FSH and TSH binding to their respective receptors: similarities, differences and implication for glycoprotein hormone specificity

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Abstract

The crystal structures of the leucine-rich repeat domain (LRD) of the FSH receptor (FSHR) in complex with FSH and the TSH receptor (TSHR) LRD in complex with the thyroid-stimulating autoantibody (M22) provide opportunities to assess the molecular basis of the specificity of glycoprotein hormone–receptor binding. A comparative model of the TSH–TSHR complex was built using the two solved crystal structures and verified using studies on receptor affinity and activation. Analysis of the FSH–FSHR and TSH–TSHR complexes allowed identification of receptor residues that may be important in hormone-binding specificity. These residues are in leucine-rich repeats at the two ends of the FSHR and the TSHR LRD structures but not in their central repeats. Interactions in the interfaces are consistent with a higher FSH-binding affinity for the FSHR compared with the binding affinity of TSH for the TSHR. The higher binding affinity of porcine (p)TSH and bovine (b)TSH for human (h)TSHR compared with hTSH appears not to be dependent on interactions with the TSHR LRD as none of the residues that differ among hTSH, pTSH or bTSH interact with the LRD. This suggests that TSHs are likely to interact with other parts of the receptors in addition to the LRD with these non-LRD interactions being responsible for affinity differences. Analysis of interactions in the FSH–FSHR and TSH–TSHR complexes suggests that the α -chains of both hormones tend to be involved in the receptor activation process while the β -chains are more involved in defining binding specificity.

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Introduction

In mammals, the glycoprotein hormone (GPH) family comprises three gonadotrophins: lutrophin (luteinising hormone, LH), follitrophin (follicle-stimulating hormone, FSH) and choriogonadotrophin (CG) and one thyrotrophin (thyroid-stimulating hormone, TSH). There are, however, only three high affinity GPH receptors as LH and CG bind the LH receptor (LHR). GPHs control several major physiological processes, with TSH regulating metabolism through the production of thyroid hormones and the gonadotrophins regulating reproductive functions (Pierce & Parsons 1981, Combarnous 1992).

The GPHs are heterodimers composed of non-covalently linked α - and β -subunits. The N-linked oligosaccharides of these hormones are necessary for proper folding, assembly, secretion, metabolic clearance and biological activity (Baenziger & Green 1988). Whereas β -subunits are hormone specific, α -subunits are identical within a species, although there may be differences in the oligosaccharides. The structure of each subunit comprises a cystine-knot motif with five

disulphide bonds in the α -subunit and six in the β -subunit (McDonald & Hendrickson 1993). The core of the cystine-knot motif involves three disulphide bridges arranged so that two disulphides link adjacent antiparallel strands of the peptide chain and form a ring penetrated by the third disulphide. On one side of the knot, there is a loop of double-stranded β -sheet-like structure; on the other, there are two hairpin loops lying in almost parallel planes (Wu *et al.* 1994, Fox *et al.* 2001).

The receptors of the GPHs belong to the rhodopsin-like class of G protein-coupled receptors (GPCRs) and are $\sim 45\%$ identical at the amino acid level (Dias 1992). In contrast to other receptors in their class, GPH receptors are characterised by a large extracellular region consisting of an N-terminal tail, a leucine-rich repeat domain (LRD) and a cystine-rich domain (CD). The structure is completed with a transmembrane domain (TMD) consisting of seven membrane spanning helices and a C-terminal tail (Szkudlinski *et al.* 2002, Farid & Szkudlinski 2004, Núñez Miguel *et al.* 2004). In both the TSH receptor (TSHR) and the FSH receptor (FSHR), the N-terminal tail contains four half-cystines (i.e. one of the two disulphide-bonded cysteines) and is

disulphide bonded to the first of ten repeats of the LRD (Fan & Hendrickson 2005, Sanders 2007a). The LRD is followed by a CD, part of which is cleaved out upon maturation in the case of the TSHR (TSHR cleavage domain; CD; Rees Smith *et al.* 1988, Chazenbalk *et al.* 1996, Rapoport *et al.* 1998, Núñez Miguel *et al.* 2003). The TMD structures of the GPHs comprise seven transmembrane helices similar to that of rhodopsin, and to date, the crystal structure of two GPCRs, with short extracellular regions, has been solved; i.e. rhodopsin (Palczewski *et al.* 2000) and β_2 -adrenergic receptor (Cherezov *et al.* 2007, Rasmussen *et al.* 2007). GPH binding to their receptors results in transduction of the signal through the TMD and activation of the heterotrimeric G proteins (Claus *et al.* 2006).

The human GPHs have closely related structures as do their respective receptors but cross-reactivity is weak if it occurs at all. Hormone and receptor co-evolution appear to have resulted in progressive increases in the specificity of GPH–receptor interactions compared with the more promiscuous interactions characteristic of their ‘ancient’ precursors (from which ‘modern’ TSH, FSH and LH/CG evolved over about 400 million years; Moyle *et al.* 1994, Farid & Szkudlinski 2004). The availability of the crystal structures of the FSHR LRD in complex with FSH (Fan & Hendrickson 2005) and the TSHR LRD in complex with a human thyroid-stimulating autoantibody (M22; Sanders *et al.* 2007a) now provides an opportunity to assess the molecular basis of the specificity of GPH–receptor binding.

Materials and methods

The procedure used to construct a model of the TSH–TSHR LRD is outlined in Supplementary Figure 1, see Supplementary data in the online version of the *Journal of Molecular Endocrinology* at <http://jme.endocrinology-journals.org/content/vol41/issue3/>. This involved initial alignment between the amino acid sequences of the FSH–FSHR and TSH–TSHR complexes and was obtained using the program FUGUE (Shi *et al.* 2001). FUGUE produces alignments by comparison of sequence and structural profiles. Models of the TSH–TSHR complex were produced using the program MODELLER (Sali & Blundell 1993), by the processes of satisfaction of spatial restraints with simultaneous optimisation of CHARMM energies (Brooks *et al.* 1983) employing conjugate gradients and molecular dynamics with simulated annealing. Comparative models were validated with PROCHECK (Laskowski *et al.* 1993) that studies the geometry of the structure of the protein, VERIFY3D (Luthy *et al.* 1992) that reports whether every amino acid presents acceptable environments and JOY (Mizuguchi *et al.* 1998) that compares the environments of residues of the modelled

structure with the environments of residues of the homologues used for modelling. The alignments were manually modified when the model was unsatisfactory (i.e. produced results below those recommended by validation programs), and the modelling and validation processes repeated. These processes were repeated until models with good geometry and conformation were obtained. The programs MNYFIT (Sutcliffe *et al.* 1987) and BATON (Burke unpublished results; Sali & Blundell 1989) were used for superimposing the coordinates of peptide chains. MODELLER was also used for energy and structure minimisation of the complexes.

For the study of van der Waals interactions between residues and shape complementarity in the interfaces of the complexes, accessible surface areas (ASA) were calculated using the Lee & Richards (1971) algorithm developed by Richmond (1984). The gap volume that gives a measure of the complementarity of the interacting surfaces was also calculated. The volume of the gaps between the two interacting molecules was calculated using the program SURFNET (Laskowski 1995). Each pair of atoms from different molecules was considered in turn, placing a sphere (maximum radius 5.0 Å) halfway between the surfaces of the two atoms, such that its surface touches the surfaces of the atoms in the pair. Checks were made to test whether any other atoms intercept this sphere, and each time an intercept was detected the size of the sphere was reduced accordingly. If at any time the size of the sphere fell below a minimum (minimum radius 1.0 Å), the sphere was discarded. If the sphere remained after all checks, its size was recorded. The sizes of all the allowable gap spheres were then used to calculate the gap volume between the two molecules. The gap volume index was defined as: Gap Volume Index = Gap Volume / Interface ASA (Jones & Thornton 1996).

The program HBPLUS was used for hydrogen bond definition (McDonald & Thornton 1994). The program uses a distance cut-off of 3.5 Å between donor and acceptor atoms and angle cut-offs of 90° for donor–hydrogen–acceptor angle and for donor–acceptor–acceptor adjacent angle. Electrostatic interactions between charged residues were obtained using an in-house program (ELECINT, Núñez Miguel unpublished) that employs the Henderson–Hasselbalch equation for obtaining atomic charges and a distance-dependent dielectric constant.

Results

Comparative modelling

In order to study the specificity of TSHR and FSHR for their respective hormones, an updated structure of the TSH–TSHR LRD complex was required. In the absence of an actual experimental structure, this was modelled

on the basis of homologous protein complexes. The LRD domains of FSHR and TSHR share 40% amino acid sequence identity (UniProtKB/Swiss-Prot; <http://www.ebi.ac.uk/swissprot/>). Furthermore, there is 100% amino acid sequence identity between the α -chains of human(h) FSH and hTSH and 44% identity between their β -chains (UniProtKB/Swiss-Prot; <http://www.ebi.ac.uk/swissprot/>), and these degrees of similarity provided a basis for a comparative model.

In our earlier study, a comparative model of the TSH–TSHR LRD complex was built based on the crystal structure of the FSH–FSHR LRD complex solved at 2.9 Å resolution (Fan & Hendrickson 2005, Núñez Miguel *et al.* 2005). In the current study (Supplementary Figure 1), the TSHR LRD in the earlier comparative model of the complex was replaced with the crystal structure of the TSHR LRD from the TSHR–M22 complex solved at 2.55 Å resolution (Sanders *et al.* 2007a). The two complexes, our earlier TSH–TSHR model and M22–TSHR crystal structure, were superimposed on each other and the receptor structures were exchanged. This resulted in leaving the structure of TSH in the new TSH–TSHR complex unchanged. The superimposed TSHR LRD structures from the M22–TSHR crystal structure and from the TSH–TSHR model after energy minimisation showed the root mean-squared deviation (RMSD) value of 0.15 Å. This is comparable with the RMSD values observed when different crystal structures of the same protein are superimposed on each other (Daopin *et al.* 1994, Echt *et al.* 2004). Consequently, the new TSH–TSHR complex consisted of the crystal structure of the TSHR LRD combined with the comparative model of the TSH in the same relative position as that observed in the crystal structure of the complex between the FSHR and FSH. Then, the model of the TSH–TSHR complex was energy minimised in order to optimise the interactions between the two components of the complex (Núñez Miguel *et al.* 2002; Supplementary Figure 1). The final model of the TSH–TSHR complex produced for the purpose of this study is shown in Fig. 1A.

In the new model of the TSH–TSHR complex described above, hTSH presents 92.7% residues in the most favourable regions of the Ramachandran plot, 7.3% residues with allowed conformations and 0.0% residues disallowed. There are no close contacts at a distance below 2.2 Å between two non-bonded atoms in the hTSH model. The VERIFY3D reports a segment with negative values (aa 40–43 β -chain) with a minimum value of -0.09 in the hTSH model. This segment is within the Keutmann's loop, a loop that has been reported to be important for binding of TSH or human choriongonadotrophin (hCG) to their respective receptors (Ryan *et al.* 1988, Morris *et al.* 1990, Szkudlinski *et al.* 2002). This is consistent with the observations that protein segments that bind other

proteins may present low values in the VERIFY3D reports as their local environments are not properly described in the isolated molecules (Luthy *et al.* 1992).

In terms of the TSHR LRD in the new model of the complex, 82.7% of the LRD residues present in the most favourable conformations of the Ramachandran plot, 17.2% residues are with allowed conformations and 0.0% residues with disallowed conformations. No close contacts below 2.2 Å distance are present and there are no negative values in the VERIFY3D report.

In order to assess FSH and TSH specificity for their respective receptors in more detail, we built models of hormone–receptor complexes in which the hormones were interchanged. First, the coordinates of the FSH–FSHR complex and TSH–TSHR model were superimposed. The two components of the model of the TSH–TSHR LRD showed similar relative positions compared with the two components in the FSH–FSHR LRD complex used as a template. Overall, 416 out of 433 α -carbons from the backbones of each structure could be superimposed with <3.0 Å deviation and this superimposition presented a RMSD of 0.88 Å. The superposition of TSH–TSHR LRD and FSH–FSHR LRD was followed by the exchange of the two hormones to obtain the complexes with the hormones exchanged (i.e. TSH–FSHR LRD and FSH–TSHR LRD). An energy minimisation was then carried out to obtain a final optimisation of the interface interactions. The favourable and unfavourable interactions including important steric clashes in the interfaces of the FSH–TSHR and TSH–FSHR were then studied.

FSH–FSHR and TSH–TSHR interfaces

Several important similarities and differences can be observed in the interfaces of the FSH–FSHR and TSH–TSHR complexes seen in the new model. In the FSH–FSHR complex, 16 FSH α chain residues and 13 FSH β chain residues produced interactions >10 Å² Δ ASA (difference between ASA values before and after complexation) on complexation with 31 FSHR residues. The Δ ASA values of 1 Å² and above are enough to indicate that a residue interacts on complexation; however, for the purpose of this study, values above 10 Å² were classed as strong interactions in the interface (Jones & Thornton 1997, Mihel *et al.* 2008). In the case of TSH–TSHR interactions, 15 TSH α chain residues and 14 TSH β chain residues produced interactions >10 Å² Δ ASA on complexation with 31 TSHR residues. Supplementary Table 1, see Supplementary data in the online version of the Journal of Molecular Endocrinology at <http://jme.endocrinology-journals.org/content/vol41/issue3/>, shows the residues from both FSH and TSH that are closer than 4 Å to receptor residues and in addition hormone residues that are involved in charge–charge interactions with distance between 4.0 and 7.0 Å from receptor

Figure 1 New model of the TSH-TSHR LRD complex built using solved crystal structures of the FSH-FSHR LRD (Fan & Hendrickson 2005) and the M22-TSHR LRD (Sanders *et al.* 2007a) complexes. (A) Three different points of view of the TSH-TSHR LRD complex. The LRD of the TSHR is shown in pink, the α -chain of the human TSH in green and the β -chain in cyan. N- and C-termini of every chain are marked. TSHR cysteines forming disulphide bond are shown as ball and stick with the carbon atoms in pink, oxygens in red, nitrogens in blue and sulphurs in yellow. (B) Electrostatic potential surfaces of FSH, FSHR LRD, TSH and TSHR LRD showing some important charged residues. The N- and C-termini of the receptor LRDs are marked. Some important interactions between hormones and receptors are shown by connecting lines to indicate the hormone-receptor interactions (see text for details). Negative patches are shown in red and positive in blue.

residues. The number of FSH and TSH residues that interact with each repeat of the FSHR and TSHR respectively is listed in Table 1. Table 1 shows the number and type of interactions in the interfaces of the two

complexes, in particular there are six salt bridges and five hydrogen bonds in the FSH-FSHR interface while four salt bridges and three hydrogen bonds are present in the TSH-TSHR interface.

Table 1 Interactions in the interfaces of follicle-stimulating hormone (FSH)-FSH receptor (FSHR) and thyroid-stimulating hormone (TSH)-TSH receptor (TSHR) complexes.(A) Number of residues from FSH and TSH (α - and β -chains) that interact with their respective receptor LRD repeats

Receptor LRD repeat and number of residues interacting											
	N-ter	First	Second	Third	Fourth	Fifth	Sixth	Seventh	Eighth	Ninth	Tenth
Hormone chain											
FSH α	—	1	2	8	11	6	6	3	—	—	—
FSH β	—	2	5	2	6	—	2	2	2	3	2
TSH α	—	1	1	6	9	6	8	2	—	—	—
TSH β	—	1	3	—	3	—	3	1	4	1	3

(B) Number and type of interactions in the interfaces of the FSH-FSHR and TSH-TSHR complexes

	Salt bridges	Hydrogen bonds	Ion pairs	Dipole-dipole	Dipole-induced dipole	Hydrophobic
Complex						
FSH-FSHR	6	5	6	21	37	20
TSH-TSHR	4	3	7	18	36	22

(C) Δ ASA, gap volumes and gap volume indices in the interfaces of the FSH-FSHR and TSH-TSHR complexes

	Interface ASA ($\text{\AA}^2$)	Gap volume ($\text{\AA}^3$)	Gap volume index ($\text{\AA}$)
FSH	1304		
FSHR	1279		
FSHR-FSH	2583	8410	3.26
TSH	1292		
TSHR	1241		
TSHR-TSH	2533	9279	3.66

The interface Δ ASA of the FSH-FSHR and TSH-TSHR complexes was 2583 and 2533 $\text{\AA}^2$ respectively (Table 1). The gap volume of the FSH-FSHR complex was 8410 $\text{\AA}^3$ compared with that of the TSH-TSHR complex of 9279 $\text{\AA}^3$ (a difference of 869 $\text{\AA}^3$; Table 1). Consequently, a gap volume index for the TSH-TSHR complex is 12% higher than that for the FSH-FSHR complex (3.26 vs 3.66 respectively; Table 1).

Electrostatic potential surfaces of the interacting faces of FSH with FSHR LRD and TSH with TSHR LRD are shown in Fig. 1B. Overall, the electrostatic potentials of FSH and TSH are similar in the areas that interact with their respective receptors (Fig. 1B) although there are some differences. In particular, FSH β R97 produces an electropositive patch while the equivalent residue in TSH β (E98) produces an electronegative patch. Also, TSH β K44 presents positive charge whereas there are no charged amino acids in the equivalent position in FSH β (Fig. 1B). There are clear differences, however, in the electrostatic potential of interacting surfaces of the FSHR and TSHR LRDs. For example, TSHR LRD presents an elongated positive patch that includes R38, K58 and R80, while in the equivalent area in the FSHR LRD negative and positive amino acids alternate (E76, R52, E50 and K74). Close to this patch, on the FSHR LRD surface there is a positively charged patch (R101), while on the TSHR LRD surface there is a negatively charged patch (E107;

Fig. 1B). Further on, the TSHR LRD K183 projects a positive area to the surface whereas the corresponding FSHR W176 is not charged. There are also differences in charge distribution at the C-termini of the LRDs. The C-terminal area of the FSHR LRD centred on K243 is positively charged, while the equivalent area on the TSHR LRD centred on E251 is negatively charged (Fig. 1B).

In the FSH-FSHR LRD crystal structure, the glycosylation sites on the FSH (FSH α N52 and N78 and FSH β N7 and N24) and on the FSHR LRD (N191) are remote from the interface (Fan & Hendrickson 2005). In our TSH-TSHR LRD model, the TSHR LRD glycosylation sites (N 77, N99, N113, N177 and N198) are also located away from the concave surface of the LRD consistent with the arrangements observed in the crystal structure of the M22-TSHR LRD (Sanders *et al.* 2007a). The predicted glycosylation sites on hTSH (TSH α N52 and N78 and TSH β N23; UniProtKB/Swiss-Prot; <http://www.ebi.ac.uk/swissprot/>) are all distant from the TSH-TSHR LRD complex interface in our TSH-TSHR LRD model.

Interface residue interactions

A detailed study of the interactions produced by residues in the interfaces of the two hormones and their respective receptors was carried out. The interactions studied included: hydrogen bonds, van

der Waals interactions, charge–charge interactions and steric clashes. Clashes were studied in the FSH–FSHR and TSH–TSHR complexes as well as in the models of complexes where the hormones were exchanged. In our analyses, 3.5 Å was considered the maximum distance for hydrogen bonds, 4 Å for van der Waals interactions and 7 Å for charge–charge interactions. For the latter, the notation we use in the present study is that charge–charge interactions at atomic distances ≤3.5 Å will be called ‘salt bridges’, at distances >3.5 and ≤4.5 Å will be called ‘ion pairs’ and those at >4.5 Å will be called ‘solvent-separated ion pairs’. A steric clash was defined by the distance between the nuclei of the two non-bonding atoms that was smaller than the sum of the van der Waals radii of the two atoms. In this study, only severe clashes indicated by an inter-atomic distance that was smaller by 0.3 Å than the sum of the van der Waals radii were considered.

Analysis of the FSH–FSHR and TSH–TSHR complexes indicated that out of a total of 43 residues from the two receptors that were involved in interactions with their respective hormones, 22 residues were equivalent (in both the receptors’ structure and the receptors’ sequence alignments). There were 13 residues from FSHR that interact with FSH for which their equivalent residues from TSHR do not interact with TSH and 8 residues from TSHR that interact with TSH while the equivalent residues from FSHR do not interact with FSH (Supplementary Table 1).

Similar interactions

In the two complexes, interactions that: a) involved equivalent residues in the receptors’ or hormones’ amino acid sequences (e.g. FSHR D202 and TSHR K209 are equivalent in sequence position because when the two sequences are aligned these two residues are aligned) and b) were of the same type (e.g. hydrogen bond etc.) and c) were between the same or homologous type of amino acids (e.g. two aspartic acids because they are the same amino acids, or one aspartic and one glutamic acid because they are homologous amino acids) were considered highly similar. Interactions were classed as similar if only two of the above criteria were met. Interactions were classed as different, if only one or none of the above criteria were met.

Our analysis indicated that only four receptor residues from each complex were involved in interactions with their respective hormones that could be considered as highly similar in the two complexes (Table 2; i.e. TSHR F130 and FSHR equivalent residue Y124; TSHR N135 and FSHR equivalent residue N129; TSHR E157 and FSHR equivalent residue D150; TSHR D160 and FSHR equivalent residue D153).

For example, FSHR D153 and the equivalent TSHR D160 were involved in similar interactions with residues from FSH and TSH respectively (Fig. 2A and Table 2). The main interactions were charge–charge interactions between the aspartic acid from the receptor and α-chain

Table 2 Similar interactions in the interfaces of the follicle-stimulating hormone (FSH)–FSH receptor (FSHR) and thyroid-stimulating hormone (TSH)–TSH receptor (TSHR) complexes

	Main interaction	FSH	FSHR	TSHR	TSH	Main interaction	Distance (Å)
Distance (Å)							
Highly similar interactions							
3.5	Aromatic	Y88α	Y124	F130	Y88α	Aromatic	3.5
3.3	Hydrophobic	M47α	N129	N135	M47α	Hydrophobic	2.7
3.3	Polar	L48α			L48α	Hydrogen bond	2.6
3.1	Hydrogen bond	V49α			V49α	Polar	2.8
3.1	Salt bridge	K91α	D150	E157	K91α	Salt bridge	2.7
3.7	Hydrophobic	V49α	D153	D160	V49α	Hydrophobic	3.9
3.4	Salt bridge	K51α			K51α	Ion pair	3.5
6.7	Unfavourable electrostatic	D93β			D94β	Unfavourable electrostatic	6.6
					K91α	Solvent-separated ion pair	5.1
					D91β	Unfavourable electrostatic	6.8
Similar interactions							
3.9	Polar	T46α	A105	T111	T46α	Polar	4.0
3.8	Hydrophobic	Y88α	L148	I155	Y89α	Hydrophobic	2.8
3.1	Polar	Y88α	W176	K183	Y89α	Polar	3.8
Similar relative locations within and without the 4 Å cut-off							
4.8	Hydrophobic	L48α	I155	P162	L48α	Hydrophobic	3.2
4.4	Polar	K40β	S172	T179	K39β	Polar	3.9

Ion–dipole and dipole–dipole interactions not involved in hydrogen bonds are described as ‘polar’. Dispersion and hydrophobic forces are described as ‘hydrophobic’. Charge–charge interactions at ≤3.5 Å distance are defined as ‘salt bridge’. Charge–charge interactions at >3.5 Å and ≤4.5 Å distance are defined as ‘ion pairs’. Charge–charge interactions at >4.5 Å distance are defined as ‘solvent-separated ion pairs’.

Figure 2 Interactions produced by (A) FSHR D153 with FSH in the FSH–FSHR complex and by TSHR D160 with TSH in the TSH–TSHR complex and (B) FSHR E34 with FSH residues in the FSH–FSHR complex and by TSHR D43 with TSH residues in the TSH–TSHR complex. Receptor LRD backbones are shown in magenta, hormone α -chains in green and β -chains in cyan. Interacting residues are shown as sticks with carbon atoms in grey, oxygens in red and nitrogens in blue. Solid lines denote interactions, dashed lines denote no interactions.

K51 from FSH or TSH. In experimental studies, TSHR mutation D160A caused a reduced response of cyclic AMP to stimulation by porcine (p)TSH (60–80% of the wild-type), while change of charge mutation (D160K) caused a strong reduction (<20% than the wild-type; Sanders *et al.* 2006). The effect of TSHR D160A substitution is likely due to disruption of the salt bridge with α TSH K51, while the D160K mutation would result in introducing an unfavourable electrostatic interaction (Fig. 2A).

FSHR Y124 and the equivalent residue F130 in the TSHR are involved in a face-to-face aromatic interaction with the α -chain Y88 of FSH or TSH (Table 2). Mutation of TSHR F130 to alanine produced no difference in pTSH binding or pTSH stimulation of cyclic AMP production (Sanders *et al.* 2006).

Another example of highly similar interaction is FSHR residue D150 and the equivalent residue TSHR E157 which form a salt bridge with K91 α of FSH or TSH respectively. These arrangements are consistent with mutation experiments. For example, mutation of human FSH at K91 α was reported to cause marked decrease in binding to the FSHR (Arnold *et al.* 1998). Furthermore, even though mutation of TSHR E157 to alanine resulted in a similar binding affinity for pTSH as

the wild-type TSHR, a decreased response to pTSH-induced cyclic AMP production (40–60% of wild-type activity) was observed (Sanders *et al.* 2006). Also, COS cells transiently expressing TSHR E157A showed an increased EC₅₀ for bTSH stimulation of cyclic AMP production compared with the wild-type TSHR (Smits *et al.* 2002) and the same mutation was nearly ten times less sensitive to hTSH stimulation of cyclic AMP production (Smits *et al.* 2003). In addition, the TSHR E157D mutation showed an increased sensitivity to hCG compared with the wild-type TSHR (Smits *et al.* 2002). Consequently, it has been suggested that an acidic residue in position 157 of the TSHR is needed to increase interaction of both TSH and hCG to the TSHR (Smits *et al.* 2002) and, as this residue is not able to discriminate one hormone from the other, it is unlikely to be involved in the receptor specificity. The situations observed in the complexes we have studied are consistent with this scenario.

Three interactions were classed as similar in the two complexes studied. In particular, FSHR residues L148 and W176 interact with the α FSH Y88 while the TSHR equivalent residues I155 and K183 interact with α TSH Y89 respectively (Table 2). In both complexes, the two

receptor residues are located between two neighbouring hormone tyrosines. The small observed differences in the receptor residue positions relative to the tyrosines are likely due to the differences in the side chain structures (FSHR L148 and W176, TSHR I155 and K183, α TSH Y88 and Y89, and α FSH Y88 and Y89). FSHR W176A mutation has been associated with a similar level of hFSH-stimulated cyclic AMP production and reduction in sensitivity to hCG (Vischer *et al.* 2003). Mutation of TSHR K183 to alanine showed an increase in pTSH binding to the receptor; however, no increase in pTSH-induced cyclic AMP production was shown compared with the wild-type TSHR (Sanders *et al.* 2006). In contrast, the TSHR K183D (change of charge) mutation showed a slight decrease in pTSH-stimulated cyclic AMP production (Sanders *et al.* 2006). The naturally occurring TSHR mutation K183R gives increased specificity for hCG and experimentally produced mutations of K183 to R, A, I, T, E, W, D, M, N and Q also showed an increased sensitivity to hCG (Smits *et al.* 2002). Analysis of the same mutations showed that there was no difference in bTSH-induced cyclic AMP activity (Smits *et al.* 2002) compared with the wild-type TSHR. However, later studies using recombinant hTSH (Smits *et al.* 2003) found that TSHRs with amino acid substitutions at position 183 displayed a five- to sevenfold increase in sensitivity to TSH. It has been proposed that TSHR residues K183 and E157 form an intramolecular salt bridge (Smits *et al.* 2002), and this is likely to reduce the intermolecular salt bridge between TSHR E157 and TSH α K91 (Table 2) by reducing the charge of TSHR E157. Substitutions at position K183 of TSHR are likely to increase TSH binding by breaking the intramolecular salt bridge and releasing TSHR E157 for strong binding with TSH.

In addition, two further situations should be noted where the distances between the interacting residues are similar in the two complexes but exceed the 4 Å cut-off in one of the complexes. For example, the distances between the FSHR I155 and S172 and α FSH L48 and β FSH K40 are just over 4 Å, while the distances between the equivalent residues in the TSHR (P162 and T179) and α TSH L48 and β TSH K39 are 3.2 Å and 3.9 Å respectively (Table 2). Mutation TSHR P162A has been reported to be associated with hypothyroidism (Sunthornthepvarakul *et al.* 1995, Costagliola *et al.* 1999). In COS-7 cells transfected with the wild-type TSHR, the maximal luciferase activity induced by recombinant hTSH was 20 times the basal level. Approximately, ten times more hTSH was required for an equal effect in cells transfected with thyrotrophin receptor with P162A mutation (Sunthornthepvarakul *et al.* 1995). Furthermore, a twofold increase in EC₅₀ has been found, when cyclic AMP accumulation in response to bTSH stimulation was measured in COS cells transfected with TSHR P162A (Costagliola *et al.* 1999). In the P162A mutant, in addition to a disruption

of the hydrophobic interaction with α TSH L48, a change in conformation is likely to be observed as prolines present restricted phi angles (the angle of rotation around the N-C α bond).

Different interactions produced by receptor residues without clear roles in specificity

In our analysis of interactions in the two complexes, the interactions involving receptor residues with hormone α -chains residues only were considered not to be important for specificity. Furthermore, the interactions involving receptor residues with the same type of amino acids in the hormone β -chains were not considered to be important for specificity. Consequently, receptor interactions involving different residues in hormone β -chains were considered likely to be involved in specificity.

Analysis of the interactions in the two complexes revealed that there were differences in interactions of equivalent residues from both receptors and their respective hormones. However, a closer inspection of the respective interactions together with the experimental evidence has led us to the conclusion that some of these interactions were unlikely to be important for defining the specificity of the FSHR and TSHR for their hormones (Table 3). For example, FSHR residue E34 forms a hydrogen bond with the hydroxyl group of FSH β Y103. The equivalent TSHR residue D43 has a shorter side chain compared with the FSHR glutamic acid side chain, consequently the hydrogen bond with TSH β Y104 cannot be formed, although a water-mediated hydrogen bond may be possible. In addition, FSHR E34 and TSHR D43 are involved in solvent-separated ion pairs with α FSH or α TSH residues R42 (Table 3 and Fig. 2B). TSHR mutant D43A has been reported to bind pTSH with similar affinity as the wild-type receptor, but this mutation caused a reduction in pTSH-stimulated cyclic AMP production (60–80% of wild-type activity; Sanders *et al.* 2006). The experimentally observed biological effect of TSHR mutation D43A is consistent with the loss of the solvent-separated ion pairs described above caused by this mutation. Although TSHR D43 may be important for a biological response to TSH, it is unlikely to be important for specificity as TSHR D43 and the equivalent FSHR E34 interact with R42 in both α TSH and α FSH. Further examples of interactions that are different in the FSH-FSHR and TSH-TSHR but do not have a clear role in hormone-binding specificity are listed in Table 3, and at least some of these have been validated by experimental evidence reported before (Clifton-Bligh *et al.* 1997, Smits *et al.* 2003, Sanders *et al.* 2006).

Furthermore, in both complexes, there are interactions where residues from one receptor interact with the cognate hormone while the equivalent residues from the other receptor do not interact with

Table 3 Comparison of interactions in follicle-stimulating hormone (FSH)–FSH receptor (FSHR) and thyroid-stimulating hormone (TSH)–TSH receptor (TSHR) complexes – different interaction produced by receptor residues without clear role in hormone-binding specificity

	Main interaction	FSH	FSHR	TSHR	TSH	Main interaction	Distance (Å)
Distance (Å)							
2.2	Hydrogen bond	Y103β	E34	D43	R42α	Solvent-separated ion pair	5.5
6.2	Solvent-separated ion pair	R42α					
3.2	Induced dipole	R42α	L55	E61	R42α	Solvent-separated ion pair	5.0
3.7	Hydrophobic	L99β			I100β	Induced dipole	2.8
3.0	Hydrophobic	Y103β			Y104β	Polar	3.7
2.5	Hydrogen bond	S43α	Q79	I85	R42α	Induced dipole	3.3
3.5	Polar	T46α			T46α	Hydrophobic	2.9
3.6	Hydrophobic	L99β					
3.0	Salt bridge	R42α	D81	V87	R42α	Induced dipole	3.6
3.6	Polar	K45α					
3.1	Induced dipole	T46α					
3.6	Polar	T86α	E99	H105	Y88α	Polar	2.7
2.6	Hydrogen bond	Y88α					
5.7	Unfavourable electrostatic	D93β	E103	R109	K51α	Unfavourable electrostatic	4.4
3.1	Induced dipole	T95β			K91α	Unfavourable electrostatic	4.4
					D94β	Salt bridge	2.7
					C95β	Polar	3.5
					I96β	Hydrophobic	3.0
3.0	Polar	T46α	K104	N110	T46α	Polar	2.7
3.9	Hydrophobic	M47α			M47α	Hydrophobic	2.7
3.3	Salt bridge	D93β			D94β	Polar	4.0
3.2	Induced dipole	T95β					
3.2	Polar	K45α	N106	R112	T46α	Polar	3.9
3.5	Polar	T46α			M47α	Hydrophobic	3.4
3.9	Polar	M47α			L48α	Hydrophobic	2.8
3.7	Polar	L48α					
6.7	Unfavourable electrostatic	R67α	K146	F153	Y89α	Induced dipole	3.0
2.9	Unfavourable electrostatic	K40β			K39β	Cation-π	2.9
4.7	Solvent-separated ion pair	K40β	D196	D203	K39β	Solvent-separated ion pair	5.8
3.4	Induced dipole	P42β			P43β	Polar	2.7
					K44β	Polar	3.9
5.1	Unfavourable electrostatic	D90β	D202	K209	D91β	Salt bridge	2.7

Ion–dipole and dipole–dipole interactions not involved in hydrogen bonds are described as ‘polar’. Ion–induced dipole and dipole–induced dipole interactions are described as ‘induced dipole’. Dispersion and hydrophobic forces are described as ‘hydrophobic’. Charge–charge interactions at ≤ 3.5 Å distance are defined as ‘salt bridge’. Charge–charge interactions at > 3.5 and ≤ 4.5 Å distance are defined as ‘ion pairs’. Charge–charge interactions at > 4.5 Å distance are defined as ‘solvent-separated ion pairs’. For explanation of cation- π interactions, see [Gavilan & Dougherty \(1999\)](#).

its hormone, but for which there is no evidence of a clear role in specificity (Table 4). For example, FSHR K179 is hydrogen bonded to FSHβ S89, forms a salt bridge with FSHβ D90, a solvent-separated ion pair with FSHβ D88, and has an unfavourable charge–charge interaction with FSHα K51. The TSHR equivalent residue N186 does not interact with TSH. Substitutions at K179 in FSHR have been reported to increase FSHR responsiveness to hCG (Smits *et al.* 2003, Vischer *et al.* 2003). The greatest increase in responses to hCG was observed when the two smallest amino acids (glycine or alanine) were introduced at FSHR K179 (Smits *et al.* 2003, Vischer *et al.* 2003). Consequently, it has been suggested that the absence of positive charge in this region would be favourable for hCG binding while a bulky residue might cause a steric hindrance (Smits *et al.* 2003). Analysis of the situations in the FSH–FSHR complex indicates that indeed it is likely that interaction of hCGβ R94 (equivalent residue to FSHβ

D88) and hCGβ R95 (equivalent residue to FSHβ S89) with the unmutated FSHR would produce strong steric clashes and unfavourable charge–charge interactions that would be eliminated when a small and uncharged residue was present in the place of FSHR K179.

TSHR residue E178 does not interact with TSH (Table 4). In experimental studies, mutation TSHR E178A caused reduction in pTSH-binding affinity to the receptor and reduced cyclic AMP production in response to pTSH stimulation (Sanders *et al.* 2006). The biological effect of this mutation was probably due to a TSHR LRD structure modification, as a similar reduction in binding affinities and cAMP activity were observed in the case of two TSHR monoclonal antibodies M22 Fab and RSR-B2 Fab interactions with the TSHR (Sanders *et al.* 2006). Further examples are shown in Table 4 and the effects of experimental mutations available to date, i.e. FSHR N178A, TSHR I60A, TSHR S79I, TSHR F134A, TSHR T159C, TSHR

Table 4 Comparison of interactions in follicle-stimulating hormone (FSH)–FSH receptor (FSHR) and thyroid-stimulating hormone (TSH)–TSH receptor (TSHR) complexes – interactions produced in one of the complexes only without clear role for specificity

	Main interaction	FSH	FSHR	TSHR	TSH	Main interaction	Distance (Å)
Distance (Å)							
3·8	Hydrophobic	L99β	Q33	K42	–	–	–
3·9	Hydrophobic	L99β	V54	I60	–	–	–
4·3	Unfavourable electrostatic	R42α	K57	H63	–	–	–
6·7	Solvent-separated ion pair	K63α	E73	S79	–	–	–
–	–	–	S128	F134	K91α	Induced dipole	3·4
3·1	Hydrophobic	L48α	T130	T136	–	–	–
3·9	Hydrophobic	L48α	G131	G137	–	–	–
–	–	–	Q145	D151	R67α	Solvent-separated ion pair	6·8
–	–	–	–	I152	R67α	Induced dipole	2·9
3·3	Polar	K91α	Q152	T159	–	–	–
5·8	Solvent-separated ion pair	R67α	E171	E178	–	–	–
–	–	–	N178	Y185	S92α	Polar	3·9
5·6	Unfavourable electrostatic	K51α	K179	N186	–	–	–
2·8	Hydrogen bond	S89β					
2·9	Salt bridge	D90β					
6·4	Solvent-separated ion pair	D88β					
–	–	–	V221	S229	K44β	Polar	4·0
3·7	Hydrophobic	P42β	I222	L230	–	–	–
3·5	Hydrophobic	P45β					
6·2	Solvent-separated ion pair	D90β	R227	Q235	–	–	–

Ion–dipole and dipole–dipole interactions not involved in hydrogen bonds are described as ‘polar’. Ion-induced dipole and dipole-induced dipole interactions are described as ‘induced dipole’. Dispersion and hydrophobic forces are described as ‘hydrophobic’. Charge–charge interactions at ≤3·5 Å distance are defined as ‘salt bridge’. Charge–charge interactions at >3·5 Å and ≤4·5 Å distance are defined as ‘ion pairs’. Charge–charge interactions at >4·5 Å distance are defined as ‘solvent-separated ion pairs’.

Y185A, TSHR N186G, TSHR S229K, TSHR Q235A and TSHR Q235S (Smits *et al.* 2003, Vischer *et al.* 2003, Sanders *et al.* 2006), are consistent with these interactions.

Interactions and clashes produced by receptor residues that may be considered involved in specificity for their respective hormones

Table 5 shows interactions produced by FSHR and TSHR residues that may be involved in specificity of binding with their respective hormones. The importance of some of these residues on hormone-binding and hormone-induced cyclic AMP production has been tested in experimentally produced mutations in the FSHR or the TSHR.

In the model of TSHR, solvent exposed residue R38 forms a solvent-separated ion pair with TSHβ E98 but is in an unfavourable interaction with TSHβ H97. However, the potential unfavourable interaction with TSHβ H97 could be moderated by some of the properties of histidines. In particular, histidines tend to have a positive charge that is, on average, one-eighth of the charge of a lysine or arginine in solution at pH 7·4; however, this may be moderated in less solvent accessible environments (see Materials and methods). The residue equivalent to TSHR R38 in the FSHR is the

smaller V29, and it does not interact with FSH (Table 5 and Fig. 3A).

FSHR residue E50 forms an ion pair with R97 from FSHβ, although both residues are exposed to solvent. The equivalent residue in TSHR, T56, does not interact with TSH (Table 5 and Fig. 3B) and the T56R TSHR variant has been reported to show no gain of function towards hCG (Smits *et al.* 2003). This experimental observation is consistent with the lack of interactions in the TSHR–TSH model, as the uncharged P103 in hCGβ at the equivalent position to TSHβ E98 and FSHβ R97 cannot interact with the new arginine at position 56 of the TSHR variant. Consequently, ion pair interactions between FSHR E50 and FSHβ R97 may be FSHR specific.

The FSH–FSHR complex appears to have an unfavourable electrostatic interaction between FSHR R52 and FSHβ R97. However, both residues are exposed to the solvent. In the case of the TSH–TSHR complex, there is an ion pair between the equivalent residue K58 from TSHR and TSHβ E98, with both residues also exposed to the solvent (Table 5 and Fig. 3C). TSHR mutation K58A would be expected to disrupt the ion pair between TSHR K58 and TSHβ E98 (Fig. 3C) but the solvent-separated ion pair between TSHR R38 and TSHβ E98 should remain intact (Fig. 3A). This is consistent with the experimental observations that showed no effect of TSHR K58A mutation on pTSH-binding affinity (Sanders *et al.* 2006). Furthermore, TSHR K58 and TSHβ E98 are located at the

Table 5 Comparison of interactions produced by receptor residues in follicle-stimulating hormone (FSH)–FSH receptor (FSHR) and thyroid-stimulating hormone (TSH)–TSH receptor (TSHR) complexes and steric clashes found in the complexes with the hormones exchanged – likely to be important for specificity

	Main interaction	FSH	FSHR	TSHR	TSH	Main interaction	Distance (Å)
Distance (Å)							
–	–	–	V29	R38	E98β	Solvent-separated ion pair	4.7
3.7	Ion pair	R97β	E50	T56	–	–	–
3.1	Unfavourable electrostatic	R97β	R52	K58	E98β	Ion pair	4.0
3.4	Polar	S85α	K74	R80	S85α	Polar	3.8
3.2	Polar	T86α			T86α	Hydrogen bond	2.9
7.0	Unfavourable electrostatic	R97β			Y88α	Hydrogen bond	3.1
3.7	Ion pair	R97β	E76	Y82	–	–	–
3.8	Induced dipole	T86α	R101	E107	–	–	–
6.7	Unfavourable electrostatic	K91α					
3.4	Polar	T95β					
3.5	Induced dipole	V96β					
3.8	Hydrophobic	A43β	K242	K250	L42β	Induced dipole	2.9
2.9	CLASH	TSH L42β			K44β	Hydrophobic	3.1
2.9	CLASH	TSH K44β					
3.8	Polar	A43β	K243	E251	K44β	Salt bridge	2.9

Ion–dipole and dipole–dipole interactions not involved in hydrogen bonds are described as 'polar'. Ion-induced dipole and dipole-induced dipole interactions are described as 'induced dipole'. Dispersion and hydrophobic forces are described as 'hydrophobic'. Charge–charge interactions at ≤ 3.5 Å distance are defined as 'salt bridge'. Charge–charge interactions at > 3.5 Å and ≤ 4.5 Å distance are defined as 'ion pairs'. Charge–charge interactions at > 4.5 Å distance are defined as 'solvent-separated ion pairs'. CLASH refers to situations when the distance between the nuclei of the two non-bonding atoms was smaller than the sum of the van der Waals radii of the two atoms.

edge of the interface and exposed to the solvent and this would be expected to reduce any electrostatic interactions drastically. A steady increase in the gain of function towards hCG was reported in a series of K58 TSHR mutations (Smits *et al.* 2003); from the least active K58R, then to K58S, K58A and the most active K58D (Smits *et al.* 2003). The increase in sensitivity to hCG may be due to a new interaction between D58 in the TSHR K58D mutant with K104 in hCGβ (equivalent position to TSHβ A99), and indeed Smits *et al.* (2003) suggested electrostatic interaction of a positively charged region in hCG with K58.

TSHR R80 gives rise to two hydrogen bonds (with TSHα T86 and TSHα Y88) and steric clashes with TSHα chain residues, while the equivalent FSHR K74, whose side chain is shorter, is not involved in hydrogen bonds with FSH residues and the steric clash is weaker. Furthermore, FSHR K74 is 7.0 Å away from FSHβ R97 producing an unfavourable long-range charge–charge interaction, while TSHR R80 is 9.3 Å away from TSHβ E98 producing a favourable long-range charge–charge interaction (Table 5 and Fig. 3D). The long side chain of TSHR R80 clashes with TSH. A variant with a slightly shorter side chain such as lysine (in the case of TSHR R80K mutation) will release the clash to some degree, while alanine in this position (TSHR R80A mutation) will release the clash completely. In the TSHR R80D mutation, an aspartic acid with a smaller side chain than arginine and lysine is present and, consequently, the distances from the oxygens of aspartic acid substituted for TSHR R80 to the TSHα residues S85, T86 and Y88 are longer, the hydrogen bonds are broken and the

distance to the TSHβ chain residues is far too long for effective electrostatic interactions.

Overall, the above described differences in the interactions between the equivalent residues, TSHR R80 and FSHR K74, together with the evidence from the mutation studies, indicate that these two residues are likely to be important for the respective receptors specificity (Smits *et al.* 2003, Sanders *et al.* 2006). R80A and R80D showed similar pTSH binding as the wild-type TSHR while cyclic AMP stimulation by pTSH was similar to the wild-type TSHR in the case of R80A and slightly reduced (80–100% of wild-type) in the case of R80D (Sanders *et al.* 2006). TSHR R80K mutation caused a slight increase in sensitivity towards hCG while R80A mutation resulted in a more marked effect (Smits *et al.* 2003).

FSHRE76 forms part of an ion pair with FSHβ R97 with FSHR E76 partially buried. In the TSH–TSHR complex, this ion pair is not present (Table 5 and Fig. 3E). However, the hCGβ chain K104 (equivalent to TSHβ A99) may form interactions with the TSHR when TSHR Y82 (equivalent to FSHR Glu 76) is mutated. This is in agreement with the reported increased sensitivity of the TSHR Y82E for hCG (Smits *et al.* 2003) as the new glutamate at position 82 of the TSHR may interact with βhCG K104. In contrast, mutation of TSHR Y82A showed no increased sensitivity to hCG (Smits *et al.* 2003). Furthermore, TSHR Y82A showed no effect on pTSH binding to the receptor or in pTSH-induced stimulation of cyclic AMP production (Sanders *et al.* 2006).

FSHR R101 is involved in an unfavourable charge-charge interaction with α FSH K91 while the equivalent residue, E107, from TSHR does not interact with TSH as all distances are too long. This position may be important for TSHR specificity because TSH has a histidine at position 97 of the β -chain while FSH has a valine at the equivalent position (Table 5 and Fig. 3F). The introduction of an arginine at position E107 in the TSHR sequence (Sanders *et al.* 2006) will produce two unfavourable charge-charge interactions with TSH α K91 and TSH β H97 and is likely to reduce TSH binding as was indeed found in the experimental studies using pTSH (Sanders *et al.* 2006).

FSHR K242 gives rise to a hydrophobic interaction with FSH β A43. The equivalent residue TSHR K250 makes unfavourable interactions by placing its positive charge within a hydrophobic core formed by TSH β residue L42 and the hydrophobic side chain of TSH β K44. Furthermore, FSHR K242 produces strong clashes with TSH β residues L42 and K44 in the TSH-FSHR complex (Table 5 and Fig. 4A). TSHR equivalent residue K250 is likely to be accommodated better with a much smaller FSH β equivalent residue A43 in the FSH-TSHR complex (Fig. 4A). In the TSHR K250A mutant, both these unfavourable interactions are lost and the mutation should have no effect on TSH-stimulating cyclic AMP production as shown in mutation experiments using pTSH (Sanders *et al.* 2006). The TSHR K250Q mutation showed no increase in sensitivity to hCG (Smits *et al.* 2003), as hCG also has a leucine (L49) at the equivalent position in FSH β (A43) and TSH β (L42).

In the TSH-TSHR complex there is a salt bridge between the solvent exposed residues E251 from the TSHR and K44 from TSH β , but in order to produce this salt bridge TSH β K44 clashes with TSHR K250. Equivalent interactions are not present in the FSH-FSHR complex. Equivalent residue FSHR K243 has a polar interaction with the oxygen of FSH β A43 (Table 5 and Fig. 4B). The TSHR E251A variant lacks the clash

Figure 3 Interactions produced by (A) FSHR V29 with FSH β residues in the FSH-FSHR complex and by TSHR R38 with TSH β residues in the TSH-TSHR complex, (B) FSHR E50 with FSH β R97 in the FSH-FSHR complex and by TSHR T56 with TSH β E98 in the TSH-TSHR complex, (C) FSHR R52 with FSH β residues in the FSH-FSHR complex and by TSHR K58 with TSH β residues in the TSH-TSHR complex, (D) FSHR K74 with FSH α residues in the FSH-FSHR complex and by TSHR R80 with TSH α residues in the TSH-TSHR complex, (E) FSHR E76 with FSH β R97 in the FSH-FSHR complex and by TSHR Y82 with TSH β E98 in the TSH-TSHR complex and (F) FSHR R101 with FSH residues in the FSH-FSHR complex and by TSHR E107 with TSH residues in the TSH-TSHR complex. Receptor LRD backbones are shown in magenta, hormone α -chains in green and β -chains in cyan. Interacting residues are shown as sticks with carbon atoms in grey, oxygens in red and nitrogens in blue. Solid lines denote interactions, dashed lines denote no interactions.

Figure 4 (A) Clashes produced by FSHR K242 with TSHβ residues in the TSH–FSHR complex and by TSHR K250 with FSHβ A43 in the FSH–TSHR complex. Clashing residues are shown as sticks with carbon atoms in magenta for receptor residues and cyan for hormone residues, oxygen atoms in red and nitrogens in blue. The van der Waals surfaces are shown as dotted surfaces. (B) Interactions produced by FSHR K243 with FSHβ A43 in the FSH–FSHR complex and by TSHR E251 with TSHβ K44 in the TSH–TSHR complex. Receptor LRD backbones are shown in magenta, hormone α -chains in green and β -chains in cyan. Interacting residues are shown as sticks with carbon atoms in grey, oxygens in red and nitrogens in blue. Solid lines denote interactions.

and the favourable charge–charge interaction, but because the charges of the two residues are exposed to the solvent the strength of the interaction is likely to be weak. In addition, TSHβ K44 is involved in several interactions with the receptor (see Tables 3 and 4) and, probably, the disruption of one of these interactions is not enough to show a significant reduction in activation. This is consistent with the experimental evidence using pTSH (Sanders *et al.* 2006).

Discussion

The FSHR and TSHR LRDs share 40% amino acid sequence identity, while the α -chains of hFSH and hTSH are 100% amino acid sequence identical and their β -chains are 44% identical (UniProtKB/SwissProt; <http://www.ebi.ac.uk/swissprot/>). This high level of amino acid sequence identity makes it highly likely that the structures of the two receptors as well as the two hormones are very similar and that they will bind to their respective receptors in similar ways. Smits *et al.* (2003) and Vischer *et al.* (2003) have reported that two mutations in the FSHR LRD make the FSHR highly sensitive to hCG

and eight mutations in the LRD of the TSHR make the TSHR highly sensitive to hCG. This small number of mutations that can change hormone receptor specificity emphasises the similarity in the way the hormones bind to their receptors. We have assumed that the FSH–FSHR LRD and the TSH–TSHR LRD complexes have similar hormone–receptor interfaces, and this allowed us to build a model of the structure of the TSH–TSHR LRD complex using the solved crystal structures of the FSH–FSHR LRD and M22–TSHR LRD complexes (Fan & Hendrickson 2005, Sanders *et al.* 2007a).

Some theoretical models of the TSH–TSHR complex (Kleinau *et al.* 2004, Moyle *et al.* 2004, Núñez Miguel *et al.* 2004) were published just before the coordinates of the FSH–FSHR complex became available (Fan & Hendrickson 2005). The structure of the concave surface and β -strands of the TSHR LRD were well defined in the three models (Kleinau *et al.* 2004, Moyle *et al.* 2004, Núñez Miguel *et al.* 2004). The model of Moyle *et al.* (2004) comprised three domains of the TSHR linked together and the LRD structure was modelled on the structure of ubiquitin ligase. The model shows TSH binding to one of the side surfaces of the LRD (between the concave and convex surfaces)

rather than to the concave surface itself. The model of Kleinau *et al.* (2004) consists of parts of the TSHR ectodomain only. The LRD structure was modelled on the structure of the Nogo receptor and showed a good approximation to the structure of the TSHR LRD, which is now known (Sanders *et al.* 2007a). The complex shows TSH interacting with the concave surface of the TSHR LRD but in a different position compared with the FSH–FSHR complex. In particular, the hormone lies parallel to the length of the LRD (Kleinau *et al.* 2004). Our earlier model (Núñez Miguel *et al.* 2004) consisted of three TSHR domains with the LRD structure based on ribonuclease inhibitor, and showing small helices, instead of small strands, in the convex surface. The hormone-binding arrangement in our earlier model was in agreement with that observed in the crystal structure of the FSHR LRD in complex with FSH (Fan & Hendrickson 2005), although TSH was in a 180° orientation compared with FSH in the Fan and Hendrickson structure. However, both TSH orientations relative to the concave surface of the LRD were considered while building the model (Núñez Miguel *et al.* 2004, 2005).

The advantage of the current model of TSH–TSHR LRD over the previously reported models relates to it incorporating the TSHR LRD crystal structure solved at 2.55 Å resolution, and TSH structure and the binding arrangements to the TSHR based on FSH–FSHR crystal structure solved at 2.9 Å resolution (Fan & Hendrickson 2005, Sanders *et al.* 2007a) and good validation parameters. Comparing the interfaces of the two complexes, it can be appreciated that the FSH–FSHR complex interface presents more favourable interactions (six ion pairs and five hydrogen bonds) than the interface of the TSH–TSHR complex (four ion pairs and three hydrogen bonds). For example, FSHR E50 produces an ion pair with FSHβ R97 while the TSHR equivalent residue T56 does not interact with TSH (Fig. 3B). The electrostatic potential surfaces of both TSHR and FSHR LRDs are complementary to the electrostatic potential of the respective interacting surfaces of the hormones (Fig. 1B). There are two striking differences in the electrostatic potentials between TSH and FSH related to different charges of the equivalent residues: TSHβ E98 and FSHβ R97 and TSHβ K44 and FSHβ A43, which interact with two receptor areas that are different on the electrostatic potential surface of the TSHR and FSHR. The negatively charged area centred on TSHβ E98 interacts with a positively charged area on the TSHR formed by TSHR R38, K58 and R80, whereas the positively charged area centred on FSHβ R97 interacts with an area where both negatively (FSHR E76 and E50) and positively (FSHR R52 and K74) charged residues are present. Further differences include a negative patch at the C-terminal region of the TSHR LRD centred on E251 involved in

interaction with TSHβ K44, whereas the corresponding area in the FSHR is positively charged (K243) and is not involved in interactions with FSH (Fig. 1B). It is of interest that the receptor residues that contribute to the differences in the electrostatic potentials between FSHR and TSHR were found likely to be involved in specificity in our study (Table 5 and Figs 3 and 4).

The interface ΔASA of the FSH–FSHR complex is 50 Å² greater than that of the TSH–TSHR complex and the gap volume index of the TSH–TSHR complex is 12% higher than the one of the FSH–FSHR complex (Table 1). Although there is no strict relationship between the interface area of the complex or the shape complementarity of the interfaces, they give a quantitative indication of the extent of the interactions within a homologous series (Prabhu & Sharp 2004). All these features are consistent with a higher binding affinity of FSH for the FSHR LRD than the affinity of TSH for the TSHR LRD, as has been shown experimentally (Simoni *et al.* 1997, Dias *et al.* 2002, Szkudlinski *et al.* 2002).

In order to assess reliability of gap volume index calculations for comparative models of the complexes, we have built two models of the FSH–FSHR complex. One of these was a comparative model of the FSH–FSHR complex based on a modelled complex between the crystal structure of TSHR LRD and the crystal structure of hCG following the same steps as for the construction of the model of the TSH–TSHR complex. A second comparative model of the FSH–FSHR complex was built using our TSH–TSHR complex as template. For both FSH–FSHR complexes, nine models were built by MODELLER and the average gap volume indices obtained were: 3.10 with a s.d. of 0.13 and 0.21 for the first and second models respectively. The obtained gap volume indices values for comparative models of the FSH–FSHR complexes were similar to that obtained from the solved crystal structure of the complex (Table 1). The average gap volume index obtained from nine comparative models of the TSH–TSHR complex was 3.64 ± 0.21 (s.d.). This indicates that the average gap volume index is fairly robust and not too dependent on the accuracy of the models.

One important feature for avoiding receptor promiscuity is that the hormone would present strong clashes with receptor residues when attempting to bind to the ‘wrong’ receptor, which would lead to a difficult if not impossible binding situation. The complexes of the TSHR and FSHR with the hormones exchanged were obtained in order to study this important feature. From all the strong clashes found in the TSH–FSHR and FSH–TSHR complexes, those produced by FSHR K242 with βTSH K44 and L42 seem to be of particular interest for specificity as shown in Fig. 4A and Table 5.

Our analyses using the criteria we selected showed that the α-chains of the hormones are involved in the majority of similar interactions in the two natural

complexes. All specificity determining situations we found involve residues from the β -chains of the hormones although the evidence from the FSH-FSHR LRD structure suggests that both α - and β -chains of GPHs are involved in binding specificity (Fan & Hendrickson 2005). It should be noted, however, that the specificity of GPH and receptor recognition was studied by sequence variation analysis by Fan & Hendrickson (2005), while in our study the interactions in the FSH-FSHR LRD complex and the TSH-TSHR LRD model were compared. We also found that there are several different interactions produced by receptor residues that appear not to be involved in specificity. In most cases, these interactions involve different receptor residues with identical residues from the α -chains of the hormones or with same type of residues in the β -chains of the hormones.

In both complexes, the α -chain of the hormone interacts mostly with the central part of the concave face of the receptor involving repeats three to six of the LRD. Hormone β -chains, on the other hand, interact more with the N-terminus and the C-terminus repeats of the concave faces of the LRDs. Because it is assumed that the β -chains of the hormones are responsible for specificity (Pierce & Parsons 1981), the receptor residues involved in specificity would appear to be located in the two termini of the LRD and not in the central area.

The analysis of interactions in the natural complexes and those in models of the complexes where the hormones have been interchanged allows some receptor residues to be identified as being very likely involved in defining receptor specificity (Table 5). These residues are located in the second, third, fourth and ninth repeats of the LRDs, and this is consistent with the suggestion that the two ends of the concave face of the receptor LRDs are important in determining specificity (Fig. 5B and C). Table 6 shows the predicted effects of mutations of these LRD residues for TSH- and FSH-binding affinity. Effects of the mutations listed in Table 6 on TSH binding to and activation of the TSHR should now be tested experimentally to assess our observations.

FSHR residues E76 and R101 have been proposed to be involved in FSH versus TSH selectivity (Fan & Hendrickson 2005, 2007). FSHR I222 has also been suggested to be involved in FSH and TSH specificity (Fan & Hendrickson 2007). Our studies also showed that FSHR residues E76 and R101 should have a role in FSHR specificity (Table 5 and Fig. 3E and F). However, FSHR I222 has hydrophobic interaction with β FSH P42 and with β FSH P45, while TSHR equivalent residue L230 does not interact with TSH. It is not clear at present whether FSHR I222 and TSHR L230 are involved in specificity or not, but because the two receptor residues are equivalent, they were not considered for specificity under the selection criteria we have used.

Especially important is the evolutionary difference in the hormones at FSH β R97, hCG β P103 and TSH β E98. In FSH, it is a positively charged amino acid, hydrophobic in hCG and negatively charged in TSH. The hormone residues at this position produce several interactions with residues from the receptors. As expected, some of the receptor positions identified as involved in specificity (TSHR residues R38 and K58 and FSHR residues E50, R52, K74 and E76) interact with these hormone residues (Table 5 and Fig. 3).

Specific binding of 125 I-labelled hTSH to the hTSHR cannot be detected easily (presumably because of a rapid dissociation rate) and, consequently, direct binding studies with the human hormone are not usually practical. Bovine and porcine TSH have been used (Sanders *et al.* 2006, 2007b) because their affinities to the hTSHR can be assessed fairly accurately; for example, pTSH-binding affinity to hTSHR has a K_a of $\sim 10^9$ L/mol (Sanders *et al.* 2006). Figure 5A shows the residues that are different between hTSH and bTSH or pTSH (UniProtKB/Swiss-Prot; <http://www.ebi.ac.uk/swissprot/>). There are 23 differences in the α -chains and 9 differences in the β -chains between hTSH and bTSH or pTSH. TSH α R67 (K64 in bTSH and pTSH) is the only one of those different residues, which undergoes a conserved substitution and interacts with the TSHR LRD. TSH α R67 produces a weak interaction with TSHR I152. Consequently, use of the results of the experimental studies with pTSH or bTSH to validate the interactions between hTSH and TSHR LRD in our model can be justified.

Substitution of α TSH residues T11, Q13, P16 and Q20 by lysines substantially increases TSHR-binding affinity and bioactivity (Szkudlinski *et al.* 1996). These α TSH residues do not interact with the TSHR LRD in our TSH-TSHR LRD complex structure. Furthermore, the equivalent α FSH residues do not interact with the FSHR LRD in the FSH-FSHR LRD complex structure (Fan & Hendrickson 2005). These observations suggest that TSH interacts with parts of the receptor in addition to the LRD and this is consistent with recent proposals by Mizutori *et al.* (2008) and Mueller *et al.* (2008). This non-LRD binding might be expected to involve hormone residues that are different between TSH hormones from different species (human, bovine and porcine).

As mentioned before, there are two areas on the surface of the structure of the TSHR LRD-presenting residues that may be involved in hormone specificity (Fig. 5C). Table 5 shows that an area at the N-terminus (TSHR residues R38, T56, K58, R80, Y82 and E107) interact with α TSH C-terminal residues and with the 'seat belt' of TSH (β TSH residues 88–105) which has been previously predicted to be important for hormone binding and specificity (Grossman *et al.* 1997a,b, Szkudlinski *et al.* 2002), while an area at the TSHR

Figure 5 (A) Space-fill representation of the structure of human TSH showing the face that interacts with the TSHR LRD. TSH α chain residues are shown in yellow and TSH β chain residues in orange. Different residues in the amino acid sequences between hTSH and bTSH or pTSH are shown in cyan (α -chain) and in green (β -chain). hTSH α R67 (marked) is the only residue out of all residues different between the three species of TSH that produces a weak interaction with the TSHR LRD. (B) Space-fill representation of the structure of human FSHR LRD and (C) TSHR LRD showing the faces that interact with their hormones. Interacting residues are shown in cyan. Residues that have been found as probably being involved in specificity are identified and shown in magenta. N- and C-termini are marked.

Table 6 Thyroid-stimulating hormone receptor (TSHR) and follicle-stimulating hormone receptor (FSHR) residues that may be important for specificity – predicted effect of mutations

Mutation	TSH-binding affinity	Interaction modifications	FSH-binding affinity	Interaction modifications
TSHR R38D	Small reduction	New unfavourable electrostatic	Small increment	New ion pair
TSHR R38E	Reduction	New unfavourable electrostatic	Increment	New ion pair
FSHR E50R	Increment	New ion pair	Reduced	New unfavourable electrostatic
FSHR E50K	Increment	New ion pair	Reduced	New unfavourable electrostatic
TSHR T56D	Small reduction	New unfavourable electrostatic	Small increment	New ion pair
TSHR T56E	Reduction	New unfavourable electrostatic	Increment	New ion pair
TSHR K58D	Small reduction	New unfavourable electrostatic	Small increment	New ion pair
TSHR K58E	Reduction	New unfavourable electrostatic	Increment	New ion pair
TSHR R80E	Small reduction	Interactions lost	Small increment	New ion pair
FSHR E76K	Increment	New ion pair	Reduced	New unfavourable electrostatic
FSHR E76R	Increment	New ion pair	Reduced	New unfavourable electrostatic
TSHR Y82D	Small reduction	New unfavourable electrostatic	Small increment	New ion pair
TSHR Y82E	Reduction	New unfavourable electrostatic	Increment	New ion pair
FSHR R101E	Small increment	New ion pair	Small increment	New ion pair
FSHR K242A	Small increment	Clash lost	Similar	Similar
FSHR K243D	Increment	New ion pair	Similar	Similar
FSHR K243E	Increment	New ion pair	Similar	Similar

C-terminus interacts with the ‘Keutmann’s loop’ (β TSH residues 32–50) also cited as important for TSH binding (Morris *et al.* 1990, Szkudlinski *et al.* 2002). Equivalent β hCG residues 38–57 were also reported to be important for hCG binding to the LHR (Ryan *et al.* 1988). It has been found that the ‘seat belt’ confers TSH/hCG specificity but is not enough to confer TSH/FSH specificity and that other parts of the hormone should also be involved in specificity (Grossmann *et al.* 1997a). In our study, we have found, as mentioned before, two hormone areas that may be involved in specificity (i.e. the C-termini of the β -chain (the seat belt) and the ‘Keutmann’s loop’). Overall, the observations in our structural comparison are consistent with current experimental evidence on TSH binding to the TSHR.

Some of the sugar molecules have been found to be necessary for the GPHs’ ability to bind and activate their respective receptors (Matzuk *et al.* 1989, Bishop *et al.* 1994, Grossmann *et al.* 1995, 1997b, Creus *et al.* 2001, Campo *et al.* 2007). In the case of the GPH receptors, glycosylation has been found important for appropriate folding and function of the TSHR and the FSHR (Davies *et al.* 1995, Rapoport *et al.* 1998, Oda *et al.* 1999). In contrast, acquisition of complex carbohydrates has not been found important for hormone binding in the case of the LH/CG receptor (Davies *et al.* 1997, Dufau 1998). The glycosylation sites on FSH (FSH α N52 and N78 and FSH β N7 and N24) and on the FSHR LRD (N191) observed in the crystal structure were remote from the interface (Fan & Hendrickson 2005). In the crystal structure of the M22–TSHR LRD complex, the glycosylation sites on the TSHR LRD (N77, N99, N113, N177 and N198) were observed and were located away from the concave surface of the LRD that interacts with

M22 (Sanders *et al.* 2007a). Analysis of the FSH–FSHR LRD structure and our model of the TSH–TSHR LRD complex showed that sugar residues from one component of the hormone–receptor complexes do not interact with the other component. Furthermore, in both complexes, the sugar residue locations are distant from the interacting interfaces and, consequently, are unlikely to be important for the interaction, at least with the LRDs.

It has been suggested that the TSHR on intact cells dimerises and that receptor dimerisation is important for TSHR biological activity (Latif *et al.* 2001, Urizar *et al.* 2005). A functional dimerisation of TSHR through the TMD has been proposed in which there is asymmetry in hormone binding by the two binding sites in the dimer and negative cooperativity between them (Urizar *et al.* 2005). Our study of interactions in the FSH–FSHR and TSH–TSHR complexes does not provide any suggestions as to whether the receptors dimerise or not. In crystallographic studies, however, two FSH–FSHR LRD complexes were found in one asymmetric unit of the crystal of the complex (Fan & Hendrickson 2005), whereas only one M22–TSHR complex was evident in the asymmetric unit of the crystal (Sanders *et al.* 2007a). Furthermore, only one molecule per asymmetric unit of crystal lattice has been found for the β_2 -adrenergic receptor (Cherezov *et al.* 2007, Rasmussen *et al.* 2007) and this can be compared with the experimental evidence that the monomeric β_2 -adrenergic receptor in a lipid bilayer is the minimal functional unit necessary for efficient signalling (Whorton *et al.* 2007). Consequently, whether the TSHR dimerises or not and if this is important for its activity have yet to be resolved.

It is surprising that many more substitutions between TSH in different species can be found in the α -chains than in the β -chains (see above). This suggests that the conserved TSH β residues that bind to the TSHR and confer TSH specificity with respect of binding to other GPH receptors have been maintained during the evolution to prevent cross-reactivity. Also, it has been proposed that residues from the hTSH α , T11, Q13, P16 and Q20, which are lysines in bTSH and pTSH are responsible for the low affinity binding of hTSH to the hTSHR (Szkudlinski *et al.* 1996; see above). On the other hand, the three human GPHs, hTSH, hCG and hFSH, share the same α -chain. This could indicate that the α -chains of the hormones tend to contribute to the activation of the receptor through interactions with the CD and/or the TMD, while the β -chains are more involved in the specificity of the hormones for their respective receptors through interaction with the LRD; however, both chains form important interactions with the LRD. Thus, in order to keep a similar activation process in the same organism, the three GPHs use the same α -chain, while the difference in hTSHR activation by hTSH and bTSH or pTSH comes from the considerable amino acid differences in their α -chains. This concept is also supported by the fact that bTSH and pTSH affinities for hTSHR are similarly high and that there are only three different residues in their α -chains.

Another intriguing issue is the remarkable similarity of binding arrangements of TSH and M22 with the TSHR (Rees Smith *et al.* 2007, Sanders *et al.* 2007a). This example of molecular mimicry by two proteins of different structures and origins to form almost identical interactions with and biological effects on the TSHR is not yet understood (Felding-Habermann *et al.* 2004, Rees Smith *et al.* 2007, Sanders *et al.* 2007a). However, our model of the TSH–TSHR complex will be very useful to analyse and compare the interactions between TSH and TSHR autoantibodies with the TSHR in detail and this work is currently under way.

The availability of the crystal structures of the M22–TSHR and FSH–FSHR complexes has allowed us to obtain an improved model of the TSH–TSHR complex that was used to study similarities and differences in interactions in the FSH–FSHR and TSH–TSHR complexes. Several amino acids that may be involved in defining receptor specificity have been identified. The experimentally evident higher affinity of FSH for its receptor than TSH for TSHR is reflected in interactions found in the respective complexes as well as the role of each hormone chain in receptor function. Our studies also suggest that the GPHs bind parts of their receptors in addition to the LRD and overall provide foundations for understanding the receptor specificity of GPHs at the molecular level.

Declaration of interest

RSR Ltd is a developer of *in vitro* medical diagnostics including kits for measuring thyroid autoantibodies. RNM, JS, JF and BRS are employees of RSR Ltd.

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