Antibody-induced modulation of TSH receptor post-translational processing

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Abstract

The post-translational processing of the TSH receptor (TSHR) includes intra-molecular cleavage with the loss of a 50 amino acid ectodomain region and the formation of two subunits (α and β), followed by likely α subunit shedding. TSHR antibodies (TSHR-Abs), which are directed at the ectodomain, may influence thyroid function by stimulating or inhibiting TSHR signaling or may bind without any such influence (the neutral group of antibodies). When we examined the characteristics of a series of monoclonal TSHR-Abs, we found that many were able to inhibit

receptor cleavage and enhance TSHR expression. This was especially apparent with the neutral type of TSHR-Abs directed to the cleaved region of the ectodomain (aa 316–366). Indeed, such inhibition appeared to be epitope dependent with TSHR-Abs directed to regions after residues 335–354 showing no such activity. We propose that this aberrant process, whereby TSHR-Abs influence antigen processing, is a novel mechanism for the maintenance and exacerbation of autoimmune thyroid disease.

Journal of Endocrinology (2007) **195**, 179–186

Introduction

The thyrotropin receptor (TSHR) is first expressed as a holoreceptor and then constitutively undergoes intra-molecular cleavage (Misrahi *et al.* 1994, Tanaka *et al.* 1999, Latif *et al.* 2004). The complete or incomplete loss of the cleaved region (residues ~316–366) is followed by the formation of a two subunit structure (Misrahi *et al.* 1994, Tanaka *et al.* 1999); the extracellular α (or A) subunit and a β (or B) subunit – made up of the remaining (sometimes variable length) ectodomain, the transmembrane loops, and the endodomain. Both subunits are stabilized by disulfide bonds and most likely additional contact points. Our earlier findings have shown that there is profound inter-molecular interaction between TSHRs resulting in dimerization and multimerization (Graves *et al.* 1996, Latif *et al.* 2001, 2002) and these observations have recently been confirmed (Urizar *et al.* 2005). We have also suggested that the dissociation of multimers into monomeric forms may be required for intra-molecular cleavage (Latif *et al.* 2004). It has also been shown that after reduction of the disulfide bonds holding the subunits together, the TSHR may shed its α subunit (Couet *et al.* 1996, Latif *et al.* 2004; Fig. 1A). This fragment of shed α subunit has never been detected in the peripheral circulation, most likely because it accumulates in draining lymph nodes or is rapidly degraded. This spontaneous and irreversible post-translational processing explains why β subunits predominate 2.5- to 3-fold more than the α subunits (Loosfelt *et al.* 1992,

Misrahi *et al.* 1994). However, the percentage of surface receptors that are cleaved at any one time varies from a minority of uncleaved holoreceptors remaining on the thyroid cell surface to a substantial proportion (Misrahi *et al.* 1994). Furthermore in transfected mammalian non-thyroid cells expressing recombinant TSHRs, such constitutive cleavage is inefficient leading to a greater density of uncleaved holoreceptors on the cell surface than seen in thyroid cells (Misrahi *et al.* 1994, Rapoport *et al.* 1998). Deletion of the cleaved region in the TSHR ectodomain does not affect TSH binding to, nor activation of, the TSHR (Chazenbalk *et al.* 1999) and, therefore, its relationship to receptor structure and dimerization remains a point of interest.

The TSHR is the major autoantigen in autoimmune thyroid disease. Autoantibodies to the TSHR (TSHR-Abs) may influence thyroid function by stimulating the TSHR and promoting excessive thyroid growth, hormone production, and hormone secretion causing Graves' disease (GD; Rees Smith *et al.* 1988, Rapoport *et al.* 1998, Ando *et al.* 2005). TSHR-Abs may also block the action of TSH and induce hypothyroidism as seen in some patients with the atrophic form of Hashimoto's thyroiditis (Rees Smith *et al.* 1988, Ando *et al.* 2005).

In this study, we show that many TSHR-Abs inhibit TSHR post-translational cleavage. These data introduce a new concept by which TSHR antibodies regulate post-translational processing of their antigen. We hypothesize that antibody-mediated persistence of excess uncleaved TSHRs may result in an increased TSHR antigenic load.

Materials and Methods

Cells and TSHR-mAbs

CHO-TSHR cells (Perret *et al.* 1990) stably expressing the human TSHR (kindly provided by Dr G Vassart, Université Libre de Bruxelles and Service de Genetique Medicale, Brussels, Belgium) were maintained as previously described (Ando *et al.* 2002). FRTL-5 cells, a rat thyroid cell line (Ambesi-Impimbatto *et al.* 1980), were maintained as previously described (Davies *et al.* 1987). Monoclonal TSHR-Abs Mount Sinai-1 (MS-1), 9F4, thyroid antibody (TAB)-8, 7G10, TAB-6, and TAB-16 are Armenian hamster IgG₂ mAbs (Ando *et al.* 2002, 2004a). Concentrations of hamster mAb used were saturating unless otherwise indicated. Monoclonal TSHR-Abs M1 and M4, used in our cleavage assay, were mouse IgG₁ (Oda *et al.* 1998, 2000) kindly supplied by Dr B Rees Smith (RSR Limited, Cardiff, UK). 2C11 was a mouse IgG₁ TSHR-mAb (Shepherd *et al.* 1999) purchased from Serotec (Raleigh, NC, USA). The biotinylation of mAbs was described previously (Latif *et al.* 2004).

TSHR peptide ELISA

Hamster serum binding to TSHR peptides was performed as previously described (Ando *et al.* 2004a). The human TSHR peptides (Morris *et al.* 1993) used were residues 307–326, 322–341, 337–356, 352–371, 367–386, and 382–401 (kindly

provided by Dr J Morris, Mayo Medical School, Rochester, MN, USA). For examination of human sera binding to peptides, we incubated serum (1:10) with peptide-coated wells for 2 h. After washing, biotinylated TAB-6 (500 ng/ml) or TAB-16 (200 ng/ml) was used to study binding inhibition. The concentrations of these labeled mAbs were determined as optical density (OD) ~1. The percentage of inhibition was expressed by the percentage of reduction of OD by sera based on that with biotinylated mAb alone. Positive was defined as an inhibition > 2 s.d. from the mean of control sera.

TSHR expression and cleavage determined by flow cytometry

TSHR cleavage has been studied mainly by chemical cross-linking of TSHRs and detection with radioiodine-labeled TSH (Rapoport *et al.* 1998). However, we have recently shown that this method is not suitable for studying the dynamic changes that occur with TSHR cleavage or expression (Latif *et al.* 2004). Therefore, to study the dynamic influence of TSHR-Abs on TSHR cleavage, we used a flow cytometric-based assay for quantitating TSHR cleavage (Ando *et al.* 2002, Latif *et al.* 2004). In brief, after incubating cells with a TSHR-mAb for 24 h, or as otherwise indicated, we aliquoted and stained CHO-TSHR cells with TSHR-mAb-M1 (binding to residues 381–385 in the β subunit) and M4 (binding to residues 322–341 in the cleaved region of the TSHR). Anti-mouse IgG₁ conjugated with FITC

(Roche Applied Sciences; 1:200) was used as the secondary Ab. The mean fluorescent intensity was then determined by a FACS calibur instrument (FACS shared facility at Mount Sinai School of Medicine, BD Bioscience Pharmingen, San Diego, CA, USA) as a measure of mAb binding. We excluded dead cells by staining with propidium iodide (5 µg/ml). In some experiments, after similarly incubating cells, we stained cells with biotinylated TSHR-mAb-9F4 (binding to epitope A in the α subunit; 5 µg/ml) on ice for 1 h followed by avidin-PE (BD Bioscience Pharmingen). The relative b9F4 binding was expressed based on b9F4 binding to control CHO cells. When we studied cleavage in FRTL-5 thyroid cells (Ambesi-Impim-bato *et al.* 1980), we withdrew TSH from the culture media for 5 days before. We also used TSHR-mAb-TAb-16 instead of M4, and probed with anti-hamster κ chain conjugated with biotin (BD Bioscience Pharmingen), due to stronger binding of TAb-16. Bovine TSH was from Sigma-Aldrich.

Membrane preparation and western blotting

In each experiment, three plates (100 mm dishes) of CHO-TSHR cells were treated with 2 µg/ml TAb-8 for 24 h. The cells were washed and were subjected to membrane preparation using digitonin as described previously (Latif *et al.* 2001). Equal amounts of the protein from treated and untreated CHO-TSHR cell preparations were exposed to 5× sample buffer and resolved on 12% SDS-PAGE. The blot was probed with an N-terminus-specific TSHR murine monoclonal antibody (A10) for the presence of uncleaved receptor. Antibody to β -actin at 1:1000 was used to show equal loading of the gel.

Data analysis

Prism 4 (Graphpad software Inc., San Diego, CA, USA) was used to analyze the data. Student's *t*-test or one-way ANOVA was used for statistical analysis. A value of $P < 0.05$ was considered significant.

Results

Antibodies to the cleaved region of the TSHR in a hamster model of GD

When we examined 18 sera from hamsters with GD, including those animals included in a previous study (Ando *et al.* 2004a), we found that the majority of sera (14/18) recognized linear epitopes on synthetic TSHR peptides corresponding to the cleaved region (residues 316–366; Fig. 2). This emphasized our previous finding that the major linear epitope in our model was in this region (designated epitope C; Fig. 1; Ando *et al.* 2004a).

Regulation of TSHR cleavage by TSHR-mAbs

Since we previously showed that under appropriate conditions TSH positively influenced TSHR cleavage (Latif *et al.* 2004), we

Figure 2 Recognition of the TSHR cleaved region by sera from a hamster model of Graves' disease. TSHR-Abs to the cleaved region in sera from a hamster model of GD as determined by TSHR peptide ELISAs. The numbers shown below each column indicate the initial AA of each synthetic peptide as described in the Materials and Methods section. Immunoreactivity (OD) was compared with peptide 382 and an irrelevant control mAb (data are mean of duplicate measurements). * $P < 0.05$ and ** $P < 0.01$.

hypothesized that TSHR-mAbs may also influence TSHR cleavage. In order to determine the influence of TSHR-mAbs on TSHR cleavage, we incubated CHO cells overexpressing TSHRs (CHO-TSHR; Perret *et al.* 1990) with hamster-derived TSHR-mAbs of known epitope specificity (Ando *et al.* 2004a; Table 1). In order to assess changes in TSHR cleavage, we used a FACS-based system previously described by this laboratory (Ando *et al.* 2002, Latif *et al.* 2004). We measured the uncleaved receptor by the binding of a well-characterized murine mAb to the unique cleaved region (M4) and the 'assumed' total (cleaved and uncleaved) TSH receptor population by a murine Ab to the β subunit (M1). The M1 antibody detected any change in total TSHR expression, such as down-regulation, which would also influence M4 binding and, therefore, served as control. We expressed TSHR cleavage as a relative ratio of M4 to M1 binding. Any reduction in this ratio was taken as an indication of enhanced cleavage and vice versa (Ando *et al.* 2002, Latif *et al.* 2004). As we previously showed, TSH decreased this ratio in a dose-dependent manner, which indicated mild enhancement of receptor cleavage (Fig. 3, inset). We next studied the influence of TSHR-mAbs to epitope A (on the α subunit) on TSHR cleavage (Fig. 3). Both TSHR-stimulating mAb (MS-1) and blocking mAb (9F4) inhibited rather than enhanced TSHR cleavage to a modest degree when present in high concentrations. We then examined the influence of a blocking TSHR-mAb known to bind to epitope B (Tab-8) and a neutral TSHR-mAb to epitope C (7G10). Both Tab-8 and 7G10 inhibited TSHR cleavage to a marked degree (Fig. 3). Tab-6 had no influence on cleavage. Therefore, in contrast to TSH, some TSHR-mAbs increased the cleavage ratio in a dose-dependent manner, indicating inhibition of constitutive cleavage.

Time dependency of TSHR-Ab effects

We also studied the time dependency of this antibody inhibition of cleavage. TSH (100 µU/ml) induced such

Table 1 Summary of thyrotropin receptor (TSHR)-mAbs used in this study and their influence on the TSHR

	Species	Function ^a	Epitope ^b	α subunit ^c	Cleavage ^d
mAb					
MS-1	Hamster	Stimulating	A, conformational ^e	Increase ^f	Inhibitory
9F4	Hamster	Blocking	A, conformational ^e	ND	Inhibitory
TAb-8	Hamster	Blocking	B, conformational ^e	Increase	Inhibitory
TAb-16 ^g	Hamster	Neutral	C, residues 322–341	Increase	Inhibitory ^h
7G10	Hamster	Neutral	C, residues 335–354	Increase	Inhibitory
TAb-6	Hamster	Neutral	C, residues 335–354	No change	No change
M4	Mouse	Neutral	C, residues 322–341	Increase	Inhibitory ^h
2C11	Mouse	Neutral	C, residues 354–359	No change	No change ^h
M1	Mouse	Blocking	Residues 381–385	No change	No change

^aThe functions of all TSHR-mAbs were studied in CHO-TSHR cells. Stimulating mAb increased the intracellular cAMP production via TSHR. Blocking mAbs inhibited labeled TSH binding to the TSHR without increasing the intracellular cAMP. Neutral mAb had neither activity.

^bEpitopes A–C correspond to those in Fig. 1b.

^cInfluence on α subunit content of the TSHR.

^dInfluence on TSHR cleavage.

^eConformational epitopes A and B are localized within the first 316 amino acids (Ando *et al.* 2002, 2004a), but distinct from each other (Ando *et al.* 2004a,b).

^fDetermined using TAb-8 labeled with fluorescent dye, relative TAb-8 binding increased over M1 binding.

^gTAb-16 is the only hamster mAb with κ light chain (Ando *et al.* 2004a).

^hDetermined by change in b9F4 binding (for details see text).

changes within 6 h (relative M4/M1; 0.825 ± 0.003, *P* < 0.05 vs 0 h). Blocking mAbs (9F4 and TAb-8; at 2 μg/ml) inhibited cleavage within 3 h (1.395 ± 0.042, *P* < 0.0005 for TAb-8 and 1.229 ± 0.037 *P* < 0.005 for 9F4) and reached a plateau at 24 h and continued to influence cleavage for at least 72 h (data not shown). However, the increase in the M4 to M1 ratio obtained in the FACS cleavage assay was not able to indicate whether inhibition of cleavage was complete or incomplete and could not assess constitutive activity.

Epitope dependency of cleavage inhibition

All but one of the hamster TSHR-mAbs used in this study; stimulating, blocking, and neutral, inhibited TSHR cleavage (Table 1). This indicated that inhibition of cleavage was not

dependent on signal transduction at the TSHR. Since these hamster mAbs bound proximal to residue 354 (Table 1), we obtained and examined two mouse mAbs binding distal to residue 354 (Fig. 4). These two mouse mAbs (2C11 and M1) which bound to linear residues 354–359 and 381–384 respectively, did not inhibit cleavage as evidenced by a lack of increase in the binding of an α subunit probe (9F4). Thus, inhibition of TSHR cleavage depended on the epitope recognized within the TSHR ectodomain proximal to residue 354. However, two of the neutral mAbs (TAb-6 and 7G10) recognizing residues 335–354 had different CDR3 sequences (not shown) but only one of them (7G10) inhibited TSHR cleavage. This suggested that residues 335–354 represented the border between residues for cleavage- and non-inhibitory Abs.

Figure 3 Inhibition of TSHR cleavage by TSHR-mAbs. Cleavage was measured using the flow cytometric cleavage assay as described in Materials and Methods. Data on the regulation of TSHR cleavage are shown after incubating cells with TSH (inset) or increasing concentrations (20–2000 ng/ml) of TSHR-mAbs (mean ± s.e.m. of more than three independent experiments). ***P* < 0.01, †*P* < 0.001, and #*P* < 0.0005.

Figure 4 Epitope dependency of cleavage inhibition. TSHR expression assessed by biotinylated TSHR-mAb-9F4 after incubating cells with two mouse TSHR-mAbs (2C11 and M1) binding distal to residue 354. M4, binding to residues 322–341, enhanced 9F4 binding and served as a positive control, but 2C11 and M1 mAbs failed to show any enhancement (data expressed as mean $\pm$ S.E.M. of duplicate measurements from three independent experiments). * $P < 0.005$.

Effects of TSHR-mAbs on recombinant TSHR expression

The α subunit is the site(s) which contains the epitope for many TSHR-Abs and also contains a region which appears to suppress the constitutive activity of the TSHR (Zhang *et al.* 2000, Vlaeminck-Guillem *et al.* 2002). Our results suggested that TSHR-Abs directed at either the α subunit or the cleaved region influenced the variety of TSHR forms present on the cell surface. However, the increase in the M4 to M1 ratio obtained in the FACS cleavage assay was not able to indicate whether inhibition of cleavage was complete or incomplete. Incomplete cleavage has been evidenced as a variation in β subunit size and sequence shown in transfected cells (de Bernard *et al.* 1999, Tanaka *et al.* 1999, Oda *et al.* 2000). Since we found that the M1 antibody increased the α subunit content of the cell by studying the cell surface binding of a TSHR-mAb probe to epitope A (biotinylated 9F4; Fig. 4), we decided to examine this approach in more detail (Ando *et al.* 2004a). Using this probe to epitope A, we examined the influence of a blocking TSHR-mAb to epitope

B and three neutral TSHR-mAbs to epitope C, described earlier, and also known a) not to interfere with the epitope A probe binding (Ando *et al.* 2004a,b) and b) not able to alter probe-binding affinity (as evidenced by consistent IC_{50} ; data not shown). Three of these mAbs also increased the probe binding in a dose-dependent manner (mean increase $\sim 50\%$; Fig. 5), although one neutral mAb (TAB-6) which did not inhibit cleavage failed to demonstrate this effect. This increased binding of the probe observed with TAB-8-treated cells was not associated with any major affinity changes in the cell surface receptors (Fig. 5B). These results suggested that only cleavage-inhibiting mAbs increased the amount of TSHR α subunit. A linear correlation between the increase in α subunit content and the degree of cleavage inhibition (Fig. 6; $r = 0.988$, $P < 0.0001$) was also compatible with inhibition of TSHR cleavage leading to increased intact TSHR expression. We tried to examine whether this change in surface receptors was detectable by immunoblotting. We treated CHO-TSHR cells with 2 μ g/ml TSHR-mAb for 24 h and measured the full-length receptor using an amino terminus-specific antibody. This showed only minimal change in TSHR-mAb binding to surface TSHRs (Fig. 7). We concluded that, most likely, the incomplete degree of TSHR cleavage in these transfected cells made it difficult to detect changes in TSHR with the TSHR-Ab probes utilized in the immunoblot (Fig. 7).

Regulation of TSHR cleavage in a thyroid cell line

In order to examine whether the data obtained in transfected cells were physiologically relevant, we studied regulation of cleavage on rat thyroid cells (FRTL-5) which express low numbers of receptor (Ambesi-Impimbato *et al.* 1980). We first examined the state of the TSHRs expressed. Total TSHR expression was detectable by a TSHR-mAb to the β subunit (M1; Table 2). We used two antibodies to the cleaved region (M4 and TAB-16) but could not observe antibody binding,

Figure 5 Dose dependency of enhanced TSHR-mAb-induced TSHR expression. (A) TSHR expression of biotinylated TSHR-mAb-9F4 is shown after incubating CHO-TSHR cells with the indicated mAb concentration (data expressed as mean $\pm$ S.E.M. of duplicate measurements in four independent experiments). * $P < 0.05$, ** $P < 0.01$, + $P < 0.001$, and # $P < 0.0005$. (B) Maximum fluorescence intensity of 9F4 binding before and after exposure of CHO-TSHR cells to mAb TAB-8.

Figure 6 Correlation between TSHR expression and TSHR cleavage. Changes in binding of TSHR-Ab to epitope A after antibody exposure compared with the changes in TSHR cleavage induced by the same antibody. Data from 11 experiments are shown from six different mAbs, each antibody represented by a different symbol.

demonstrating that constitutive cleavage in the cell line was almost complete after culture with TSH (Table 2). After exposure of FRTL-5 cells to TSHR-mAbs (9F4 and Tab-8), we were then able to detect cleaved region antibody binding compatible with cleavage inhibition (Table 2).

Discussion

TSHR-Abs have been considered as pathogenic in autoimmune thyroid disease because they activate or inactivate TSHR signaling (Rees Smith *et al.* 1988, Rapoport *et al.* 1998, Ando *et al.* 2005) although the clinical significance of neutral TSHR-Abs, which have no such actions, has been uncertain (Ando *et al.* 2005). We observed that almost all monoclonal TSHR-Abs regulated TSHR post-translational changes on the cell surface including the neutral variety by effectively inhibiting cleavage

of the TSHR. This phenomenon was epitope dependent and restricted to mAbs binding to amino acids 1–354. Such regulated post-translational processing by TSHR-mAbs was also observed in a rat thyroid cell line implying a functional significance to this post-translational influence.

There have been extensive biochemical investigations of TSHR intra-molecular cleavage (Rapoport *et al.* 1998, Tanaka *et al.* 1998, 2000, de Bernard *et al.* 1999, Siffroi-Fernandez *et al.* 2001) but the physiologic role, if any, for such processing has not been resolved. We have shown that TSH enhanced TSHR cleavage (Ando *et al.* 2002, Latif *et al.* 2004) as well as subsequent shedding of the α subunit *in vitro* (Couet *et al.* 1996, Latif *et al.* 2004). This regulation was not secondary to accumulation of intracellular cAMP (Couet *et al.* 1996, Latif *et al.* 2004). We recently found that TSH was not able to enhance cleavage of the TSHR ectodomain tethered with a short lipid tail (our unpublished data), excluding TSH as a cleaving enzyme. In the present study, we examined the influence of TSHR-Abs on TSHR cleavage using an mAb to epitope A (conformational epitope on the α subunit). Since epitope A is present in both the uncleaved and cleaved TSHRs, our data indicated that cell surface expression was increased when TSHR-Abs inhibited TSHR cleavage. Previous evidence using a truncated TSHR, mimicking residual β subunits, showed that truncated receptors had faster internalization and thus a shortened functional half-life (Quellari *et al.* 2003). Therefore, we concluded that TSH and TSHR-Abs had an opposite influence on this process and also that the constitutive and irreversible TSHR post-translational cleavage and shedding, involved TSHR degradation.

We have previously shown constitutive TSHR multimerization *in vivo* (Graves *et al.* 1996) and *in vitro* (Latif *et al.* 2001, 2002) and also that TSHR cleavage appeared to follow only monomeric, but not multimeric TSHR activation (Latif *et al.*

Figure 7 TSHR expression by immunoblot. Membranes prepared from TSH- and antibody-treated cells were resolved on 10% SDS-PAGE and blotted on to PVDF. The membrane was probed with an amino terminal TSHR-mAb (A10; Morgenthaler *et al.* 1997) at 1:5000 dilution. (A) Lane 1, untreated cells; lane 2, treated with Tab-8 antibody (2 μ g/ml) for 24 h; and lane 3, treated with TSH (1000 μ U/ml) for 24 h. * P < 0.01. (B) Densitometric representation of the TSH holoreceptor bands indicated in (A).

Table 2 Influence of blocking thyrotropin receptor (TSHR)-mAbs on TSHR expression in rat thyroid cells

	MAb	MFI pre-treatment	MFI post-treatment	
			12 h	24 h
TSHR target				
β subunit	M1 probe	69.48 $\pm$ 2.61		
	9F4		79.30 $\pm$ 2.12	*84.22 $\pm$ 3.33*
	Tab-8		88.60 $\pm$ 3.01	*85.10 $\pm$ 1.67*
Cleaved region	Tab-16 probe	13.32 $\pm$ 0.53		
	9F4		38.24 $\pm$ 0.45	[†] 32.80 $\pm$ 2.74 [†]
	Tab-8		47.79 $\pm$ 1.52	*46.27 $\pm$ 1.00*

The amount of the β subunit and the cleaved region of the TSHR as determined by binding of M1 (binding to residue 381–385) and Tab-16 (residues 322–341) before and after incubating cells with the indicated blocking TSHR-mAb (at 20 μ g/ml) in the post-treatment columns. Note the increase in the cleaved region binding on treatment with TSHR-mAbs 9F4 and Tab-8. Data are expressed as maximum fluorescence intensity (MFI). *, [†], and * indicate $P < 0.05$, $P < 0.01$, and $P < 0.001$ respectively, against pre-treatment.

2004). Our thyroid-stimulating monoclonal Ab (MS-1), which inhibited TSHR cleavage, induced a slowing of TSHR mobility suggesting the induction of TSHR dimers/multimers (Latif *et al.* 2004). Therefore, we propose that cleavage-inhibiting TSHR-Abs stabilize the TSHR ectodomain by maintaining dimeric or multimeric TSHR forms due to their IgG bivalency. The mechanism by which multimerization may inhibit intramolecular cleavage of the TSHR is unknown. However, our data suggest that a structural inhibition of the cleavage enzyme(s) is the most likely. Evidence for this conclusion comes from the fact that the TSHR-Ab-induced inhibition of cleavage was epitope dependent. The detailed molecular explanation for such activity will need to await the identification and structure of the cleavage enzyme(s) involved in this phenomenon. However, it is likely that intact TSHR bound to a TSHR-Ab with cleavage-inhibiting activity is more resistant to degradation. Therefore, such TSHRs would have a higher chance of a subsequent interaction with additional TSHR-Abs with different epitope(s). We hypothesize that this action of multiple TSHR-Ab binding may have consequences *in vivo*. Such interactions may lengthen and sustain TSHR autoantigen expression, which would increase the autoantigenic load on the cell surface. Hence, inhibition of TSHR cleavage may play a role in maintaining and/or exacerbating GD.

In summary, we showed that TSHR-Abs binding to the ectodomain (the first ~ 341 amino acids), irrespective of their function, inhibited TSHR cleavage and likely increased the antigenic load of uncleaved TSHR. This concept of antibody-mediated regulation of the post-translational processing of a major autoantigen is novel and may be a more generalized mechanism involved in the maintenance and exacerbation of autoimmune disease.

Acknowledgment

This study was supported in part by NIH grants DK069713, DK052464, the VA Merit Award program and the David Owen Segal Endowment. The authors declare that there is no

conflict of interest that would prejudice the impartiality of this scientific work.

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Received in final form 23 July 2007

Accepted 9 August 2007

Made available online as an Accepted Preprint
10 August 2007