TSH receptor oligomers associated with the TSH receptor antibody reactome

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Abbreviations: TSH – thyroid stimulating hormone, TSHR – TSH receptor, GPCR - G protein coupled receptor, MD - molecular dynamics, ECD – TSHR extracellular domain, LRD – TSHR leucine-rich domain, TMD – TSHR transmembrane domain DPPC – dipalmitoylphosphatidylcholine

Abstract

The TSH receptor (TSHR) and its many forms are the primary antigens of Graves' disease as evidenced by the presence of TSHR antibodies of differing biological activity. The TSH holoreceptor undergoes complex post-translational changes including cleavage of its ectodomain and oligomer formation. We have previously shown that the TSHR exists in both monomeric and dimeric structures in the thyroid cell membrane and have demonstrated, by modeling, that the transmembrane domains (TMD) can form stable dimeric structures. Based on these earlier simulations of the TSHR-TMD structure and our most recent model of the full-length TSHR we have now built models of full length TSHR multimers with and without TSH ligand in addition to multimers of the extracellular leucine-rich domain (LRD) – the site of TSH and autoantibody binding.

Starting from these models we ran molecular dynamics (MD) simulation of the receptor oligomers solvated with water and counterions; the full-length oligomers also were embedded in a DPPC bilayer. The full length TSHR dimer and trimer models stayed in the same relative orientation and distance during 2000 ns (or longer) MD simulation in keeping with our earlier report of TMD dimerization. Simulations were also performed to model oligomers of the LRD alone; we found a trimeric complex to be even more stable than the dimers.

These data provide further evidence that different forms of the TSHR add to the complexity of the immune response to this antigen which in patients with autoimmune thyroid disease generate an autoantibody reactome with multiple types of autoantibody to the TSHR.

INTRODUCTION

Everybody carries an array of autoantibodies (the reactome) generated against self and which may be disease modifying or clinically silent until their activity is unmasked (1). One of the best known examples of a self antigen with multiple types of autoantibodies is found in hyperthyroid Graves' disease (2). In this condition autoantibodies with greatly varying biological activity are found directed against the receptor for thyroid stimulating hormone (TSHR) expressed primarily on the surface of the thyroid follicular cell. The TSHR is a class A GPCR (2) with 764 amino acids comprising a large, heavily glycosylated, ectodomain (ECD) connected to a seven-helix transmembrane domain (TMD) (3,4) (**Figure 1A**). The ECD consists of two domains, the well-structured leucine-rich domain (LRD) where TSH and autoantibodies bind and a flexible linker (or hinge) region (LR). Cleavage and shedding of the ECD from the membrane-anchored TMD has been well characterized in vitro (5) and release of the ECD is considered to form a primary autoimmune target (6).

We have previously shown, by biochemical and biophysical methods, that TSHRs in native, as well as transfected cells, exist in multiple forms including both dimeric and oligomeric units and that oligomerization may be regulated by exposure to TSH ligand (4,7,8). Studies have also shown that TSHR dimerization may have physiological consequences including a role in receptor negative co-operativity (9) and G-protein selection and activation favoring $G_{\alpha q}$ (10). We have also shown previously that dimerization involves contact between the TSHR ECDs (11) and experimental data with truncated TSHRs have indicated that the TMD alone continues to dimerize and has a major role in TSHR dimerization and oligomerization (12). In all, therefore, there are at

least 4 different TSHR forms expressed and this structural complexity may help explain the diverse reactome that patients may express (2).

Recent progress in TSHR crystallization (13) and modeling studies (14,15) have now allowed us to examine structures of these different forms to enhance our understanding of this dynamic post translational processing and reference (16) presents a review of the use of molecular simulations in the study of protein structures. We first modeled TSHR dimerization by using a Brownian Dynamics approach (17). We recently confirmed this model by running Molecular Dynamics of this TSHR TMD dimer model embedded in a DPPC membrane and fully solvated with water and counterions (18). We have now developed in silico models of the full-length TSHR, both with and without TSH ligand present (15), and an improved version (19) helped by experimental data from cryo-EM studies (6,13). The current work combines our models of the full-length TSHR with that of the TMD dimer using Molecular Dynamics (MD) to first examine the stability of the full-length TSHR dimer. In addition, to obtain models for potential TSHR oligomers, we combined two dimer models to produce a trimer model and examined its stability in the aqueous membrane environment using MD after inserting it into a DPPC membrane, with solvent water and counterions added. There is also experimental evidence that the LRD of the TSHR alone can oligomerize (6) and our own study showed that Y116 on the α helix of the TSHR LRD was a potential site (11). To understand the structural implication of these ectodomain forms, we also performed simulations of potential LRD oligomers obtained by removing the TMD and LR from our full-length model.

It should be pointed out that our simulations only demonstrate the kinetic stability of these oligomers. In order to computationally demonstrate thermodynamic stability we would need to use a more exacting type of simulation requiring greater computer power.

MATERIALS AND METHODS

Initial TSHR dimer model: The starting structure of the full-length TSHR-TSH complex (**Figure 1A**) was aligned, based on their TMD, to each monomer of our TMD dimer simulation (20); the TSH was omitted for clarity. It should also be noted that in our experience incorrectly positioned dimers actually separate within ~500 ns. The resulting complex, however, showed serious steric clashes of the ECDs of the two monomers, prompting us to search for a different conformation of the TSHR's ECD. The search involved the scanning of the MD trajectory of the TSHR-TSH simulation (19), looking for the conformation that, when aligned to the TMD monomers, results in the shortest distance that was still above 3 Å. The conformation selected was the structure at 707 ns. The final dimer model used the ECD of the aligned TSHR but kept the TMD from the dimer model that included the positions of internal waters, obtained as generic sites (21) calculated with the program MMC (22).

Initial TMD and TSHR trimer models: To extend the dimer to trimer we first generated a trimer of the TMD by aligning monomer 1 of the TMD dimer to monomer 2 of a copy of the TMD dimer. The resulting trimer formed a triangular structure, again without significant steric clashes. As a 2000 ns long simulation of this TMD trimer, embedded in

a membrane, indicated that this trimer structure is stable we proceeded with aligning the TSHR-TSH conformation at 707 ns to each of the three monomers to produce the starting trimer TSHR structure.

Initial LRD dimer and trimer models: As the ECD is cleaved to form structures of primarily the leucine rich domain (LRD) (23) we limited our study to the oligomerization of the LRD. The initial structures for the LRD dimer and trimer were obtained by removing residues > 270. We also generated two additional dimer structures, the first by placing the two monomers in the conformation proposed previously (6): the interface is the concave side of the LRD and one monomer is rotated by 90 degrees and the second structure that was generated by a Monte Carlo simulation based on continuum solvent.

Monte Carlo simulation: The simulation used two rigid copies of the LRD and the solvent was represented with a distance-dependent dielectric constant (24). The simulation was run with the program MMC (22).

Setting up molecular simulations: Each model, with internal water molecules in the TMD, was sent to the CHARMM-GUI server (25) which inserted it into a DPPC bilayer and added the rest of the solvating waters and counterions. The simulations used the Charmm-36 force field (26) and the TIP3P water model (27). The number of counterions (K^+ and Cl^-) were established to ensure electroneutrality and an ionic strength of 0.3 m/L. The system generated used periodic boundary conditions with hexagonal prism as the

unit cell for the TMD and TSHR oligomers and truncated octahedron for the LRD oligomers. For systems including the membrane CHARMM-GUI also provided inputs for a six-step equilibration protocol that progressively released constraints on the protein and lipids. The MD simulations were run using the program NAMD (28) using 2fs time step.

Analysis: The contacts between different extracellular domains of the oligomers studied were specified using mutual proximity for the contact criterion (29): a pair of atoms, each one on different domains, is considered in contact if atom 1 on domain 1 is the nearest to all atoms in domain 2 and atom 2 is the nearest to all atoms in domain 1. The smallest contact distance is also the shortest distance between the two domains; if that minimum is above a threshold then the two domains are not in contact. A pair of residues is considered to be in contact if at least one contact atom pair involves these two residues. The interactions involving the TMDs were tracked by looking at hydrogen bonds. Hydrogen bonds are defined as X···H-Y where X and Y are polar heavy atoms, the X···H-Y angle is above 120° and the X-Y distance is below 2.52, 2.52, 3.24, and 3.15 Å for N-H, O-H, P-H, and S-H, respectively. Since this definition ignores the actual charges, it includes salt bridges as well. The calculation and plotting of residues in mutual proximity or forming hydrogen bonds were performed by the program Simulaid (30).

RESULTS:

Multiple forms of the TSHR are expressed in the thyroid: Western blotting of thyroid cell membranes is well known to reveal multiple forms of the TSHR (**Figure 1B**). Lysate from a TSHR expressing cell was resolved in 4-15% SDS-PAGE gel and the PVDF transferred protein was probed with TSHR specific antibody M1 to reveal the different

forms of the TSH receptors as indicated here. These forms may be secondary to multiple mRNAs resulting from splicing (31,32) as well as secondary to post translational processing (33) or receptor degradation (34). The sizes of the different forms detected are very likely to be influenced by the degree of proteolysis and also by receptor glycosylation. **Table 1** indicates the possible sizes of the holoreceptor, the ectodomain after cleavage and the truncated TMD all of which may exist in different predicted oligomeric forms.

The simulations: The simulated systems, as generated by CHARMM-GUI, are described in **Table 2** with the system size parameters of the simulations performed. Most simulations were run for a minimum of 2000 ns and the trajectories generated were analyzed separately to obtain information concerning their structure and stability.

Dimeric forms of the TSHR monomer with and without TSH ligand: The initial structure of the full length TSHR dimer simulation is shown in **Figure 2A.** The concern here is always for steric “clashes” where a clash is defined as heavy atoms with the potential for overlap and being closer than the sum of their Van der Waals radii. In fact, the model showed that the relative orientation of the LRDs still allowed TSH to bind to both monomers without encountering any such clashes (**Figure 2B**). These simulated TSHR dimers were found to be stable during the MD runs and no lipid molecules entered the interface, as observed when the trajectories were animated using VMD (35).

Trimeric forms of the TSHR monomer with and without TSH ligand: In addition to the dimeric model we also show the initial_structures of potential full-length trimeric forms of the full length TSHR monomer (**Figure 3)**. The apo structure is shown in such a way that the interface of monomers 1 and 3 are facing the viewer, showing that there are again no steric clashes (**Figure 3A**). After modeling with TSH bound to the trimeric holoreceptor structure, **Figure 3B** shows the top view (i.e., from the extracellular side), again highlighting the fact that TSH could continue to bind to each of the three full length monomers without causing steric clashes. Significantly, the ECD orientations in the dimer and a trimer models were such that the inclusion of the TSH ligand resulted in no significant steric “clashes” and continued our previously reported enhanced stabilization of the LR (19)

Dimeric contact evaluation: **Figure 4** shows the residue-residue LRD^1-LRD^2 contact history between the two full length monomers in the apo TSHR dimer. In the figure (and in the **Figures 5** and **6** below) each significant residue-residue contact is represented by a line that may only span part of the time course and be broken at places, indicating that at those times the line was broken that pair of residues lost contact. Residue pairs that were in contact less than 10% of the simulation time were omitted. Contacts were well maintained throughout the 2000 ns simulation with 10 of 16 residue pairs registering >30% occupancy. **Figure 5** shows the influence of TSH on these LRD^1-LRD^2 contact histories illustrating the lower number of reported contact switches indicating improved stability (17 compared with 26). **Figure 6** shows the contacts

between just TSH and the LRD[1] illustrating another stabilizing factor to the holoreceptor dimer.

Trimeric contact evaluation: The MD simulations showed that the stability of the full length trimer was greater than that seen with the dimer. The percent of the time contacts formed between residue pairs are shown in **Supplementary Tables 1**, **2** and **3** (36) for the domain pairs LRD-LRD, TSH_{α}-LRD, TSH_{β}-LRD, respectively (TSH_{α} and TSH_{β} being the two domains of TSH). Each column represents contacts between two monomers, be it the dimer or the trimer. Since the contact points between the trimer monomers are similar to those of the dimer they were omitted for brevity. The extracellular domains showed some flexibility as observed in the bond history plots.

Transmembrane interactions: The interactions between the TMDs were characterized by looking for hydrogen bonds that the TMDs formed with other domains in the system. They are summarized in **Supplementary Table 4** (36). The table shows all residue pairs involving the TMDs that were found forming hydrogen bonds in at least one of the oligomers studied. The analysis included the TMD dimer described earlier (20).

Dimers formed by LRDs alone: A 2000 ns simulation of the LRD dimer starting from the conformation obtained from the full-length TSHR model (**Supplementary Figure 1A** (36)) at first dissociated but later settled into a stable conformation (**Figure 7A)**. The contact history (**Figure 8**) also shows that after dissociation it first sampled a conformation

that was different from the one the simulation settled in. We also started a simulation from the model conformation (**Supplementary Figure 1B** (36)) suggested earlier (6)**.** In this simulation the LRDs rapidly dissociated and the secondary structures unraveled, even when the two monomers were still in contact. This and the early dissociation of the model from the full-length dimer (that, somewhat unexpectedly, later formed a stable dimer, as discussed above) prompted us to look for additional LRD dimer model structures using Monte Carlo simulation. The simulation involved 100,000 trial moves (translation and rotation of one of the LRDs) and after about 50,000 steps the simulation settled into a conformation shown in **Supplementary Figure 1C** (36) which we then used to start another MD simulation. This MD simulation produced a surprising behavior because it kept forming contacts between the monomers but the beta sheets unraveled while helices started to form. After ~600 ns the simulation settled to fluctuate around a new conformation and presumably became misfolded into a molten globule. **Figure 7B** shows this structure at the end of the run and **Supplementary Figure 2** (36) shows the secondary structure history of this dimer.

LRD trimers: Simulations started from the LRD structure of the full-length trimer conformation (**Supplementary Figure 1D** (36)) stayed together from the beginning although rapidly rearranged during equilibration from the initial structure – see the inset in **Figure 8**. This is in contrast to the LRD dimer behavior that first dissociated and only found the final conformation after forming a different dimer. This difference in behavior indicates that the LRD trimer is more stable than the dimer. The overall conformation, however, changed significantly with time and became more planar and the contacts

rearranged. Comparing this conformation with the model proposed earlier (6) we note that they both formed a pyramidal structure but in the present model there were more contacts between the monomers and they were distinct from the contacts previously proposed.

Antibody accessibility: A previous study (6) discussed the interaction of the TSHR LRD with the TSHR monoclonal antibody (mAb) M22. Using the crystal structure of M22 bound to the TSHR LRD (37) we overlaid the LRD structure with the monomers of the current dimer and trimer structures so we could assess the accessibility of these oligomers by the M22 mAb. We found that one of the monomers in the dimer (**Figure 9A**) and two of the monomers in the trimer (**Figure 9B**) could easily accommodate M22 without any steric clashes.

DISCUSSION:

Patients with hyperthyroid Graves' disease (GD) have multiple types of autoantibodies to the TSHR which can be referred to as the "GD autoantibody reactome". What drives the initiation and maturation of the "GD autoantibody reactome" is the TSHR protein and its different structural higher order forms. We previously reported the existence of such higher order forms, including dimers and oligomers, in native thyroid membranes (38) by immunoblot blot analysis (8) and further showed their interacting surfaces by biochemical methods such as FRET (12) in heterologous cells. Constitutive holoreceptor dimers and oligomers as seem in the present studies (**Figure 1B**) and our

277 published work (7,8) are not unique to the TSHR (9,38). In addition, the higher order
278 forms have functional roles in negative co-operativity (9,39), in regulating early events
279 during receptor maturation, in intracellular trafficking (12,40) and in $G\alpha q_{11}$ signaling (41).
280 We have previously shown that monomeric TSHR and higher order complexes can also
281 bind TSH receptor autoantibodies (42) and may be regulated by TSH and stimulating TSH
282 receptor antibodies within lipid rafts (43,44). The diverse autoantibody reactome that we
283 see in GD may result in thyroid stimulation, thyroid blockade or thyroid cell death by
284 inducing thyroid cell stress (45). Hence, the TSHR is a highly complex GPCR in its post
285 translational processing and signaling cascade. These observations have led to a large
286 body of work investigating the structure and function of the TSHR and our current
287 modeling study provides further evidence of the different structural forms that can result
288 from modelling and extended Molecular Dynamic (MD) simulation studies and shows its
289 relevance to TSH and stimulating autoantibody binding.

290 Using our published complete full length model of the TSHR holoreceptor (19)
291 aided by studies of ECD from cryoEM (46) we performed modelling followed by robust
292 MD simulation on the various TSHR forms. In fact the holoreceptor homodimerized with
293 high stability and, importantly, retained a structure allowing TSH and TSHR antibodies to
294 bind (**Figure 2**). Furthermore our current modelling confirmed our previous experimental
295 and computational data examining TMD dimers as the major dimerization interface of this
296 receptor and also suggested that possibility of ECD-ECD interaction as shown our study
297 (11) and by others (47). As a corollary to our previous observations this modelling has
298 shown that TSHR full length monomers were able to form stable trimeric structures in
299 addition to dimers adding to the complex diversity of different forms of the TSHR

300 expressed at the cell surface that we see on protein analysis. This analysis led us
301 examine the ECD especially the ability of the LRD to interact to produce diverse forms.

302 The TSHR has a large ECD incorporating the LR of which part is known to be
303 cleaved but the integrity of the ECD is retained via multiple cysteine bonds (5). The
304 cleaved peptide is thought to be subsequently shed via proteolysis by protein isomerases
305 resulting in an antigenic form of the TSHR which may be present in the circulation or local
306 lymph nodes. This prompted us examine the possibility of diverse ECD structures in our
307 models. Having the structure of the LRD from the crystal structure (48) and from our own
308 modelling (19) we examined LRD–LRD interaction using molecular dynamics simulations
309 and found that the LRD, like the TMD, was capable of forming dimers and trimers while
310 retaining their overall structure (**Figures 7A and 7C**). However, a misfolded dimer was
311 also observed (**Figure 7B**). These simulations demonstrated considerable stability for
312 both dimeric and especially trimeric forms of the LRD. However, our models of the dimeric
313 and trimeric LRD were quite different in the contact sites and orientation to that previously
314 reported using an earlier modeling approach (6).

315 The 2000 ns MD simulation of the LRD trimer suggested strong stability of this
316 antigenic form and this model showed that the ECD retained the propensity to bind two
317 stimulating TSHR antibody molecules. In the MD simulations we found that the trimeric
318 forms were more stable and retained the ability in-silico to bind TSH and human
319 stimulating TSHR antibody M22 suggesting that they retained the structural integrity for
320 binding a stimulating antibody and were potent antigenic proteins (47).

321

Furthermore, the trimeric structure of the LRD may also help explain the enhanced immunogenicity of the TSHR ECD protein initiated by immunization with the adeno 289 TSHR construct (3,49,50) so successful at immunizing mice for the induction of GD (51). Similarly, in cell free protein production of influenza hemagglutinin (HA) for vaccine development it has been shown that trimeric structures are more immunogenic than monomeric counterparts (52) suggesting the importance of looking at diverse forms of the TSHR protein as shown in the present studies.

In summary, we have succeeded in examining in-silico the multiple forms of the TSHR by demonstrating stable structures of the holoreceptor dimer and trimer and the cleaved ECD form of the LRD also forming stable dimers and highly stable trimers. The study suggests that these diverse forms of the TSHR expressed by thyroid cells as well as extrathyroidal tissue sites are important players in the inflammatory response to the TSHR and offer opportunities to strategize and develop therapeutic blockade of TSHR action for treatment of Graves' disease.

Figure Legends

Figure 1:

A: **Model of the full length TSHR** generated as described in reference(19): The LRD is shown in red, the highly flexible LR is shown in green and the signal transducing membrane embedded TMD is shown in blue. B: Different forms of the TSHR revealed by Western blotting of lysates prepared from CHO-TSHR cells and probed with TSHR specific antibody (M1)

Figure 2:

Starting structures for the MD dimer simulations with and without TSH. A: apo TSHR dimer: The full length TSHR monomers are shown in red and blue, resp. B: TSHR-TSH dimer. The full length TSHR monomers are shown in red and blue, resp., TSH is shown in yellow.

Figure 3:

Starting structures for the MD trimer simulations with and without TSH. A: apo TSHR dimer: The full length TSHR monomers are shown in red, green and blue, resp. B: TSHR-TSH dimer. The full length TSHR monomers are shown in red, green and blue, resp., TSH is shown in yellow.

Figure 4:

History of residue-residue contacts between the LRD-LRDs of the two monomers during the TSHR dimer simulation. Each line represents a residue pair; lines are broken when

the contact is broken. The first and last appearance of the contact is shown as a small disc.

Figure 5:
History of residue-residue contacts between the LRD-LRD of the two monomers during the TSHR-TSH dimer simulation.

Figure 6:
History of residue-residue contacts between the TSH of monomer 1 and the LRD of monomer 2 during the TSHR-TSH dimer simulation.

Figure 7:
History of residue-residue contacts between the two LRD monomer for the simulation started from the full-length TSHR dimer conformation.

Figure 8:
Final LRD dimer and trimer conformations. A: dimer based on full-length TSHR dimer. B: dimer generated with continuum solvent Monte Carlo. C: trimer based on the full-length TSHR

Figure 9:

Conformations of the antibody M22 obtained by superimposing the LRD domain of the M22-LRD crystal structure on one of the monomers of the dimer (Figure 9A) and on two of the monomers of the trimer (Figure 9B).

Data availaibility:

Some or all datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.

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540