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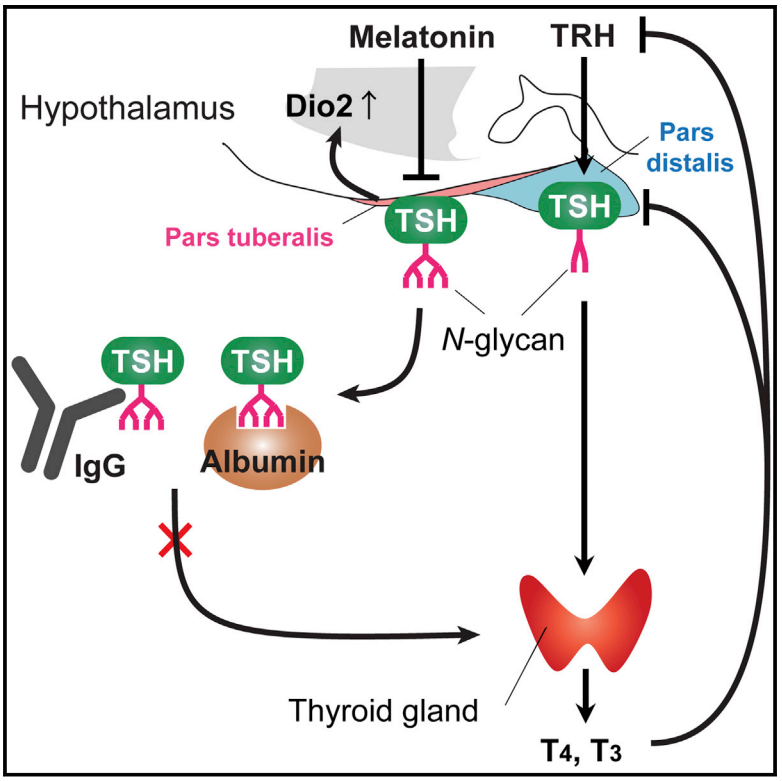
Tissue-Specific Posttranslational Modification Allows Functional Targeting of Thyrotropin

Graphical Abstract



Highlights

Tissue-specific glycosylation imparts specificity to thyroid-stimulating hormone

Pars tuberalis-TSH (PT-TSH) does not stimulate the thyroid gland

PT-TSH has tissue-specific *N*-glycans and forms macro-TSH in blood

Macro-TSH circulating in blood does not stimulate thyroid hormone secretion

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In Brief

Pars distalis-derived thyroid-stimulating hormone (TSH) stimulates the thyroid gland to produce thyroid hormones, whereas pars tuberalis-derived TSH acts on the hypothalamus to regulate seasonality. It had not been clear how these two TSHs avoid functional crosstalk. Ikegami et al. now show that this regulation is mediated by tissue-specific glycosylation.

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Report

Tissue-Specific Posttranslational Modification Allows Functional Targeting of Thyrotropin

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SUMMARY

Thyroid-stimulating hormone (TSH; thyrotropin) is a glycoprotein secreted from the pituitary gland. Pars distalis-derived TSH (PD-TSH) stimulates the thyroid gland to produce thyroid hormones (THs), whereas pars tuberalis-derived TSH (PT-TSH) acts on the hypothalamus to regulate seasonal physiology and behavior. However, it had not been clear how these two TSHs avoid functional crosstalk. Here, we show that this regulation is mediated by tissue-specific glycosylation. Although PT-TSH is released into the circulation, it does not stimulate the thyroid gland. PD-TSH is known to have sulfated biantennary *N*-glycans, and sulfated TSH is rapidly metabolized in the liver. In contrast, PT-TSH has sialylated multibranched *N*-glycans; in the circulation, it forms the macro-TSH complex with immunoglobulin or albumin, resulting in the loss of its bioactivity. Glycosylation is fundamental to a wide range of biological processes. This report demonstrates its involvement in preventing functional crosstalk of signaling molecules in the body.

INTRODUCTION

Thyroid-stimulating hormone (TSH) (thyrotropin) is a pituitary hormone that stimulates the thyroid gland to produce thyroid hormones (THs) (Szkudlinski et al., 2002). TSH is a noncovalently linked heterodimeric glycoprotein consisting of α and β subunits. The α subunit is common to other glycoprotein hormones such as luteinizing hormone (LH) and follicle-stimulating hormone (FSH), whereas the β subunit is unique to each hormone (Pierce and Parsons, 1981). TSH is synthesized in thyrotrophs of the pars distalis (PD) of the pituitary gland (Magner, 1990) (Figure S1A). Synthesis and secretion of PD-TSH are positively regulated by hypothalamic thyrotropin-releasing hormone (TRH). Circulating PD-TSH acts on thyrocytes through the TSH receptor (TSHR) to stimulate synthesis and release of THs, and circulating TH inhibits PD-TSH by a negative-feedback loop (Szkudlinski et al., 2002). In addition to this well-known action, an unexpected function of this molecule was recently discovered: TSH derived from the pars tuberalis (PT) of the pituitary gland (PT-TSH) is a primary factor regulating seasonality (Hanon et al., 2008; Nakao et al., 2008; Ono et al., 2008). Various physiological and behavioral activities, such as reproduction and migration, are regulated by changes in day length. Long-day (LD) stimulus induces the synthesis of PT-TSH, which acts on TSHR expressed in ependymal cells within the mediobasal hypothalamus (MBH) to induce expression of the *Dio2* gene. *Dio2* encodes type 2 deiodinase, a TH-activating enzyme, which converts the precursor thyroxine (T_4) to bioactive triiodothyronine (T_3) within the MBH, serving as the key regulator of seasonality (Yoshimura et al., 2003).

The PT surrounds the hypophysial stalk and extends along the ventral surface of the median eminence (Wittkowski et al., 1999) (Figure S1A). The pituitary gland receives blood supply via the hypothalamohypophysial portal circulation: capillaries arising

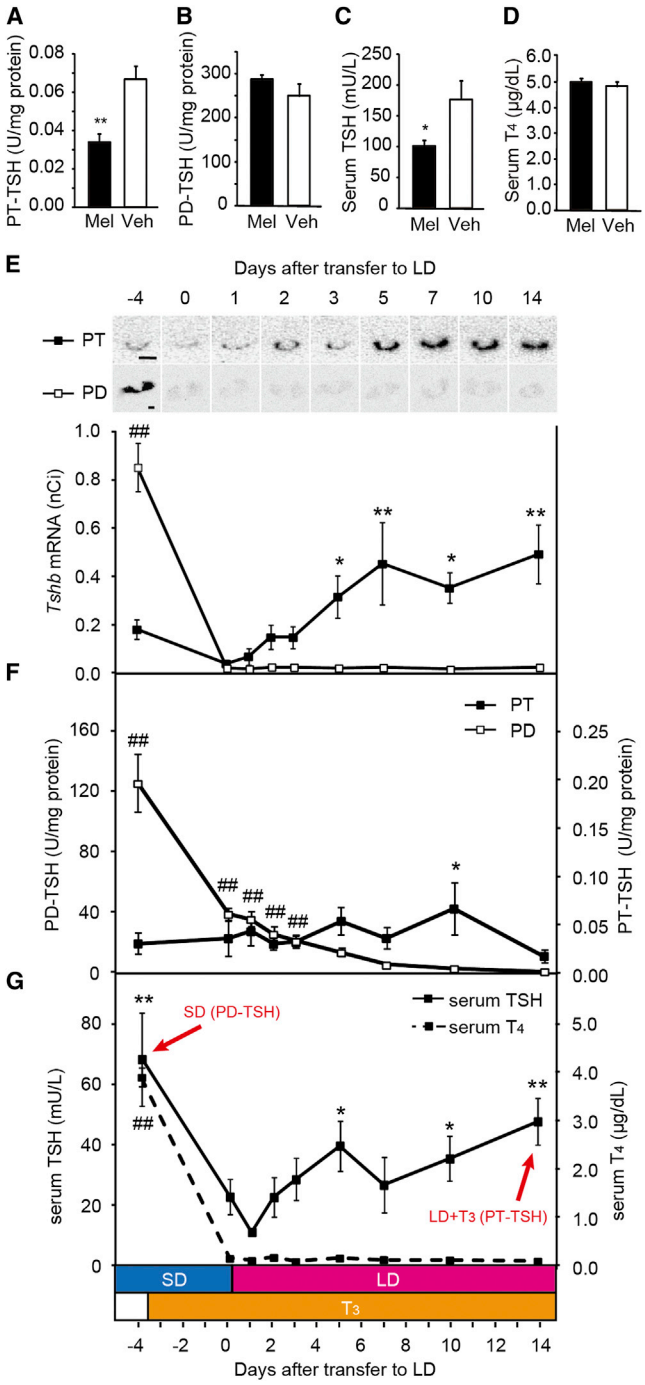

Figure 1. PT-TSH Secreted into the Peripheral Circulation Has Little Bioactivity

(A–D) Effects of melatonin on TSH concentration in PT (A), PD (B), and serum (C) and on serum T_4 level (D). *$p < 0.05$; **$p < 0.01$ (t test, n = 6–12).

(E–G) Effects of changing day length and T_3 administration on *Tshb* mRNA (E), TSH concentration in PD and PT (F), and serum TSH and T_4 levels (G). T_3, daily T_3 administration. *$p < 0.05$; **$p < 0.01$; $^{\#\#}p < 0.01$ versus smallest value (ANOVA, Fisher's least significant difference post hoc test, n = 5–9). Values are means ± SEM. Scale bars, 200 μm.

from the primary plexus project down the PT to form the portal veins, and a secondary capillary plexus arising from the portal vein supplies the PD before draining into the general circulation. Previously, it had been unclear how PD-TSH and PT-TSH avoid functional crosstalk and thereby maintain their distinct functions. Here we report that tissue-specific glycosylation is central to this mechanism.

RESULTS

PT-TSH Is Independent of TRH Regulation

Several lines of evidence suggest that PD-TSH and PT-TSH are regulated through different mechanisms. In marked contrast to PD-TSH, PT-TSH is thought to be independent of TRH regulation because PT cells lack TRH receptor (TRHR) (Bockmann et al., 1997). To verify this hypothesis, we measured *Tshb* expression and TSHβ immunoreactivity in the PD and PT of TRH null mice. Although an amount of *Tshb* mRNA was detected, TSHβ immunoreactivity was absent in the PD of TRH null mice (Figures S1B and S1C), consistent with a previous report (Yamada et al., 1997). In contrast, *Tshb* expression and TSHβ immunoreactivity were not affected by TRH deficiency in the PT (Figures S1B and S1C), confirming that PT-TSH is independent of TRH regulation.

Circulating PT-TSH Exhibits Little Bioactivity

In mammals, photoperiodic information received by the eye is transmitted to the pineal gland via the circadian pacemaker, the suprachiasmatic nucleus (Reiter, 1980). Melatonin is secreted from the pineal gland at night, and its secretion pattern encodes the signal of night length (Reiter, 1980). Photoperiodic regulation of TSH has been reported in the PT (Wittkowski et al., 1988), and MT1 melatonin receptor has been observed in PT thyrotrophs but not in the PD (Klosen et al., 2002). Furthermore, melatonin suppresses expression of *Tshb* in the PT via MT1 (Ono et al., 2008; Yasuo et al., 2009). Thus, unlike PD-TSH, PT-TSH is controlled by melatonin. To confirm this notion, we examined the effects of melatonin on levels of TSH in the PT, PD, and serum of C57BL mice using a radioimmunoassay (RIA). We used C57BL mice because the effect of melatonin is more apparent in this strain, which is genetically deficient in melatonin synthesis, than in melatonin-proficient strains (Ebihara et al., 1986; Ono et al., 2008). As expected, melatonin injections decreased TSH level in the PT (Figure 1A), but had no effect on the PD (Figure 1B). When we measured TSH level in the peripheral blood, we found that serum TSH was suppressed by melatonin and appeared to reflect the PT-TSH profile (Figure 1C). This result was surprising because the TSH concentration within the PD was ~10,000 times higher than that in the PT for the latter to affect the TSH level in the peripheral blood. A series of electron microscopic analyses revealed that PT thyrotrophs are distinct from PD thyrotrophs (Bergmann et al., 1989; Sakamoto et al., 2000). For example, PD thyrotrophs have numerous dense secretory granules, whereas PT thyrotrophs have only a small number of granules. Because PT thyrotrophs contain well-developed Golgi apparatus, large numbers of microvesicles, and small numbers of secretory granules, they resemble hyperfunctional PD thyrotrophs of thyroidectomized rat (Baker and Yu, 1971; Böckers et al., 1995). Thus, it is believed that PT

thyrotrophs constitutively release TSH (Sakamoto et al., 2000). Because the serum T_4 concentration rises in response to TSH (Andersen et al., 2002), we next measured the serum T_4 level. Unexpectedly, however, serum T_4 levels were not influenced by differences in serum TSH (Figure 1D), suggesting that PT-TSH has little bioactivity in the circulation. To further confirm these observations, we controlled the source of TSH in the circulation by taking advantage of the differing regulatory mechanisms of PD-TSH and PT-TSH (Figures 1E–1G). When melatonin-proficient CBA mice were raised under short-day (SD) conditions, expression of *Tshb* was suppressed in the PT, but not in the PD (Figure 1E). We then gave these SD mice daily T_3 injections to suppress PD-TSH. It is well established that this T_3 treatment does not interfere with the action of TSH at the level of the TSHR on the thyrotroph, and this method is often used to measure the bioactivity of TSH in vivo (McKenzie, 1958; Moeller et al., 2003). As a result, levels of *Tshb* mRNA and TSH were dramatically reduced in the PD (Figures 1E and 1F). Importantly, serum TSH level mirrored the decline in PD-TSH level (Figure 1G). We then transferred these PD-TSH-suppressed mice to LD to induce production of PT-TSH. Although the increase in TSH protein in the PT was modest, probably due to the constitutive-release pathway (Figure 1F), *Tshb* was markedly induced in the PT by LD stimulus (Figure 1E), as reflected by a significant increase in the serum TSH level under LD (Figure 1G). These results present additional clear evidence for the existence of different mechanisms that regulate PD-TSH and PT-TSH. It is important to note that the serum T_4 level, which reflects TSH bioactivity, did not recover even after the restoration of serum TSH level by the induction of PT-TSH by LD (Figure 1G). Taken together, these data demonstrate that TSH of PT origin circulating peripherally has little bioactivity.

Differential Glycosylation of the Two TSHs

To investigate the mechanism underlying the differing bioactivities of PD-TSH and PT-TSH, we first searched for possible differences in their cDNA sequences, but found none (data not shown). Therefore, we next performed western blotting (WB) of PT and PD homogenates to analyze the sizes and mobilities of TSH from each source. Under reducing conditions, PD-TSH and bovine TSH (bTSH; used as a control) were detected as TSHβ and TSHα monomers (Figure 2A). In contrast, TSHβ and TSHα were detected in PT homogenate at molecular weight that corresponds to the predicted size of a heterodimer (Figure 2A). These results suggested that the ability to dissociate the heterodimer into α and β subunits differs between PT-TSH and PD-TSH. When we examined the WB under nonreducing conditions, all TSHs (PT-TSH, PD-TSH, and bovine) were detected as heterodimers (Figure 2B). However, the molecular weights of PT-TSH (~40 kDa) and PD-TSH (~37 kDa) were different (Figure 2B). To determine the reason for this difference, we next focused on the glycosylation of TSH. Deglycosylation by PNGase F, which cleaves *N*-linked glycans, yielded immunoreactive bands with the same low molecular weight (~34 kDa) for all TSHs (Figure 2C). In contrast, *O*-glycanase did not affect apparent molecular weight (Figure 2C). To further elucidate the detailed structures of the linked oligosaccharides, we immunopurified TSH using antibody against TSHβ. Immunoprecipitated (IP)-PT-TSH was detected as two immunoreactive bands, as shown in Figures 2B and 2D, whereas PD-TSH was detected as a single band. Possible structures of oligosaccharides were determined by positive-ion MALDI-TOF-MS using PNGase F-digested IP-PD-TSH and IP-PT-TSH (Figure 2E). In the PD, glycans were mainly biantennary and sulfated complexes, as previously reported for bTSH (Wheeler and Harvey, 2001). Although PT-TSH shared some of these biantennary *N*-glycans with PD-TSH, tetra-antennary and triantennary multibranched *N*-glycans associated with sialic acid were also identified in the PT. Because negatively charged sulfates affect the intensity of the positive-ion mass spectrograph (Wheeler and Harvey, 2001), we could not quantitate the relative amount of each *N*-glycan in this analysis. However, this result clearly suggested that PT-TSH has multibranched, sialylated *N*-glycans. These results were further confirmed by lectin blot (LB) analysis using *Maackia amurensis* [MAM] lectin, which recognizes Siaα2-3Galβ1-4GlcNAc, and *Phaseolus vulgaris* (red kidney bean) [PHA-L_4] lectin, which recognizes glycans with triantennary or greater branching (Figure 2F). Furthermore, WB and immunohistochemistry demonstrated colocalization of sialyltransferase (ST3GalIV) and TSHβ only within the PT (Figures S2A and S2B). Although *N*-acetylglucosaminyl-transferase V (GnT-V) was highly expressed in the pituitary, as previously reported (Togayachi et al., 2001), its colocalization with TSHβ was only observed in the PT (Figures S2C and S2D). All of these results suggested that tissue-specific expression of glycosyltransferases causes the difference in oligosaccharides between PD- and PT-TSH.

PT-TSH Forms the Macro-TSH Complex in the Blood

Based on the findings described above, we predicted that PT-specific glycosylation is the cause of the low bioactivity of PT-TSH in the peripheral circulation. This TSH can bind serum immunoglobulin (IgG) to form a higher molecular weight complex known as "macro-TSH" that has little bioactivity in humans and rats (Klug and Adelman, 1977; Spitz et al., 1981). Therefore, we analyzed serum TSH from SD mice and LD mice treated with T_3 (LD+T_3), which contain mainly PD-TSH and PT-TSH, respectively (Figures 1G and 3). In the WB of sera derived from LD+T_3 mice, we observed presence of macro-TSH (Figure 3A). Using dot blot analysis on fractions (fr) obtained from gel filtration chromatography, we were able to distinguish macro-TSH from free TSH (Figure 3B). Most TSH in the serum of SD mice was in the free form (fr 28–32; Figure 3B). In contrast, in LD+T_3 mice, macro-TSH (fr 18–22) was present along with some free TSH (fr 26–32) (Figure 3B). The largest amount of free TSH was observed in fractions 29 and 30 of LD+T_3 and SD mice, respectively. This difference was undoubtedly the result of differences in glycosylation of PT-TSH and PD-TSH. In addition to TSH, we measured the relative amount of IgG subclasses and IgM in each fraction. IgG2b and IgG3 appeared to have an additional shifted peak at fractions 19 and 20 of the LD+T_3 serum, where macro-TSH was detected (Figure 3B). To separate IgG subclasses that form macro-TSH, we performed additional dot blots using fractions collected during protein A affinity chromatography. IgG-unbound TSH was observed in the flow-through of fractions 1 and 2, whereas IgG-TSH complex was detected in fractions 30, 37, and 46 (Figure 3C). Lack of these signals in

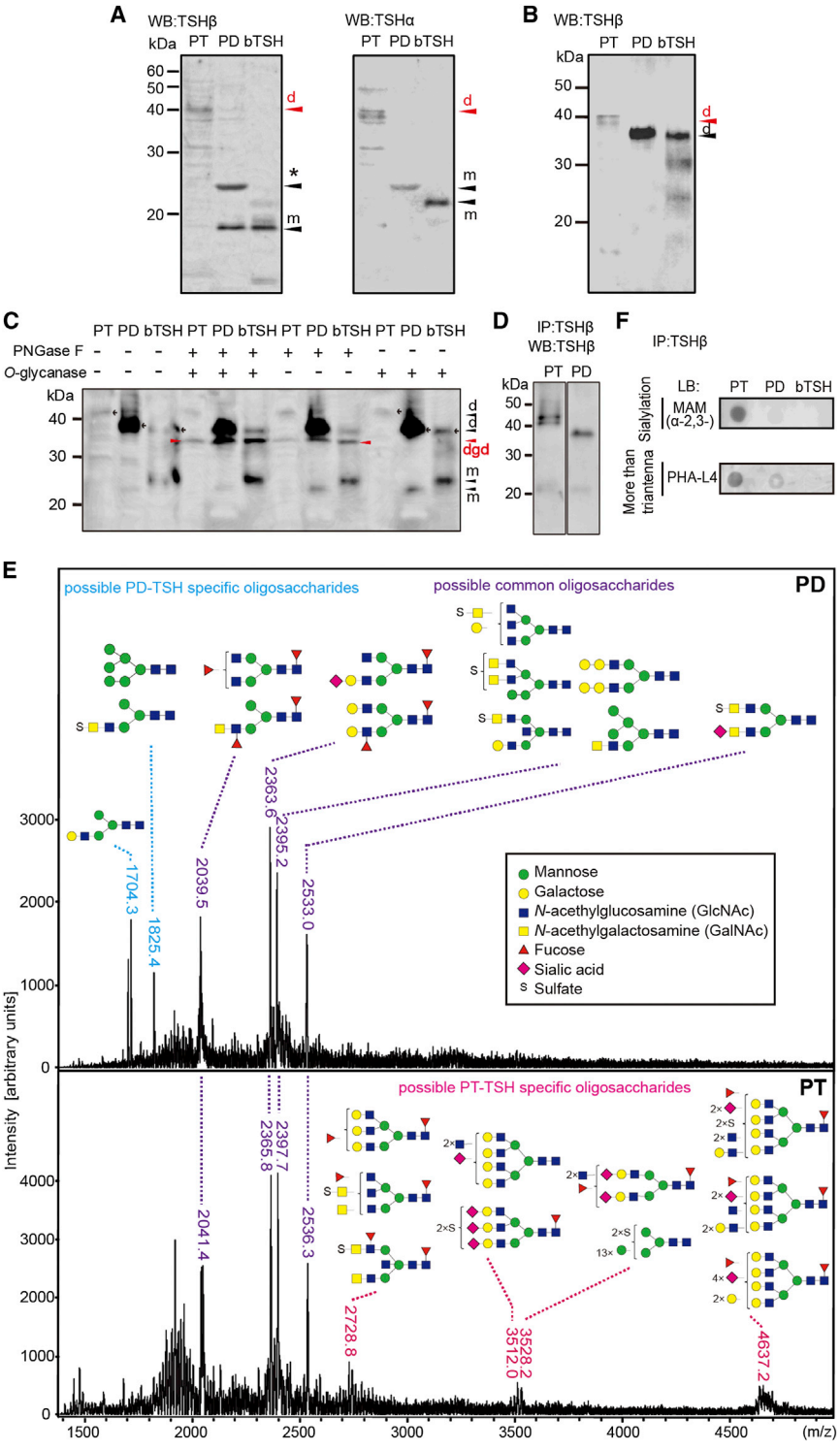

Figure 2. Glycosylation Is Different between PT-TSH and PD-TSH

(A) WB of PT and PD homogenates with TSHβ and TSHα antibodies under reducing and denaturing conditions. *m*, monomeric band; *d*, dimeric bands; *, nonspecific bands.

(B) WB of PT and PD homogenate under nonreducing conditions.

(C) Deglycosylated TSHs were analyzed under nonreducing conditions. dgd, deglycosylated form; arrow, glycosylated form.

(D) WB of IP-TSH under nonreducing conditions.

(E) Positive-ion MALDI-TOF-MS of *N*-glycans cleaved from IP-TSHs. Blue, PD-TSH-specific *N*-glycans; red, PT-TSH-specific *N*-glycans; purple, common *N*-glycans.

(F) LB analysis of TSHs.

immunodeficient NOD/SCID mice also supported the involvement of IgG in the formation of macro-TSH (Figure 3C). SD mice formed less macro-TSH than LD+T_3 mice. However, they still formed some macro-TSH with IgG when compared with NOD/SCID mice (Figure 3C). IgG2b was detected in fractions 37 and 46, whereas IgG2a and IgG3 were observed in fraction 30 (Figure 3C). IgG subclass isotyping revealed that TSH bound to IgG2b was approximately 2-fold higher in LD+T_3 mice than in SD mice (Figure 3D, bottom), although its serum concentration did not differ significantly between the two groups of animals (Figure 3D, top). Taken together, these results suggested that PT-TSH predominantly binds IgG2b.

Based on the results presented above, we next performed WB analysis to determine whether some serum factor(s) can also bind PT-TSH. Indeed, addition of serum from CBA mice to PT homogenate decreased the relative amount of unbound TSH (Figure 3E). However, we also observed absorption of PT-TSH by the serum of immunodeficient NOD-SCID mice (Figure 3E), indicating the existence of another TSH-binding protein in the peripheral circulation. WB of TSHβ in IgG-depleted serum from CBA mice (fr 2 in Figure 3C) revealed a band of high molecular weight (~100 kDa) (Figure 3F, left). We also detected a faint band of a similar size in NOD-SCID mice (Figure 3F, right). Although the signal intensity of this TSHβ band was weak, the result was plausible because NOD-SCID mice are known to have low serum TSH levels (Pohlenz et al., 1999). When we performed WB using an antibody against albumin, we observed an immunopositive signal in the IgG-depleted serum (fr 2 in Figure 3C) in both strains of mice (Figure 3G). Furthermore, albumin was IP from the flow-through (fr 2 in Figure 3C), and albumin and TSHβ were detected by WB in both strains of mice (Figure 3H), providing additional confirmation of the existence of an albumin-TSH complex in

the peripheral circulation. When we removed albumin from the IgG-free flow-through (fr 2 in Figure 3C) by immunoprecipitation, the albumin-TSH complex was no longer detectable (Figure 3I). These results indicated that PT-TSH forms macro-TSH with IgG or albumin. It is suggested that the binding of TSH to IgG is extremely high in humans and rats (Erhardt and Scriba, 1977; Kourides et al., 1978; Mori et al., 1984; Sakai et al., 2009; Vieira et al., 2006). For example, incubations with the surfactant Triton-X, the strong denaturants mercaptoethanol and quinidine hydrochloride, or the enzyme trypsin do not dissociate the IgG-TSH complex. In fact, SDS used in the WB did not dissociate TSH from IgG in the present study (Figures 3A and 3F–3H). We also examined the effect of the denaturant dithiothreitol (DTT), which failed to dissociate the IgG-TSH complex (Figure 3J). Denaturant treatments often shift the mobility of immunoreactive band owing to altered protein structures. Although the immunoreactive band of IgG-TSH was shifted by the DTT treatment, detection of IgG2b at the same molecular weight confirmed the existence of IgG-TSH complex even after DTT treatment (Figure 3J). In contrast to aforementioned treatments, only acidification below pH 3 has been reported to dissociate the macro-TSH complex in humans (Sakai et al., 2009; Vieira et al., 2006). Therefore, we also examined the effect of acidification on the stability of the mouse IgG-TSH complex. However, acidification at pH 3 by citrate also did not dissociate the IgG-TSH complex (Figure 3J). These results clearly suggested that the IgG-TSH complex is extremely stable and that PT-TSH is trapped by IgG for long periods of time in circulation.

Formation of Macro-TSH Strongly Reduces Bioactivity

Glycosylation influences the bioactivity of glycoproteins (Baenziger and Green, 1988; Szkudlinski et al., 2002). Therefore, we postulated that differences in *N*-glycans could affect the bioactivities of two TSHs. To validate this hypothesis, we examined the bioactivities of TSH extracted from PD and PT homogenates by the in vitro cyclic AMP accumulation assay, using Chinese hamster ovary (CHO) cells stably expressing TSHR (Perret et al., 1990). Unexpectedly, however, we observed no difference in the bioactivities of PT-TSH and PD-TSH (Figure 4A), indicating that variation in the TSH-linked *N*-glycans does not itself influence bioactivity. In contrast, the bioactivity of serum TSH obtained from LD+T_3 animals (containing PT-TSH) was lower than that of serum TSH from SD animals (containing PD-TSH) in both primary cultured MBH and thyrocytes (Figure 4B). When we compared the bioactivity of free TSH (fr 30 in Figure 3B) with macro-TSH (fr 20 in Figure 3B), the macro-TSH fraction exhibited little bioactivity in primary cultures (Figure 4C). To validate this observation, we investigated whether addition of serum to homogenate could abolish the bioactivity of PT-TSH. Although the addition of serum to PD homogenate had no effect, the addition of serum to PT homogenate significantly reduced the bioactivity in primary cultures (Figure 4D).

DISCUSSION

Previous studies indicated that TSH secreted from the PT acts on the adjacent ependymal cells to regulate seasonal physiology and behavior (Hanon et al., 2008; Nakao et al., 2008; Ono et al., 2008). In this study, we showed that PT-TSH is also detected in the systemic circulation (Figure 1). This result was surprising because TSH contents within the PT is 10,000-fold lower than the contents within the PD. Constitutive release of TSH from the PT seems to explain why we detected considerable amounts of PT-TSH in the circulation. Furthermore, we demonstrated differences between the oligosaccharide modifications of PD-TSH and PT-TSH (Figure 2). Glycosylation affects the half-life of circulating glycoprotein (Baenziger and Green, 1988; Strott, 2002); for example, the short half-life (~0.5–1.0 hr) of TSH is regulated by rapid hepatic clearance. Hepatic receptors specifically interact with sulfated TSH, resulting in a pulsatile secretion profile of the TSH level in serum, as in the case of LH (Baenziger and Green, 1988; Strott, 2002; Szkudlinski et al., 1993, 1995). Although both LH and FSH are regulated by pulsatile GnRH secretion, only LH exhibits episodic variation. This is because FSH has sialylated *N*-glycan and lacks the sulfate modification, resulting in a longer half-life (~17 hr) (Wide et al., 2009). Similarly, sialylated TSH escapes from specific receptor-mediated metabolic clearance in the liver (Szkudlinski et al., 1993). Therefore, it is possible that PT-TSH, which has multiantennary sialylated *N*-glycans has a much longer half-life than PD-TSH. Indeed, when we performed an in vitro TSH clearance assay using mouse primary liver-cell culture, the half-life of PT-TSH (18.7 hr, $y = e^{-0.037x}$) was far greater than that of PD-TSH (1.45 hr, $y = e^{-0.479x}$) (Figure S3). Thus, it seems plausible that the prolonged half-life of PT-TSH is another reason why higher levels of PT-TSH can be detected in the serum.

Notably, patients and rodents with hypothalamic hypothyroidism exhibit elevated concentration of circulating TSH with altered oligosaccharides (Beck-Peccoz et al., 1985; Helton and Magner, 1995; Persani et al., 1998; Taylor and Weintraub, 1989). Hypothalamic hypothyroidism results in the appearance of multiantennary *N*-glycans in circulating TSH (Taylor and Weintraub, 1989). In thyroidectomized or thyroid tumor-induced hypothyroid mice, sulfation is reduced, and sialylation and multiantennary glycans were elevated in circulating TSH (DeCherney et al., 1989; Gesundheit et al., 1986). Because expression of the gene encoding α2-3 sialyltransferase is elevated in the PD of hypothyroid mice, induction of this gene in PD may partially explain the increased sialylation of TSH during hypothyroidism (Helton and Magner, 1995). However, it is possible that the serum TSH with altered oligosaccharides observed in patients and rodents with hypothalamic hypothyroidism could be partially derived from the PT.

The bioactivity of PT-TSH extracted from PT homogenate was indistinguishable from that of PD-TSH. However, formation of macro-TSH with IgG or albumin strongly reduced the bioactivity of PT-TSH in the systemic circulation (Figure 4). Circulating PT-TSH formed macro-TSH predominantly via binding to IgG2b (Figure 3). Because these results were obtained in specific pathogen-free animals, we propose that the IgG molecules involved in the macro-TSH complex are naturally occurring antibodies (NAbs). NAbs, in contrast to antibodies induced by exogenous antigens, are physiological antibodies generated in healthy animals without prior contact to specific antigens (Lutz, 2012). Although IgM is the primary NAb, IgG is also produced as a NAb (Aksentijevich et al., 1991). The majority of NAbs are

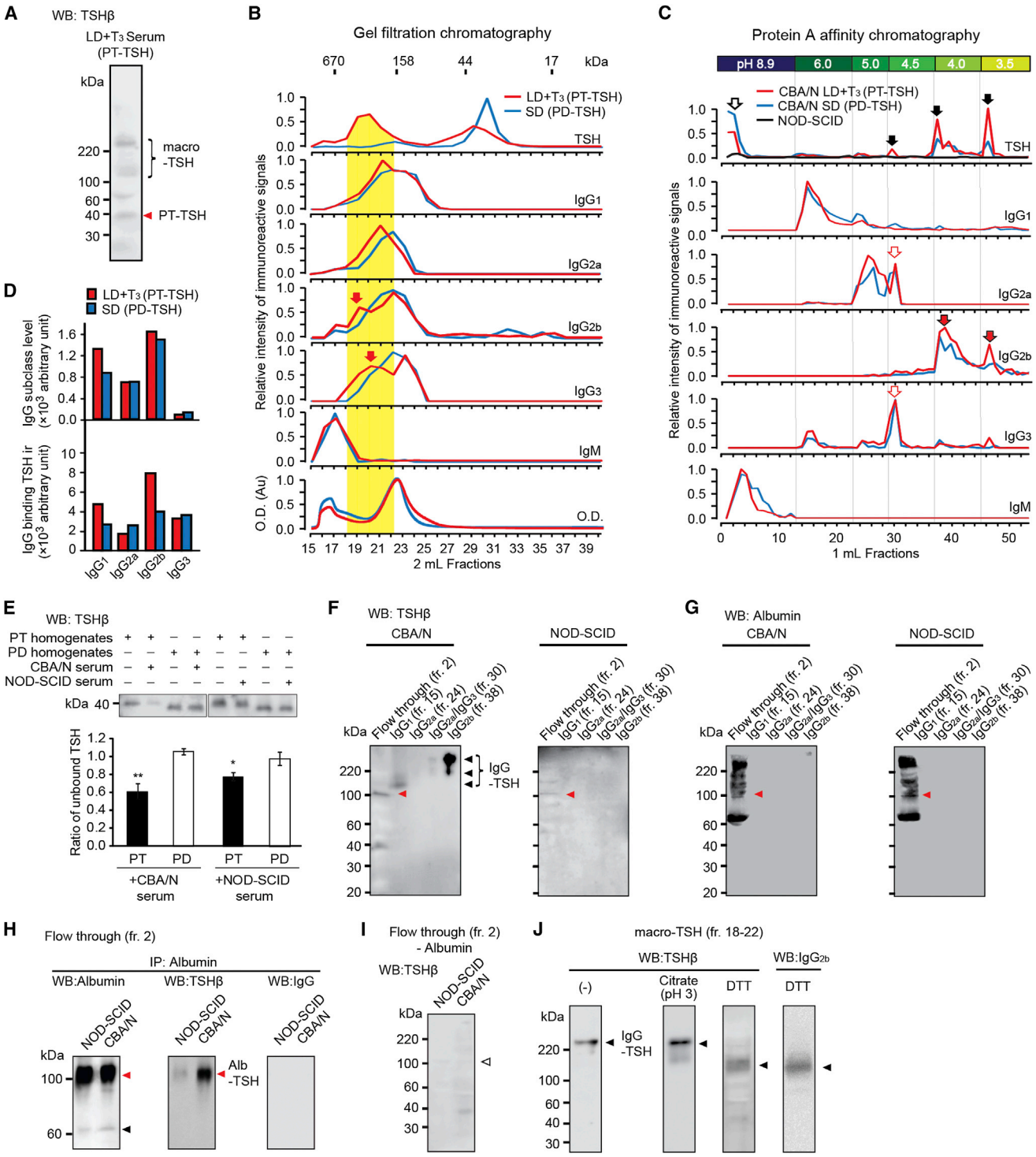

Figure 3. TSH Forms in the Circulation Macro-TSH with IgG or Albumin

(A) WB of serum TSH from LD+T_3 mice (see Figure 1).

(B) Measurements of TSH, IgGs, and IgM in fractions from gel-filtration chromatography. Yellow shading, macro-TSH; arrow, additional peak.

(C) Separation of IgG subclasses by protein A affinity chromatography. Black open arrow, free TSH; black solid arrow, macro-TSH; red solid arrows, IgG2b; red open arrows, IgG2a, IgG3.

(D) IgG isotyping of serum and macro-TSH.

(E) Adsorption test of TSH with serum. *$p < 0.05$, **$p < 0.01$ versus PD (means ± SEM, t test, $n = 4$).

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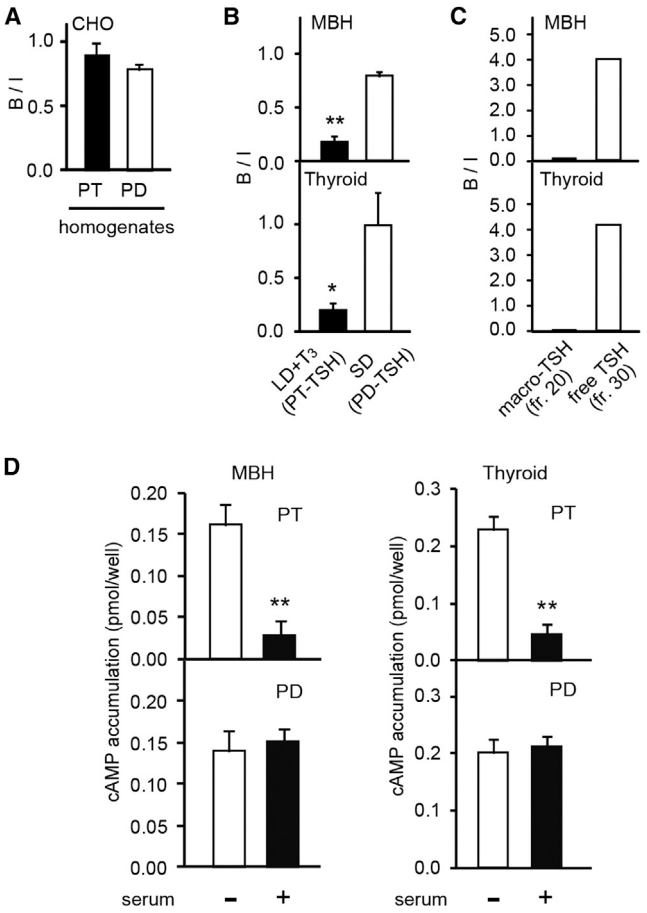

Figure 4. Formation of Macro-TSH Strongly Reduces Bioactivity of PT-TSH

(A) Bioactivity of TSH from PT or PD homogenates, analyzed using TSHR-expressing CHO cells. Ratio of TSH bioactivity to immunoreactivity (B/I) was evaluated by cyclic AMP accumulation.

(B) Bioactivity of serum TSH from LD+T_3 and SD animals, examined using primary cultured MBH and thyrocytes.

(C) Bioactivity of macro-TSH (fr 20 of Figure 3B) and free TSH (fr 30), examined using primary cultured MBH and thyrocytes.

(D) Effect of serum incubation on bioactivity of PT-TSH and PD-TSH. All values are means + SEM, n = 3–5. *$p < 0.05$, **$p < 0.01$ (t test).

polyclonal and therefore bind to various antigens and epitopes. One well-known function of NAbs is the clearance of apoptotic and damaged cells and proteins. The results of this study suggest that NAbs are also involved in the functional diversification of a hormone, TSH. In addition to IgG, PT-TSH also bound to serum albumin. Many metabolites and drugs bind to circulating albumin, and albumin-bound drugs often become inactive (Müller and Wollert, 1979). Albumin is the most abundant protein in serum and has a half-life of 19 days in humans. Therefore, it seems reasonable to conclude that the albumin-TSH complex also contributes to the long half-life of PT-TSH in the circulation.

In this study, we demonstrated that the PT-TSH heterodimer is more stable than the PD-TSH heterodimer (Figure 2A). We confirmed this result under both reducing and denaturing conditions (Figures S4A and S4B). Although glycosylation affects the stability of some protein dimers (Walker et al., 2013), deglycosylation of PD-TSH and PT-TSH did not change the stability profiles of the respective heterodimers (Figure S4C). Therefore, we also investigated whether other types of modifications might play a role in determining stability. Transglutaminases are enzymes that catalyze the formation of a covalent isopeptide bond (Lorand and Graham, 2003). Among seven transglutaminase (*Tgm*) genes, we observed expression of *Tgm2* in the PT. Thus, the activity of *Tgm2* may govern in the different structural stability of PT-TSH (Figure S4D).

In summary, we showed here that tissue-specific glycosylation prevents functional crosstalk between PT-TSH and PD-TSH. Glycosylation is critical to a wide range of biological processes, and the importance of tissue-specific glycosylation has come to be recognized (Seppälä et al., 2009). This report demonstrates the involvement of tissue-specific glycosylation in preventing functional crosstalk between signaling molecules in vivo.

EXPERIMENTAL PROCEDURES

Animal Experiments

This study was approved by the Committee on Animal Experiments of the Graduate School of Bioagricultural Sciences and Research Institute of Environmental Medicine, Nagoya University.

In Situ Hybridization

Coronal sections (20 μm thick) were prepared using a cryostat (Leica Microsystems, CM3050S). In situ hybridization was performed using antisense 45-mer nucleotide probes (Table S1), as previously described (Ono et al., 2008). Control sections were hybridized in the presence of excess unlabeled probe.

Immunohistochemistry

Immunohistochemistry to detect TSHβ was performed using rabbit polyclonal antibody to mouse/rat TSHβ (1:10,000) (provided by Dr. Albert F. Parlow, the National Institute of Diabetes and Digestive and Kidney Diseases [NIDDK]), as previously reported (Ikegami et al., 2009) (Figure S1). For double-immunofluorescence staining, PT and PD slices were incubated with rabbit polyclonal antibody against TSHβ and goat polyclonal antibody against ST3GalIV (1:2,000) or GnT-V (1:2,000) (Santa Cruz Biotechnology) for 1 hr at room temperature and then reacted with Alexa Fluor 546-conjugated donkey antigoat IgG (1:400) and Alexa Fluor 488-conjugated goat antirabbit IgG (1:400) (Molecular Probes) and mounted in DAPI/mounting medium (LK-096A, Kreatech).

RIA

Serum and tissue TSH was measured using a sensitive, heterologous, disequilibrium double-antibody precipitation RIA as previously described (Pohlenz et al., 1999). The sensitivity of this assay was 9 mIU/L. Serum T_4 concentrations were measured by an antibody-coated solid phase RIA (Simens Medical Solutions, Diagnostics) using 25 μl of serum. The sensitivity of this assay was 0.2 μg/dl.

(F and G) WB of TSH (F) and albumin (G) in fractions from protein A affinity chromatography. Arrowhead, albumin-TSH complex.

(H) Detection of IP-albumin-TSH complex in sera. Red, albumin-TSH complex; black, free albumin.

(I) Macro-TSH disappeared when albumin was removed from the IgG-free flow-through fraction. Arrowhead, position of albumin-TSH.

(J) WB of TSHβ and IgG2b in macro-TSH fractions under acidification condition (pH 3) by citrate or denature condition by DTT. Arrowhead, position of IgG-TSH.

WB

WB was performed as previously reported (Ikegami et al., 2009). Membranes were incubated with the following primary antibodies: rabbit polyclonal antibodies against TSHβ (1:10,000), TSHα (1:10,000, provided by Dr. Albert F. Parlow, the NIDDK), and albumin (1:2,000; abcam); HRP-conjugated goat polyclonal antibody against mouse IgG (1:20,000; abcam) and mouse IgG2b (1:40,000; abcam); and goat polyclonal antibodies against ST3GalIV (1:2,000) and GnT-V (1:2,000). Deglycosylation of samples was performed under nonreducing conditions using GlycoPro Enzymatic Deglycosylation kit (PROZYME), as previously reported (Zou et al., 2008).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.10.006.

AUTHOR CONTRIBUTIONS

T.Y. conceived the research. T.Y., K.I., and S.R. designed the research. K.I., X.L., Y.H., H.O., W.O., Y.I., and T.N.O. performed experiments and analyzed data. M.Y., C.S., K.K., Y.M., M.I., Y.S., and T.Y. provided new materials and advised the research. K.I, T.Y., and S.R. wrote the paper. All of the authors reviewed and commented on the manuscript.

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