucleic acid
CDR	complementarity determining region
CHO	Chinese hamster ovary
DNA	deoxyribonucleic acid
ECD	extracellular domain
Fab	fragment, antigen binding
FSH	follicle stimulating hormone
FSHR	follicle stimulating hormone receptor
HC	heavy chain
IgG	immunoglobulin G
L	litre
LC	light chain
LH	luteinising hormone
LHR	luteinising hormone receptor
LRD	leucine rich domain
MAbs	monoclonal antibodies
NIBSC	National Institute for Biological Standards and Control
TMD	transmembrane domain
TRAb	TSH receptor autoantibodies
TSH	thyrotropin
TSHR	thyrotropin receptor
TSHR260	TSHR extracellular fragment aa 22-260
U	unit

INTRODUCTION

Autoimmune thyroid disease

Autoimmune thyroid disease (AITD) is the most common autoimmune disorder with the prevalence of Graves' disease calculated at approximately 1,000/100,000 population and hypothyroidism (Hashimoto's disease) at 790/100,000 (1). In addition post-partum thyroiditis that affects approximately 5-9% of women after delivery is related to autoimmune responses towards the thyroid gland (2). The main features of Graves' disease are hyperthyroidism, goiter and ophthalmopathy and in some patients pretibial myxoedema (3). Graves' ophthalmopathy is characterized by the enlargement of the orbital tissues often associated with mononuclear cell infiltration which lead to proptosis, oedema, chemosis, and in severe cases vision impairment (4).

Autoimmune background of Graves' disease is reflected in the presence of serum autoantibodies to the specific thyroid antigens; thyroglobulin, thyroid peroxidase and the TSH receptor (TSHR) (5,6). TSHR autoantibodies (TRAbs) are serological markers of Graves' disease and are detectable in over 90% of patients when measured using sensitive assays (7). The majority of TRAbs have the ability to stimulate thyroid hormone synthesis (stimulating TRAbs) and are responsible for hyperthyroidism of Graves' disease (5).

Current treatment options for Graves' disease include: antithyroid drugs (ATDs; methimazole, carbimazole and propylthiouracyl), reduction of thyroid volume by surgery (subtotal thyroidectomy) and ablation of the thyroid with radioactive iodine (8-10). ATDs have been in clinical use since 1940s and provide effective control of hyperthyroidism in the majority of patients. ATDs reduce thyroid hormone synthesis by the thyrocytes by way of inhibiting iodine organification and iodotyrosyl coupling. In addition propylthiouracyl decreases the conversion of thyroxine to triiodothyronine on the periphery (8). Treatment with ATDs results in decrease of circulating thyroid hormone levels and alleviation of the clinical symptoms. Furthermore the levels of TRAbs usually

fall during the treatment with ATDs. The mechanism of ATDs' effect on TRAb levels is not clear. It may be that controlling thyroid over-activity may lead to reduction of expression of the TSHR in the thyroid and decrease of antigen driven autoantibody production by the lymphocytes (3). Also it has been proposed that ATDs have the direct immunosuppressive effect on the lymphocytes (3). However the most likely mechanism may be related to restoration of immunological regulation as the euthyroid state is achieved either by ATDs or thyroid surgery. Hyperthyroidism is associated with aberration of the autoimmune responses which leads to increased production of thyroid stimulating autoantibodies and worsening of hyperthyroidism (11). This mechanism was first proposed by Volpe (12) and recently supported by Laurberg (11). Understanding of this cycle has an important impact on the strategies to control Graves' disease with the main focus on controlling thyroid hormone overproduction as quickly as possible. Any improvement in achieving this goal would mean an improvement in controlling Graves' disease course, remission and relapse.

Almost 90% of patients with Graves' disease present with eye signs but only in approximately 5% of patients the symptoms are serious and require special interventions (4). The orbits in Graves' ophthalmopathy show inflammatory infiltration of the connective tissue and extraocular muscles although the muscle fibres often remain intact. Enlargement of the muscles is due to a build up of glycosaminoglycans (hyaluronic acid) and associated oedema. Accumulation of glucosaminoglycans and oedema together with an increase in orbital fat volume are responsible for enlargement of the connective tissue compartment (4). It is now well accepted that autoimmune responses against the TSHR expressed in the orbital tissues are involved in the pathogenesis of Graves' ophthalmopathy. The TSHR expression has been demonstrated in orbital fibroblasts and preadipocytes (13) and furthermore the evidence of humoral and cellular autoimmune responses to the TSHR in the orbit have been now shown (4,13).

Currently there is no specific treatment for Graves' ophthalmopathy. In most cases the symptoms resolve with the control of thyroid over-activity. Cigarette smoking aggravates the symptoms and the disease tends to be more severe in smokers than in non-smokers (4). In severe cases the treatment is based on controlling the symptoms and preventing

the optic nerve damage by use of glucocorticoids, orbital irradiation or surgical decompression. More recently trials with rituximab, a monoclonal antibody against CD-20 antigen on the B cells have shown some encouraging results (14). However treatment with rituximab is not targeting specifically the mechanisms involved in the pathogenesis of Graves' ophthalmopathy and is associated with possible side effects. Consequently, specific and effective new therapies for Graves' ophthalmopathy are clearly needed.

TSH receptor autoantibodies

There are two types of TRAbs. In the majority of patients TRAbs bind to the TSHR, mimic the biological activity of TSH and stimulate cyclic AMP pathway and thyroid hormone synthesis (stimulating TRAbs, TSHR agonists). In some patients TRAbs bind to the receptor but do not activate cyclic AMP and act as TSHR antagonists (blocking TRAbs) (5).

The main feature of TRAbs is high affinity binding to the TSHR and the ability to inhibit TSH binding to the receptor. Consequently the hypothalamic-pituitary-thyroid feedback mechanism is no longer effective and the symptoms of thyroid over-activity or under-activity develop depending on the type of TRAbs involved. The ability of TRAbs to inhibit TSH binding has been exploited in developing sensitive assays to measure TRAbs in patients' sera (7).

The TSHR epitopes for stimulating and blocking TRAbs and TSH have been a subject of numerous studies (5,6). These studies progressed when the TSHR cDNA sequence has become available (6). Studies on binding of TRAbs and TSH to the recombinant TSHR expressed in Chinese Hamster ovary (CHO) cells and measurement of cyclic AMP response have provided valuable information. Furthermore it was possible to study the binding and biological activities of the TSHR-luteinizing hormone receptor (LHR) chimeric proteins or the TSHRs containing amino acid mutations (6,15). Studies using mouse monoclonal antibodies (MAbs) to the TSHR provided further details on the binding sites on the TSHR for TRAbs and TSH (16). Taken together these studies indicated that patient serum TSHR autoantibodies with thyroid stimulating activity, thyroid blocking activity and TSH itself all bind to the "TSHR binding pocket" on the extracellular domain (ECD) of

the TSHR formed by discontinuous regions of the TSHR aa 246-260, 277-296 and 381-385 (16). The three ligands compete effectively with each other for the binding site on the receptor. Consequently, the binding sites for TSH and TRAbs (stimulating and blocking) on the TSHR ECD overlap extensively however there are subtle differences in TSHR amino acids involved in the interactions (16).

Understanding of the interactions of TSH and the antibodies which have different biological activities with the TSHR is important for understanding the mechanisms of the TSHR activation and to develop new strategies to control Graves' disease.

TSH receptor structure

The TSHR is a G protein coupled receptor expressed on the basal membrane of thyroid epithelial cells. It has a key role in regulation of thyroid function and is a major thyroid autoantigen in AITD (5). The TSHR belongs to a family of the glycoprotein hormone receptors together with LHR and follicle stimulating hormone receptor (FSHR). The glycoprotein hormone receptors show homology at the cDNA and amino acid (aa) levels however are characterised by distinct hormone binding specificity (15).

The TSHR molecule is organised in three major domains in addition to the N-terminal and the C-terminal arms. The TSHR N-terminal arm extends between aa 1-35, however aa 1-21 are the signal sequence and are not present in the mature processed molecule (15). The TSHR ECD consists of the leucine rich repeats domain (LRD; aa 36-281) and the cleavage domain (CD; aa 282- 409). The TSHR transmembrane domain (TMD; aa 410-699) is organised in 7 membrane spanning regions characteristic of the G protein coupled receptors and the last TSHR aa 700-764 form the cytoplasmic tail of the protein (17).

Although biologically active recombinant TSHR could be expressed in CHO cells obtaining highly purified receptor preparations suitable for crystallisation and X-ray diffraction proved very difficult (6). One of the reasons was that the TSHR preparations (native and recombinant) tend to be very unstable during purification processes. In the absence of

the structural information the TSHR comparative model was built by Núñez Miguel et al (17). In this model the LRD was based on the structure of porcine ribonuclease inhibitor and shown to have a characteristic horseshoe shape with opposing concave and convex surfaces. The CD was modelled using a tissue inhibitor of matrix metalloproteinases-2 as a template and the TMD using a structure of bovine rhodopsin as a template (17). This comparative TSHR model was then used to build a model of the TSHR—TSH complex. The interactions observed in the complex were validated using results from experimental studies on the TSH-TSHR binding. Although these studies provided valuable insight how TSH and TSHR may interact there were however limitations in particular in understanding the interactions between the TSHR and TRAbs.

Consequently solving the structure of the TSHR in complex with TSH or TRAb remained to be an important goal in the studies on the TSHR.

Human monoclonal autoantibodies to the TSHR

Over 50 years ago Adams and Purves showed that Graves' serum immunoglobulins had the ability to stimulate the thyroid (18). Nearly 20 years after this milestone observation Smith and Hall demonstrated that thyroid-stimulating immunoglobulins were the autoantibodies to the TSHR (19). However it took many years for the scientific community to accept the concept that autoantibodies to the TSHR indeed had a key role in the pathogenesis of Graves' disease (5,6). In particular the mechanism of TSHR activation by stimulating TRAb was difficult to study unless the MAbs with the properties of serum autoantibodies were available (6). Following the cloning of the TSHR many efforts in different laboratories took place to produce mouse MAbs that would have at least some characteristics of human TRAbs (6,20). The most important criteria were that the MAbs had the ability to inhibit TSH binding to the receptor and to stimulate cyclic AMP production in TSHR-expressing CHO cells (6,20). Different immunisation strategies including DNA immunisation were used over the years without success and it was even suggested that antibody activation of the TSHR needed at least two antibodies binding at the same time (21). However MAbs with strong thyroid stimulating and TSH binding inhibiting activities were produced in mice and hamsters

(21). These MAbs had high affinity for the TSHR (approx. 10^{10} L/mol) and competed effectively in dose dependent manner with TSH, each other and patient TRAbs with stimulating or blocking activities (21).

Production of animal MAbs to the TSHR that had some of the characteristics of the human TRAbs represented a major step forward in the studies on the TSHR. However the most challenging goal in thyroid research was to isolate a thyroid stimulating immunoglobulin from a patient with Graves' disease. One of the reasons for this challenge was that these antibodies are present in serum at very low concentrations (5,6,22). Isolation of human MAbs to the TSHR remained elusive despite many attempts in different laboratories. Some human MAbs were produced but failed to fulfil the essential criteria set out by McLachlan and Rapoport (6,22). These criteria included: 1) MAbs should be of IgG class, 2) MAbs should be active at nanogram per millilitre concentrations (show high affinity for the TSHR), 3) MAbs activity should be removed by incubation with specific antigen (TSHR), 4) MAbs should be active when purified to homogeneity and 5) recombinant Fab expressed from the heavy chain (HC) and the light chain (LC) V region sequences of MAbs should show comparable activity to native antibody.

The first human MAbs of this type are described in this thesis.

AIMS OF THE STUDY

The overall aims of this study were to understand the interactions of the TSHR autoantibodies with the TSHR at the molecular level and to provide new perspectives for the management of thyroid diseases. In particular:

- Isolation and characterisation of a human monoclonal autoantibody to the TSHR with thyroid stimulating activity
- Isolation and characterisation of a human monoclonal autoantibody to the TSHR with thyroid blocking activity
- Preparation and purification of a stable complex of the TSHR with a thyroid stimulating autoantibody for crystallisation and X-ray diffraction analysis
- Analysis of the binding arrangements between the TSHR and a thyroid stimulating autoantibody in the solved crystal structure
- Preparation and purification of a stable complex of the TSHR with a thyroid blocking autoantibody for crystallisation and X-ray diffraction analysis
- Analysis of the binding arrangements between the TSHR and a thyroid blocking autoantibody in the solved crystal structure
- Analysis of the interactions between TSHR and TSH using a comparative model built based on the crystal structures of the FSHR-FSH and the TSHR-thyroid stimulating autoantibody complexes
- Comparison of the interactions between the TSHR and thyroid stimulating autoantibodies, thyroid blocking autoantibody and TSH
- Providing new perspectives for the management of thyroid diseases

THE STUDIES

Human monoclonal thyroid stimulating autoantibody

There were many attempts in different laboratories including our own over many years to isolate human MAb to the TSHR (6). It was widely appreciated that the availability of such reagents would lead to progress in understanding the pathogenesis of Graves' disease and would have an impact on disease management. For these reasons isolation of a human thyroid stimulating autoantibody became a holy grail for thyroidologists (6,22).

The first human thyroid stimulating autoantibody was isolated in our laboratory from the peripheral blood lymphocytes of a 19-year old male patient (**Publication 1**). The patient presented with hyperthyroidism (free thyroxine at 14.9 pmol/L and TSH at <0.01mU/L) associated with high serum TRAbs levels (400U/L in the TSH binding inhibition assay and 900U/L in the stimulation of cyclic AMP assay) and was diagnosed with Graves' disease. In addition the patient suffered from type 1 diabetes mellitus and was on insulin twice daily. At the time of blood donation the patients was treated with carbimazole at 60mg a day. The study was approved by the Gwent Ethical Committee and the patient gave written consent for the study. The lymphocytes were separated from 20 mL of the peripheral blood using centrifugation on the Ficoll-Paque gradient, immortalised with Epstein Barr virus and cultured using standard techniques. The presence of TSHR autoantibodies in the lymphocyte culture supernatants was measured using a sensitive inhibition of TSH binding assay. The colonies from the positive wells were stabilised by fusion with mouse/human hybrid cell line (K6H6/B5) followed by cloning by limiting dilution. After 4 rounds of cloning and screening (in all approximately 16,500 wells were screened) a single stable colony producing TSHR MAb (M22) was isolated.

M22 is an IgG1/lambda antibody and binds to the TSHR with a high affinity (5×10^{10} L/mol). Furthermore binding affinity of M22 Fab is essentially the same as the intact immunoglobulin (**Publication 1**). Both M22 IgG and Fab are potent inhibitors of

¹²⁵I-labelled TSH binding to the TSHR with over 90% inhibition evident at ng/mL concentrations. In the experiments as little as 10 ng/mL of M22 IgG and 5 ng/mL of Fab showed approx. 20% inhibition. In contrast donor serum IgG showed inhibition of ¹²⁵I-labelled TSH binding to more than 90% at µg/mL concentrations (**Publication 1**).

M22 IgG and Fab are powerful stimulators of cyclic AMP in CHO cells expressing TSHR. As little as 3 ng/mL of M22 IgG or Fab was able to increase cyclic AMP production to about 5x basal level and concentrations of 10 ng/mL cause approx. 10x basal stimulation. Monoclonal M22 IgG was found to be approx. 3000x more potent than donor serum IgG. M22 IgG shows similar cyclic AMP stimulating activity as porcine TSH however M22 IgG and Fab have greater potency compared to human TSH. For example 200 pmol/L of M22 Fab increased cyclic AMP to 40x basal while human TSH showed similar level of stimulation at 20,000 pmol/L (**Publication 1**).

Binding of ¹²⁵I-labelled M22 IgG or Fab to the TSHR is inhibited in a dose dependent manner by sera from patients with Graves' disease. Sera from patients containing both types of TRAbs (with thyroid stimulating activity and with blocking activity) competed effectively with labelled M22 binding to the TSHR (**Publication 1**). Furthermore M22 has the ability to inhibit binding of mouse MAbs with TSHR stimulating activity (**Publication 1**).

Overall M22 is the first human MAb to the TSHR with all the characteristics of patient serum autoantibodies. The availability of M22 opened new opportunities to study the interactions of the TSHR with TRAbs and with TSH itself.

Crystal structure of the complex of the TSHR with the stimulating human MAb M22 at 2.55 Å resolution

Since the cloning of the TSHR in 1989 recombinant preparations of the receptor have been produced in different expression systems (15). However only mammalian cell systems have proven to be suitable for expressing biologically active full length TSHRs (15). One of the reasons was that the TSHR has a complex structure consisting of a large ECD, a cysteine-rich CD and a TMD with 7 helices embedded in the lipid bilayer (17).

Furthermore experimental observations indicated that the receptor is susceptible to proteolysis and denaturation and very unstable under purification process conditions (5,6).

Availability of pure preparations of a thyroid stimulating MAb M22 allowed developing new strategies for the TSHR purification. Experimental evidence indicated that M22 binds to the TSHR with a very high affinity (**Publication 1**). Further studies have shown that M22 dissociation rate from the TSHR is slow with essentially no dissociation after 180 min compared to 50% of bound porcine TSH dissociating in approximately 20 min (23). Consequently strong and stable binding of M22 could protect the TSHR from degradation during purification. To explore this strategy the TSHR extracellular fragment aa 22-260 (TSHR260) with a 6-histidine C-terminal tag was expressed in High Five insect cells using baculovirus system (**Publication 2**). TSHR cDNA construct coding for the N-terminal aa sequence 1-260 was used which includes the natural signal sequence (aa 1-21) resulting in expression of TSHR aa 22-260 protein (**Publication 2**). High Five cells were infected with baculovirus stock and cultured using standard procedures. M22 Fab was added to cell cultures 96 hours post infection to a final concentration of 2 µg/mL.

TSHR260-M22 Fab complex was purified from the insect cell culture supernatants using a series of steps. The purified complex was then deglycosylated, separated from glycosidases and impurities and concentrated to 32 mg/mL. The integrity of the complex was analysed by gel filtration chromatography, polyacrylamide gel electrophoresis and the N-terminal amino acid sequencing (**Publication 2**). Purified TSHR260-M22 Fab complex was then used for crystallisation trials. The TSHR260-M22 Fab complex formed crystals in 8% polyethylene glycol 8000, 0.1mol/L 2-(N-morpholino)ethanesulfonic acid pH6.0 and 0.25 mol/L zinc acetate and diffracted X-ray to 2.55 Å resolution (**Publication 2**).

The solved structure provided for the first time the molecular detail of the TSHR itself. The TSHR260 structure forms a slightly curved helical tube with the opposed concave and convex surfaces constructed from leucine-rich repeat motifs. The inner surface of the tube is lined with hydrophobic residues. The concave surface of the structure is made

up by 10-stranded beta-sheets while the convex surface is formed by eight small strands comprising of beta sheets. All five known glycosylation sites on the TSHR260 contain sugar residues and all are present on the convex surface of the molecule. The TSHR N-terminal C 31 and C 41 are disulfide bonded (Figure 1) (**Publication 2**).

In the complex M22 Fab embraces the TSHR260 concave surface at 90 degrees to the TSHR tube axis. Almost the entire concave surface of the TSHR is involved in the interactions with M22 Fab. The TSHR residues from all 10 leucine-rich repeats form interactions with M22 Fab. Both the HC and the LC of M22 are involved in the interactions with the TSHR and the interacting residues are contributed from the hypervariable regions with the majority from LC CDR2, HC CDR2 and HC CDR3. M22 LC combines predominantly with the C-terminus whereas M22 HC combines predominantly with the N-terminus of the TSHR260 concave surface. The area buried in the interface is very large covering 2500 Å². There is a strong network of interactions in the complex; there are 22 hydrogen bonds and salt bridges, 17 nonhydrogen polar bonds, and 14 hydrophobic contacts. All glycosylation sites are distant from the interface (Figure 1). (**Publication 2**).

The electrostatic surface potential of the TSHR260 concave surface shows predominantly negatively charged patches at the C-terminus and predominantly positively charged patches at the N-terminus. The distribution of the charged patches on the surface of M22 Fab is complementary to that present on the TSHR260. In the complex the negatively charged region on the surface of M22 combines with the positively charged region at the TSHR260 N-terminus while the positively charged patch of M22 surface combines with the TSHR260 C-terminal negative patches (Figure 2)(**Publication 2**).

In 2005 the crystal structure of the FSHR ECD in complex with FSH at 2.9 Å resolution was described by Fan and Hendrickson (24). The binding arrangements in the TSHR260-M22 complex and in the FSHR-FSH complex were compared and showed that they were remarkably similar (**Publication 2**). In both complexes the area buried in the interface is very large (2500 Å² and 2600 Å² respectively) and when the two structures were superimposed there was essentially no variation on the core atoms between the LRDs

(Publication 2).

The binding arrangements in the TSHR260-M22 Fab complex were validated by mutation experiments. In particular, TSHR mutations R80A, E107A, R109A, K129A, K183A, Y185A and R255A had an effect on M22 (but not TSH) cyclic AMP stimulating activity and all these residues were found to form strong interactions with M22 in the solved structure of the complex. Furthermore, these residues were engaged in 9 out of 22 hydrogen bonds and salt bridges in the network of interactions (**Publication 2**). Further validation of the binding arrangements between TSHR260 and M22 Fab was provided by amino acid mutations in M22 Fab itself (25). Recombinant M22 Fab was expressed in *E. coli* and showed the same biological activity as the native Fab. Single amino acid mutations were produced using standard methods and mutated M22 Fab was used to validate interactions found in the complex (25). M22 HC R28D mutation caused a loss of stimulating activity. This M22 residue is involved in forming three salt bridges with the TSHR D151, polar interactions with TSHR F153 and I152 as well as hydrophobic contacts with TSHR I152 and F153. M22 mutated at HC D52K was unable to stimulate the receptor and in the solved complex this residue is found in strong electrostatic interactions with TSHR R80 and polar interactions with TSHR H105 (25).

Some of the interactions in the TSHR260-M22 Fab complex are of particular interest. For example, TSHR R38, K58, R80, H105 and K129 form a positively charged area interacting with a negatively charged cavity on the M22 antigen binding surface. A small molecule designed to interfere with this area would be expected to inhibit autoantibody binding to the TSHR. Furthermore, mutation experiments indicate that TSHR R255 is critical for stimulation by all thyroid stimulating antibodies (including M22, serum TRAbs and animal stimulating MAbs) but not by TSH. Small molecules designed to interfere in interactions in the region around R255 should act as inhibitors of stimulating antibodies (**Publication 2**).

Comparison of the interactions of the TSHR with the stimulating human MAb (M22) and TSH

The sequence and structural homologies among the glycoprotein hormones and among the glycoprotein hormone receptors themselves allow building the comparative models with a great degree of confidence (**Publication 3**). The crystal structure of the FSHR LRD in complex with FSH at 2.9 Å resolution (24) showed similarities in the binding arrangements with the TSHR LRD-M22 structure (**Publication 2**). The crystal structure of FSH has been solved in 2001 (26) and was used to build a comparative model of TSH (17). Consequently, a comparative model of the TSHR LRD-TSH complex was built based on the structures of the FSHR LRD-FSH complex (2.9 Å resolution) and the TSHR LRD-M22 complex (2.55 Å resolution). In the TSHR LRD-TSH model the structure of the TSHR LRD was from the TSHR LRD-M22 complex and the binding arrangements between TSH and the TSHR were based on the FSHR LRD-FSH complex (27).

The binding arrangements between the TSHR and M22 or TSH were analysed in the TSHR LRD-M22 and the TSHR LRD-TSH complexes. In the complexes both TSH and M22 position themselves in a very similar way with respect to the TSHR LRD. Both TSH and M22 embrace almost the entire concave surface of the TSHR LRD with very similar accessible surface areas in the interface; 2514 Å² and 2533 Å² for TSHR-M22 and TSHR—TSH complexes, respectively (**Publication 3**). However the network of interactions between TSHR and M22 is stronger than between TSHR and TSH and involves a greater number of hydrogen bonds and salt bridges (n=22 compared to n=7 respectively) with fewer hydrophobic interactions (n=14 and n=22 respectively) (**Publication 3**). Furthermore the gap volume index which is a measure of strength with which the molecules interact was greater in the TSHR LRD-TSH complex compared to the TSHR LRD-M22 complex indicating that the interactions between the TSHR and TSH are weaker. These observations are consistent with the lower binding affinity of human TSH for the TSHR compared to the high affinity binding of M22 to the receptor (**Publication 3**).

The interactions between TSH and M22 involve the residues from all 10 beta strands in the TSHR LRD (**Publication 3**). There is however a remarkable pattern in which TSH α and

β chain residues interact with the TSHR leucine rich repeats compared to the interactions of M22 HC and the LC. M22 LC and TSH β chain form more interactions with the repeats at the two ends of the LRD than with the central part while M22 HC and TSH α chain interact mostly with the repeats in the central part of the LRD (**Publication 3**). Furthermore M22 HC and TSH α chain residues usually interact with the same TSHR residues and M22 LC and TSH β chain residues interact with another set of the same residues (**Publication 3**). These observations indicate that M22 LC mimics the TSH β chain in its interactions with the TSHR LRD while M22 HC mimics the interactions of the TSH α chain (**Publication 3**). This example of molecular mimicry must be related to strong evolutionary mechanisms which are not yet understood.

Analysis of the TSHR LRD-M22 complex showed the complementarity between the electrostatic surface potential of interacting faces of TSHR and M22 (**Publication 2**). Similar observations are also true in the case of the TSHR LRD-TSH complex (**Publication 3**). Furthermore, M22 and TSH have similar electrostatic surface potential in the areas that interact with the concave surface of the TSHR LRD (Figure 2) (**Publication 3**).

Experimental studies and the analysis of the TSHR LRD-M22 complex indicated that the area around TSHR R255 is essential for biological activity of TSHR stimulating antibodies but not TSH and forms strong interactions with M22 (**Publication 2**). This area is exposed in the TSHR—TSH complex and may be a good candidate for the development of small molecule inhibitors that would block autoantibody binding yet permit TSH binding. (**Publication 3**).

Isolation of a thyroid stimulating human MAb (K1-18) and a thyroid blocking human MAb (K1-70)

In search for more human MAbs which would represent a repertoire of TRAbs present in patient sera peripheral lymphocytes were isolated from 54-year old female patient with hypothyroidism (**Publication 4**). The patient had 8 year history of AITD and had originally presented with hyperthyroidism which was treated with methimazole. Four years prior to the lymphocyte collection the patient developed hypothyroidism and was treated with thyroxine. At the time of blood collection she was in euthyreosis and

was positive for TRAbs at 160 U/L National Institute for Biological Standards and Control; NIBSC (90/672) measured by inhibition of TSH binding to the TSHR, thyroid peroxidase autoantibodies at >500U/mL (NIBSC 66/387) and was negative for thyroglobulin autoantibodies (**Publication 4**). The donor serum showed a weak cyclic AMP stimulating activity as well as a weak TSHR blocking activity. The patient's lymphocytes were immortalised with Epstein Barr virus, cultured and screened for TSHR binding activity as described for M22 (**Publication 1**). This produced two stable distinct clones secreting antibodies with TSH binding inhibiting activity; K1-18 (IgG1/kappa) and K1-70 (IgG1/lambda) (**Publication 4**).

Both K1-18 and K1-70 are powerful inhibitors of TSH binding to the receptor with as little as 10 ng/mL giving approx. 20% inhibition and 100ng/mL 80-90% inhibition (**Publication 4**). K1-18 and K1-70 were approx. 10,000 more active than the K1 donor serum in inhibition of TSH binding to the receptor. Furthermore inhibiting activities of K1-18 or K1-70 Fabs were similar to intact IgGs. The binding affinity of K1-18 for the TSHR was 6.7×10^9 L/mol and in the case of K1-70 the affinity was 3.9×10^{10} L/mol with the respective Fab giving similar affinity values (**Publication 4**). In addition both MAbs are strong inhibitors of patient serum TRAbs binding to the TSHR (**Publication 4**).

K1-18 and K1-70 differed however in terms of their effect on TSHR cyclic AMP activity. K1-18 has the ability to stimulate cyclic AMP activity in CHO-TSHR cells with concentrations below 10 ng/mL giving a clear stimulation and the maximum responses at the similar concentrations to that observed with M22 IgG. The stimulatory potency of K1-18 IgG is however lower than M22 IgG when compared to the NIBSC 90/672 at 155 U/mg and 286 U/mg respectively (**Publication 4**). This may be consistent with the lower binding affinity of K1-18 compared to M22 (**Publication 1**). In contrast to K1-18 the other MAb K1-70 does not have the ability to stimulate cyclic AMP production. It has the ability to block TSH stimulation in TSHR transfected CHO cells in a dose dependent manner at ng/mL concentrations with complete inhibition at 100 ng/mL (**Publication 4**). Furthermore K1-70 IgG has the ability to inhibit TSHR-stimulating activities of K1-18, M22 and patient serum stimulating autoantibodies (**Publication 4**). K1-70 IgG has no effect on TSHR constitutive activity (**Publication 4**). Analysis of the V region genes of K1-

18 and K1-70 showed that the two antibodies have evolved separately from each other and were derived from different B cell clones (**Publication 4**).

Taken together K1-18 and K1-70 are distinct TSHR autoantibodies with different biological activities and have the characteristics of patient serum TRAbs. Isolation of these two distinct MAbs from a single blood sample of one patient provides the evidence for the first time that a patient can produce stimulating and blocking autoantibodies to the TSHR at the same time (**Publication 4**).

Crystal structure of the complex of the TSHR with the blocking human MAb K1-70 at 1.9 Å resolution

The relationship between the binding sites on the TSHR and the biological activities of TSHR autoantibodies has been controversial (5,6,21). The experimental evidence suggested that the epitopes for the stimulating and blocking autoantibodies are located on the TSHR ECD and overlap extensively (21). Availability of K1-70 provided an opportunity to study the interactions of TSHR blocking autoantibodies with the receptor at the molecular level.

The approach to produce and purify stable complexes of the TSHR260 with K1-70 Fab was similar to that described in the case of TSHR260-M22 Fab complexes (**Publication 2**). The purified complex after deglycosylation and final polishing was concentrated to 10 mg/mL and set for crystallisation trials. The TSHR260—K1-70 Fab complex formed best crystals at 16% polyethylene glycol 3350 and 0.2 mol/L sodium malonate pH 5.0. A single crystal diffracted X-ray to 1.9 Å resolution (**Publication 5**).

The TSHR260—K1-70 Fab structure shows that the structure of the TSHR260 is very similar to that in the TSHR260-M22 Fab complex (**Publication 2**). When the structures of the TSHR260 from the two complexes are superimposed there is essentially no deviation on the core atoms (**Publication 5**). This provides further evidence that there is no movement in the TSHR260 structure on complexation with stimulating or blocking autoantibodies. Due to the higher resolution of the TSHR260—K1-70 Fab complex the

electron densities for the N-terminus of the TSHR have become available. This provided details of disulphide bonding at the N-terminus; C24 is bonded with C29 and C31 is bonded with C41. Consequently in the TSHR the 1st and the 2nd cysteines and the 3rd and the 4th cysteines are bonded and form the N-terminal cap of the LRD. These arrangements are different than in the FSHR (**Publication 5**) and (24).

The binding arrangements between the TSHR260 and K1-70 Fab are remarkably similar to those with M22 (and TSH) (Figure 1). However K1-70 Fab positions itself on the TSHR LRD more N-terminally than M22 Fab and is bound at an approx. rotation of 155 degrees with respect to the position of M22 Fab. Furthermore the orientation of K1-70 and M22 Fab HCs and LCs are opposite (Figure 1). The area buried in K1-70 Fab binding interface with the TSHR260 is very large covering 2565 Å² and this is similar to that observed for M22 or TSH in complex with TSHR (**Publications 2 and 3**). The network of interactions in the TSHR260—K1-70 Fab complex involves 25 hydrogen bonds and salt bridges, 19 water-mediated hydrogen bonds, 11 polar interactions and 19 hydrophobic contacts. The electrostatic surface potential of the interacting faces of TSHR260 and K1-70 Fab shows polarity in terms of charge distribution. In the complex the TSHR260 areas with predominantly negative charge combine with the positively charged areas on K1-70 Fab binding surface and the positively charged area on the TSHR260 combines with the negative patches on the K1-70 Fab surface. The negatively charged patches on the antigen binding surface of K1-70 are contributed from the HC and the positively charged patches from the LC (**Publication 5**). The charge contributions from the HC and LC on the antigen binding surface of M22 are opposite (**Publication 2**).

Although K1-70 Fab and M22 Fab show very similar binding arrangements with the TSHR260 there is a major difference in relation to the involvement of the N- and the C-termini in the interactions. K1-70 Fab binds more towards the N-terminus of the TSHR260 concave surface and the interactions extend between TSHR D36 and D203. In contrast the binding sites for M22 Fab extend further towards the C-terminus and span the region between TSHR D36 and N256 (**Publications 2 and 5**). K1-70 Fab interacts with the TSHR residues from the leucine repeats 1-7 and there is only one interaction with the repeat 8. In contrast M22 Fab interacts with the residues from leucine rich

repeats 1-10 (**Publications 2 and 5**).

The TSHR260—K1-70 Fab structure was validated in experiments using TSHR containing amino acid mutations and recombinant native and mutated K1-70 Fab. In particular TSHR mutations K58A, I60A, E61A, Y82A, R109A and K183A had an effect on K1-70's ability to inhibit TSH stimulation of cyclic AMP production in CHO-TSHR cells. In the structure K58, Y82 and R109 are involved in 7 out of 25 hydrogen bonds and salt bridges while K58, I60, E61, Y82, R109 and K183 are involved in numerous other interactions (**Publication 5**). Furthermore, mutation of K1-70 HC W97A and HC N100A, LC R94D, HC Y99A and HC N32A resulted in loss of the ability of K1-70 Fab to inhibit TSH-induced stimulation of cyclic AMP production. These five K1-70 Fab residues were found to be involved in the interactions with the TSHR in the complex (**Publication 5**).

The high resolution crystal structures of the TSHR in complex with a stimulating and blocking autoantibody and the availability of a comparative model of the TSHR—TSH complex provides an opportunity to compare how the three ligands interact with the receptor.

DISCUSSION

Human MAbs to the TSHR have a unique role in the studies on Graves' disease and stringent criteria had been proposed for isolation of the MAbs (22,28). In the case of all three TSHR human MAbs described here (M22, K1-18 and K1-70) these criteria have been met. In particular; the MAbs are of IgG1 subclass, are potent TSH binding inhibitors at ng/mL concentrations, are potent stimulators of cyclic AMP (M22 and K1-18) or a blocker of TSH mediated cyclic AMP stimulation (K1-70) at ng/mL concentrations, the activity of MAbs is adsorbed by TSHR, the MAbs are active when purified to homogeneity and the MAbs are active when expressed as recombinant Fabs (**Publications 2 and 4**) (29). Consequently, M22, K1-18 and K1-70 are representative of the TRAbs present in patients' sera.

An insight into antibody V region genes usage should be helpful in understanding the mechanisms of autoimmune responses in Graves' disease. The HC and LC V regions from all three MAbs (M22, K1-18 and K1-70) show the evidence of somatic mutations indicating that the MAbs have been subject to antigen-driven maturation (**Publications 1 and 4**). M22, K1-18 and K1-70 HC V region genes are derived from the same germline family (VH5). It may well be that this small gene family has a significant role in the formation of TSHR autoantibodies with different biological activities (**Publications 1 and 4**). Analysis of the V regions of K1-18 and K1-70 has provided a compelling evidence that the two antibodies (stimulating and blocking) present in the same patient at the same time have evolved separately from each other and must have originated from different B cell clones (**Publication 4**). Further studies on the molecular origin of M22, K1-18 and K1-70 may contribute to unravelling the genetic background, inheritance and evolution of autoimmune response to the TSHR.

Isolation of two MAbs (K1-18 and K1-70) with different biological activity (stimulating and blocking) from the same blood sample provides the first evidence that a patient can produce both types of autoantibodies at the same time (**Publication 4**). This is consistent with the observations that in some patients the symptoms of hyper- and hypothyroidism vary over time (5). Consequently, the clinical symptoms depend on the relative

concentrations and activities of blocking and stimulating TRAbs at any one time. The interaction of TSHR with TSH itself may also contribute to the clinical presentation. Finally, the ability of the thyroid gland to respond to the stimulating activities of TRAbs or TSH is involved. Understanding how different factors may affect thyroid function should be helpful in the clinical management of patients with AITD.

Human MAbs to the TSHR have already found some practical applications. Use of pure preparations of M22 has led to improving the assays for detection of TRAbs in patient sera with the development of the third generation sensitive assays (7). Furthermore M22 IgG serves as a standard for calibration of various assays in different laboratories and allowed replacement of the depleted NIBSC reference preparation 90/672 (<http://www.nibsc.ac.uk/>).

Solving the crystal structure of the TSHR bound to a human thyroid stimulating autoantibody provides opportunities for designing new strategies for management of Graves' disease (**Publication 2**). Remarkable similarities between the binding of TSH and M22 with the TSHR could present a problem for developing the specific inhibitors of thyroid stimulating autoantibodies binding to the receptor (**Publication 3**). However the thyroid stimulating autoantibody inhibitors should block binding of TRAbs yet still allowing for TSH to bind and provide physiological control of thyroid gland function. The candidate area for such inhibitors is on the TSHR region around R255 that was found critical for stimulating activity of all stimulating antibodies (human and animal) but not TSH. The area including TSHR N208, Q235, R255, and N256 is involved in the interactions with M22 but is exposed in the TSHR—TSH complex and a small molecule targeting this area could block binding of a stimulating autoantibody yet allow TSH to bind (**Publications 2 and 3**).

Small molecule inhibitors with the ability to interfere in interactions between autoantibodies and the TSHR would act at the beginning of the pathogenic pathway in Graves' disease. This would represent a major change in the approach to control thyroid over-activity which is currently centred further down the chain of events ie on inhibiting thyroid hormone synthesis, reduction of thyroid volume or destruction of the thyroid

gland with radioisotopes. The pharmacological approach to control hyperthyroidism has not changed since the 1940s and developing new generation of drugs would be a major advance (3). In addition to control Graves' thyrotoxicosis the new generation drugs should be helpful in controlling Graves' ophthalmopathy in situations when inhibiting thyroid stimulating antibodies to the TSHR in the orbit would be of an advantage.

Analysis of the solved structures of the TSHR-M22 and TSHR—K1-70 complexes together with analysis of the model of TSHR—TSH complex (**Publications 2, 3 and 5**) provided valuable insight into how the different ligands interact with the receptor. There are remarkable similarities in overall binding arrangements between the TSHR and the stimulating and blocking autoantibodies and TSH itself. All the ligands interact with the concave surface of the TSHR LRD and the interface areas are very large in all three complexes. There are however differences in the type of interactions; both types of autoantibodies are involved in numerous hydrogen bonds and salt bridges and fewer hydrophobic interactions than TSH. Stimulating MAb M22 and TSH showed striking similarities in the way M22 HC and LC mimic binding of TSH α and β chains to the receptor (**Publication 3**). This evidence for molecular mimicry is of great interest from the evolutionary point of view. It would be important to understand what evolutionary mechanisms were involved in driving the immune system to develop an autoantibody that can act like a hormone at both levels; the biological activity and molecular interactions with the TSHR. Although the blocking MAb K1-70 shows overall similar interactions with the TSHR as M22 and TSH it shows some differences (**Publication 5**). K1-70 binding involves the TSHR residues in the N-terminal and the central parts of the concave surface of the LRD and K1-70 does not interact with the residues in the 9th and 10th leucine rich repeat with only one interaction in the 8th repeat (**Publication 5**). Remarkably the TSHR residues in the center of the concave face of the LRD interact with M22, K1-70 and TSH (**Publication 5**). It has been demonstrated that the human MAbs M22 and K1-70 have the characteristics of patient serum TRABs with stimulating and blocking activities respectively (**Publications 1 and 4**). Consequently the molecular details of their interactions with the TSHR are very likely to be a true picture of the interactions in general ie that the stimulating TRABs mimic TSH in their interactions with the TSHR while the blocking TRABs do not mimic TSH or the stimulating autoantibodies

in the interactions with the TSHR.

Human MAbs to the TSHR may also have important applications in clinical use. For example stimulating autoantibodies such as M22 or K1-18 may be alternatives to recombinant TSH in the diagnosis, monitoring and facilitating treatment of patients with thyroid cancer (30). Furthermore human TSHR MAbs may be used for thyroid (or thyroid cancer metastases) imaging or for targeted drug delivery. A thyroid blocking autoantibody such as K1-70 may have clinical applications in treatment of thyrotoxicosis in the presence of stimulating autoantibodies. Furthermore K1-70 has a great potential to control Graves' ophthalmopathy when blocking the activity of the TSHR would be of an advantage (4,13). At present when the technology to produce antibodies for *in vivo* clinical applications is well advanced these perspectives appear to be quite realistic (31).

Availability of human MAbs to the TSHR and the molecular detail of the interactions of the TSHR with human MAbs and TSH open new avenues towards a better understanding of the TSHR function, the autoimmune response towards the TSHR and towards developing new therapeutic strategies for management of thyroid diseases.

CONCLUSIONS

- 1 Thyroid stimulating human monoclonal antibodies (M22 and K1-18) and thyroid blocking human monoclonal antibody (K1-70) with the characteristics of patient serum autoantibodies have been isolated.
- 2 The molecular details of the interactions of a thyroid stimulating human monoclonal antibody (M22) with the TSHR have been solved at 2.55Å resolution.
- 3 The molecular details of the interactions of a thyroid blocking human monoclonal antibody (K1-70) with the TSHR have been solved at 1.9Å resolution.
- 4 The molecular details of the interactions of TSH with the TSHR have been modelled with confidence.
- 5 M22 (stimulating autoantibody) and K1-70 (blocking autoantibody) and TSH itself interact with an extensive part of the TSHR concave surface.
- 6 The stimulating autoantibody (M22) mimics TSH binding closely.
- 7 The interactions of a blocking autoantibody (K1-70) with the TSHR do not mimic TSH.
- 8 The blocking autoantibody K1-70 interacts less with the C-terminal part of the TSHR concave surface than the stimulating autoantibody M22.
- 9 The central part of the TSHR concave surface interacts with the stimulating and blocking autoantibodies and with TSH.
- 10 The interactions of M22 and K1-70 with the TSHR most probably represent the interactions of the stimulating and blocking autoantibodies in general.
- 11 The crystal structure of the TSHR-M22 complex provides the rational basis for the design of small molecule inhibitors of thyroid stimulating autoantibodies.
- 12 The crystal structure of the TSHR—K1-70 complex provides the rational basis for the design of small molecule inhibitors of thyroid blocking autoantibodies.
- 13 The thyroid stimulating human monoclonal autoantibodies (such as M22 or K1-18) have potential *in vivo* applications as alternatives to recombinant human TSH, thyroid imaging and for targeted drug delivery.
- 14 The thyroid blocking human monoclonal autoantibodies (such as K1-70) have potential in *in vivo* applications as new therapeutics for Graves' ophthalmopathy.

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FIGURE LEGENDS

Figure 1 Comparison of the relative positions of the thyroid stimulating autoantibody M22 and the thyroid stimulating hormone receptor (TSHR)-blocking monoclonal autoantibody (K1-70) on the thyroid stimulating hormone receptor leucine rich repeat domain (TSHR-LRD)

A Crystal structure of TSHR260 in complex with thyroid stimulating monoclonal autoantibody M22 Fab. TSHR is in cyan, M22 light chain (LC) is in green, and the M22 heavy chain (HC) is in blue. The N-linked carbohydrates observed in the structure are shown in yellow and carbohydrate bound asparagines residues are labelled

B Crystal structure of the TSHR260 in complex with the TSHR-blocking monoclonal autoantibody K1-70 Fab. The TSHR-LRD is aligned in the same orientation as the TSHR LRD from the complex with M22 Fab (A). The relative positions of M22 and K1-70 Fabs bound to TSHR corresponds to a rotation about the TSHR vertical axis of approximately 155° reflecting their different binding sites.

(from P Sanders *et al.* Crystal structure of the TSH receptor (TSHR) bound to a blocking-type TSHR autoantibody. *Journal of Molecular Endocrinology* 2011 **46**: 81-99).

Figure 2 Electrostatic potential of the interacting surfaces of M22, TSHR LRD and TSH showing some important charged residues. The N- and C- termini of TSHR LRDs are marked. Some important interactions between the

receptor and the ligands are shown by connecting lines. Negative patches are shown in red and positive in blue.

(from R Núñez Miguel *et al.* Thyroid stimulating autoantibody M22 mimics TSH binding to the TSH receptor leucine rich domain: a comparative structural study of protein-protein interactions. *Journal of Molecular Endocrinology* 2009 **42**: 381-395).

Figure 1

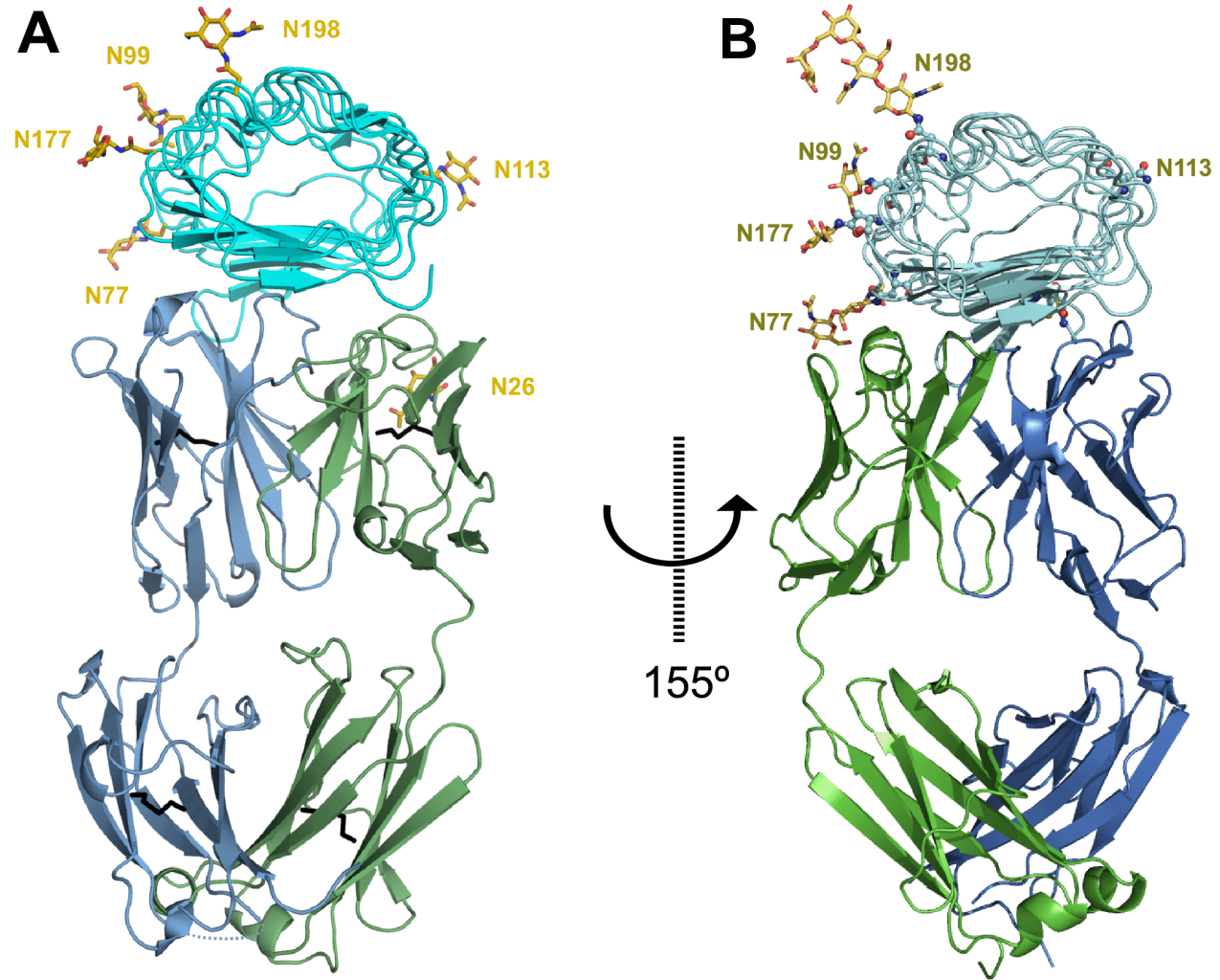
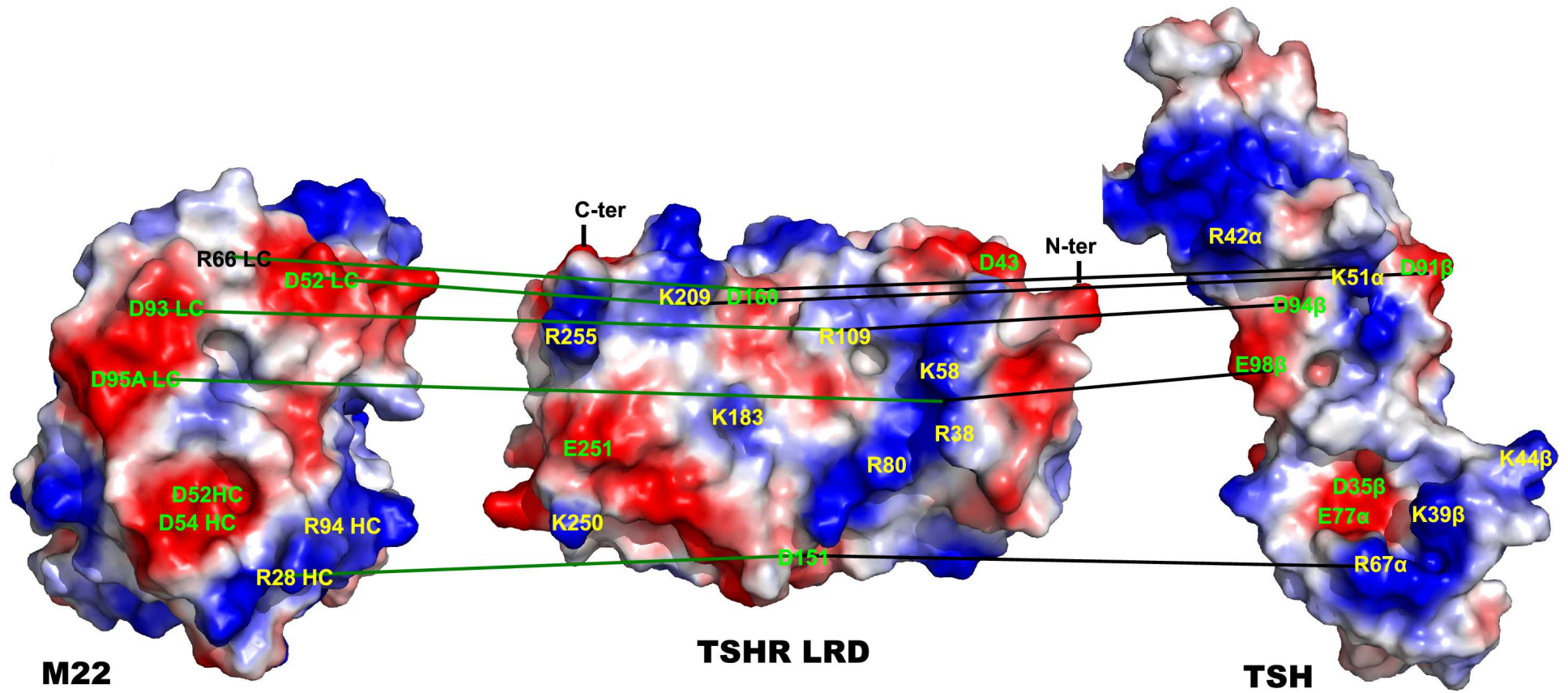


Figure 2



ABSTRACT

Isolation of the first human thyroid stimulating monoclonal autoantibody (MAb; M22) has been a major breakthrough in thyreology. M22 binds to the TSH receptor (TSHR) with a high affinity (5×10^{10} L/mol) and has the ability to inhibit TSH binding to the receptor at ng/mL concentrations. M22 at very low concentrations (ng/mL) also has the ability to stimulate cyclic AMP in the CHO cells expressing TSHRs. Both M22 IgG and Fab have comparable TSH binding inhibiting and cyclic AMP stimulating activities. The activity of M22 is preadsorbed with TSHR preparations and recombinant M22 Fab are as active as the hybridoma secreted MAb. Consequently M22 is representative of patient serum TSHR autoantibodies.

Availability of M22 allowed purification and crystallization of the TSHR-M22 complex and the solved structure (2.55 Å resolution) showed for the first time how a thyroid stimulating autoantibody binds to the TSHR. Furthermore a structure of a TSHR fragment (amino acids 22-260) has become available. These developments have allowed building a model of the TSHR—TSH complex. Analysis of the interactions between M22 and TSH indicated that remarkably M22 heavy and light chains mimic TSH α and β chains in the interactions with the TSHR.

Further two human MAbs were isolated from one blood sample from a patient at the same time; a thyroid stimulating K1-18 and a thyroid blocking K1-70 MAbs with high binding affinity for the TSHR (6.7×10^9 L/mol and 3.9×10^{10} L/mol respectively). This was the first evidence that TSHR autoantibodies with different biological activities can be produced by a patient at the same time.

A complex of the TSHR bound to the blocking MAb K1-70 was solved at 1.9 Å resolution. Binding of K1-70 with the TSHR showed great similarities to binding of a stimulating MAb M22. However, K1-70 forms interactions only with the N-terminal and the central parts of the concave surface of the TSHR leucine rich domain (LRD) in contrast to M22 and TSH that bind to the whole surface from the N- to the C-terminus. Furthermore K1-70 does not mimic TSH in its interactions with the TSHR.

Availability of human MAbs to the TSHR and the molecular detail of the interactions of the TSHR with human MAbs and TSH open new avenues towards a better understanding of the TSHR function, the autoimmune response towards the TSHR and towards developing new therapeutic strategies for management of thyroid diseases.

STRESZCZENIE

Podstawą pracy habilitacyjnej jest cykl badań poczyniony od izolacji pierwszego ludzkiego przeciwciała stymulującego tarczycę (M22) a następnie blokującego przeciwciała (K1-70). Dostępność tych przeciwciał pozwoliła na otrzymanie kompleksu receptora dla TSH (TSHR) związanego z M22 oraz z K1-70 i poznanie jak białko receptorowe wiąże się z przeciwciałem stymulującym i blokującym. Postępy w poznaniu struktury receptora pozwoliły na zbudowanie wiarygodnego modelu kompleksu wiązania pomiędzy TSHR i TSH.

Izolacja ludzkiego stymulującego tarczycę przeciwciała monoklonalnego (MAb;M22) była przełomowym osiągnięciem w tyreologii. Po raz pierwszy był to niezaprzeczalny dowód na rolę jaką przeciwciało wiążące się z wysokim powinowactwem (5×10^{10} L/mol) z TSHR odgrywa w patofizjologii nadczynności tarczycy w chorobie Graves-Basedowa. Wysoko oczyszczone M22 ma zdolność hamowania wiązania TSH z receptorem w bardzo niskich stężeniach (ng/mL). M22 ma także zdolność stymulowania aktywności cylicznego AMP w ng/mL stężeniach. Zarówno M22 IgG i fragment Fab mają zdolności hamowania wiązania TSH i stymulacji cyklicznego AMP. Aktywność M22 jest eliminowana po adsorpcji z preparatami TSHR. Ponadto rekombinowane M22 Fab ma takie same właściwości jak natywne przeciwciało. Wszystkie te cechy spełniają kryteria konieczne do uznania ludzkiego przeciwciała receptorowego za reprezentatywne dla przeciwciał obecnych w surowicy chorych.

Dostępność M22 pozwoliła na krystalizację kompleksu M22 z TSHR i poznanie na poziomie molekularnym jak te dwie cząsteczki wiążą się ze sobą. Dodatkowo po raz pierwszy struktura części zewnątrz-komórkowej TSHR została poznana z dokładnością do 2.55 Å. Opierając się na strukturze kompleksu TSHR-M22 oraz strukturze kompleksu

FSHR-FSH było możliwe zbudowanie modelu kompleksu TSHR—TSH. Pozwoliło to na przeprowadzenie szczegółowej analizy wiązania TSH z TSHR i porównanie wiązania TSH i M22 z receptorem. Badania te wykazały zadziwiające podobieństwo pomiędzy interakcją stymulującego przeciwciała i TSH z receptorem; wiązanie łańcucha lekkiego i ciężkiego M22 naśladuje wiązanie łańcucha β i α TSH.

Kolejnym etapem w tym cyklu badań była izolacja równocześnie z jednej próbki krwi od jednego pacjenta dwóch przeciwciał dla TSHR mających odmienne aktywności biologiczne. Te przeciwciała to K1-18 MAb o zdolności stymulowania tarczycy oraz K1-70 o zdolności blokowania tarczycy. Dostraczyło to pierwszy dowód że stymulujące i blokujące przeciwciała dla TSHR mogą być obecne w tym samym czasie w surowicy chorych a obraz kliniczny zależy od sumy ich aktywności i zdolności tkanki tarczycowej do odpowiedzi. Obydwa przeciwciała mają wysokie powinowactwo wiązania z TSHR (6.7×10^9 L/mol dla K1-18 i 3.9×10^{10} L/mol dla K1-70), są aktywne w stężeniach ng/mL i posiadają cechy przeciwciał występujących w surowicy pacjentów z chorobami tarczycy.

Cykl tych badań został uzupełniony krystalizacją i poznaniem struktury kompleksu TSHR z blokującym MAb K1-70 z dokładnością do 1.9 Å. Wiązanie K1-70 z TSHR wykazuje wiele podobieństw do wiązania M22 z główną różnicą polegającą na interakcji z N-terminalną i centralną częścią receptora w przeciwieństwie do M22 i TSH które są zaangażowane w interakcje z całą powierzchnią od N-terminus do C-terminus. W odróżnieniu od stymulującego MAb M22 przeciwciało blokujące K1-70 nie naśladuje TSH w sposobie wiązania z TSHR.

Poznanie struktury molekularnej kompleksu TSHR ze stymulującym MAb M22 i blokującym MAb K1-70 otwiera nowe perspektywy dla leczenia chorób tarczycy. Przykładem nowych leków kontrolujących nadczynności tarczycy mogą być swoiste inhibitory wiązania przeciwciał stymulujących (oraz blokujących) TSHR syntezowane na podstawie interakcji zaobserwowanych w strukturze kryształu. Stymulujące przeciwciała dla TSHR jako takie mogą mieć zastosowanie w praktyce klinicznej dla ulepszonej diagnostyki i monitorowania raka tarczycy mając podobne zastosowanie do rekombinowanego TSH. Dodatkowo przeciwciała wiążące się silnie z TSHR mogą mieć

zastosowanie w połączeniu z izotopami promieniotwórczymi albo lekami chemioterapeutycznymi do leczenia opornych przypadków raka tarczycy i przerzutów. Przeciwciała blokujące mogą mieć zastosowanie w leczeniu nadczynności tarczycy w specjalnych sytuacjach ale głównie w leczeniu ophthalmopatii tarczycowej dla której obecnie nie ma skutecznej i swoistej terapii. Wraz z rozwojem technologii produkowania przeciwciał do stosowania *in vivo* osiągnięcie tych horyzontów w przyszłości może być całkowicie realne.

STATEMENT

All the authors have given their written consent to include the five publications in this thesis.