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Sialylation of Human Thyrotropin Receptor Improves and Prolongs Its Cell-Surface Expression

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ABSTRACT

Glycosylation of the thyrotropin receptor (TSHR) has been shown to be essential for correct protein folding and for cell-surface targeting. In a recent study, we detected increased expression of β-galactoside α(2,6)-sialyltransferase (SIAT1) in toxic thyroid adenomas where gain-of-function mutations of the TSHR have been invoked as one of the major causes. To investigate the physiological meaning of these findings, we designed experiments to evaluate the consequences of sialylation for the expression of the TSHR. Hence, we investigated the effect of coexpressing the TSHR and different sialyltransferases (SIAT1, SIAT4a, and SIAT8a) for cell-surface expression of the receptor. Coexpression of each of the three SIAT isoforms and the TSHR in COS-7 cells increased TSHR expression on the cell surface in the range of 50 to 100%. Moreover, Western blot analysis with lectins specific for α(2,3) and α(2,6)-linked sialic acids and lectin-binding enzyme-linked immunosorbent assay support a direct effect on TSHR cell-surface expression mediated by sialic acid transfer to the TSHR. Finally, we treated living COS-7 cells after cotransfection of TSHR and SIAT8a with neuraminidase for 30 min to remove covalently linked sialic acid. Subsequent loss of TSHR cell-surface expression suggests that sialylation prolongs the resting time of the TSHR on the cell surface. Our data demonstrate for the first time that the transfer of sialic acid can improve and prolong cell-surface expression of a transmembrane receptor.

Thyrotropin (TSH) signaling through its receptor mediates the paracrine control of thyroid function. The thyrotropin receptor (TSHR) belongs to a subgroup of G protein-coupled receptors comprising the TSHR, the follicle-stimulating hormone receptor, and the lutropin-choriogonadotropic hormone receptor. These three G protein-coupled receptors are characterized by large, heavily glycosylated extracellular domains that form the hormone-binding sites (Sanders et al., 1997). In the human thyroid, the TSH-bound TSHR is first synthesized as an ~84-kDa polypeptide chain to which high mannose type carbohydrates are attached in the endoplasmic reticulum (100-kDa fragment). These are further processed to mature, complex-type carbohydrates in the Golgi apparatus (120-kDa fragment) (Rapoport et al., 1996; Nagayama et al., 1998). Whereas the 100-kDa band in principle contains mannose-type sugars, the 120-kDa band in principle contains complex-type sugars like fucose-linked [α(1–6)]*N*-acetylglucosamin, [α(1–3)]*N*-acetyllactosamin, [β(1–4)]*N*-acetylglucosamin, and α(2,3)-linked sialic acids (Oda et al., 1999). Radioligand binding assays showed that 80% of radiolabeled TSH was bound to the 120-kDa band (Oda et al., 1999). Analysis of recombinant TSHR led to the conclusion that the TSHR-ectodomain contains six glycosylation sites (Asn77, Asn99, Asn113, Asn177, Asn198, and Asn302), at least four of them have to be glycosylated to express a functional TSHR (Nagayama et al., 2000).

An amino acid substitution of Asn113 to Gln113 disrupted TSH binding and TSH-stimulated cAMP synthesis, very likely because of altered TSHR conformation caused by impaired glycosylation (Nagayama et al., 2000). However, deglycosylation of the native TSHR by PNGase F treatment does not affect autoantibody binding to the TSHR (Atger et al., 1999). The importance of glycosylation has been also

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ABBREVIATIONS: TSH, thyrotropin; TSHR, thyrotropin receptor; SIAT1, β-galactoside α(2,6)-sialyltransferase; SIAT4a, β-galactoside α(2,3)-sialyltransferase; SIAT8a, β-galactoside α(Z,8)-sialyltransferase; AFTN, autonomously functioning thyroid nodule; ST, normal surrounding thyroid tissue; wt, wild type; GFP, green fluorescent protein; RT-PCR, reverse-transcriptase polymerase chain reaction; Tg, thyroglobulin; CTN, cold thyroid nodule; DMEM, Dulbecco's modified Eagle's medium; IP, inositol phosphate; b, bovine; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorting; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; TBST, Tris-buffered saline/Tween 20; PBST, phosphate-buffered saline/Tween 20; MALII, *Maackia amurensis* lectin 2; SNA, *Sambucus nigra*.

shown for the follicle-stimulating hormone receptor (Davis et al., 1995). In contrast, acquisition of complex carbohydrates has not been found to be important for hormone binding in the case of the lutropin-choriogonadotropic hormone receptor (Davis et al., 1997; Dufau, 1998). Binding of calnexin (a lectin-like molecular chaperone, which facilitates correct protein folding) (Helenius, 1994) has been demonstrated for the immature forms (high mannose-type carbohydrates) of all three glycoprotein hormone receptors (Rozell et al., 1998; Mizrachi and Segaloff, 2004).

In addition to forming part of the binding site for the ligand, oligosaccharides synthesized and processed in the endoplasmic reticulum or the Golgi apparatus are crucial for protein folding and intracellular trafficking, respectively (Nagayama et al., 1998). In case of the TSHR, inhibition of N-linked glycosylation blocks cell-surface expression (Nagayama et al., 2000). Moreover, TSHR expressed in *Escherichia coli* is not glycosylated and therefore is not able to bind TSH (Huang et al., 1992).

Sialic acid is a frequent component in complex carbohydrates that has been demonstrated to affect the biological activity or the metabolic half-life of many proteins (Kaneko et al., 2004; Otto et al., 2004). This is also evident for the TSHR ligand and the thyroid hormone synthesis template and storage molecule thyreoglobulin (Tg) (Ronin et al., 1986; Grollman et al., 1993; Medeiros-Neto et al., 1993; Persani et al., 1998; Trojan et al., 1998). An increased sialylation of TSH in hyperthyroid patients (Miura et al., 1989) leads to a prolonged plasma half-life and prolonged bioactivity (Helton and Magner, 1995; Szkudlinski et al., 1995). TSH demonstrates the ability to down-regulate not only steady-state TSHR mRNA levels (Saji et al., 1992) but also SIAT1 mRNA levels (Grollman et al., 1993). Sialylation of membrane proteins, including the thyroid peroxidase, may participate in the TSH-regulated vectorial transport and maturation of Tg (Grollman et al., 1993). After stimulation with TSH, an increased sialylation of Tg was observed in porcine thyroid glands and follicles (Ronin et al., 1986). In thyrotrophs of hypothyroid mice, an increased expression of SIAT1 and SIAT4a was observed (Helton and Magner, 1995), whereas in liver cells stimulated with thyroid hormones T_3 and T_4, expression of SIAT1 and SIAT4a was decreased (Feng et al., 2000). The expression and activity of sialyltransferases are known to be regulated by thyroid hormone through negative feedback and hypothalamic thyrotropin-releasing hormone (TRH) and by sleep-related mechanisms (Persani et al., 1998).

Recently, we have shown a clear increase in mRNA expression of SIAT1 in autonomously functioning thyroid nodules (AFTNs) versus normal surrounding thyroid tissue (ST) (Eszlinger et al., 2004). Because constitutive activation of the TSHR is an important step in the etiology of AFTNs (Krohn and Paschke, 2001), and because this increased SIAT1 expression in AFTNs might therefore be related to TSHR signaling, these findings prompted us to further evaluate the role of sialylation for TSHR function as well as SIAT expression in thyroid pathologies. We designed our experiments to study the consequences of SIAT action for the surface expression of the TSHR. Moreover we quantified SIAT1, -4a, and -8a mRNA expression in thyroid nodular disease and primary cultures of thyrocytes.

Materials and Methods

Expression Plasmids. A sequence-verified human TSHR cDNA cloned into pSVL (courtesy of Dr. G. Vassart, Brussels, Belgium) was used. Respectively, for lectin binding ELISA, we used a TSHR-GFP fusion protein (courtesy of Dr R. Latif, New York) in which the human TSHR sequence lacking the stop codon was ligated into the mammalian expression vector pEGFP-N1 (BD Biosciences Clontech, Palo Alto, CA). For SIAT8a, we used the pcDNA3.1 GeneStorm Expression-Ready Clone RG001497 (Invitrogen, Carlsbad, CA). The full-length cDNAs encoding the human SIAT1 and SIAT4a were subcloned into the eukaryotic expression vector pcDNA3.1/V5-His C (Invitrogen, Paisley, UK).

Tissue Samples. All AFTNs were identified by ultrasound and scintigraphy. All preoperatively identified nodules were also identified at surgery and postoperatively characterized by histology according to the World Health Organization criteria (Hedinger, 1988). Somatic TSH receptor mutations in the hot nodules were determined previously by denaturing gradient gel electrophoresis and subsequent direct sequencing of the positive polymerase chain reaction fragments (Trulzsch et al., 2001). AFTNs without a TSHR mutation were screened for mutations in the exons 7 to 10 of the $G_s\alpha$ protein by direct sequencing (forward primer, 5′-agt tgg caa att gat gtg agc-3′; reverse primer, 5′-tct cta taa aca gtg cag acc-3′). However, no mutations in the $G_s\alpha$ protein were found. Informed consent for the analysis was given by the patients.

Primary thyrocyte cultures were obtained at surgery from specimens of non-nodular thyroid tissues of consecutive patients undergoing thyroid resection for treatment of their cold thyroid nodules (CTNs). All patients with a CTN were euthyroid with normal TSH levels and negative thyroid antibodies.

RNA Isolation. Total RNA was isolated from 15 AFTNs, 22 CTNs, and 4 primary thyrocyte cultures using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Later, the total RNA was purified with an RNeasy kit (QIAGEN GmbH, Hilden, Germany) according to the RNA clean-up protocol. The quality and quantity of the total RNAs was examined on agarose gels and photometer (Ultrospec 3300 pro; Biochrom Ltd, Cambridge, UK).

Real-Time RT-PCR. One microgram of total RNA was used to prepare double-stranded cDNA (Moloney murine leukemia virus reverse transcriptase; Invitrogen) primed with oligo(dT). The quantification of three genes (SIAT1, SIAT4a, and SIAT8a) by real-time RT-PCR was performed using a Light Cycler (Roche Diagnostics, Mannheim, Germany) as described previously (Eszlinger et al., 2004). The nucleotide sequences of the primer and polymerase chain reaction conditions are available on request. The determined ratios were normalized to the ratio of the housekeeping gene β-actin.

Cell Culture and Transfection. The influence of sialyltransferase expression on functional properties of the TSHR was investigated in cell culture. COS-7 cells were grown in DMEM supplemented with 10% fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37°C in a humidified 5% CO_2 incubator. Cells were transiently transfected in 24-well plates (0.5×10^5 cells/well) with 0.5 μg of DNA per well [TSHR-pSVL (Libert et al., 1989) and SIAT-pcDNA3.1/V5-His] for cAMP accumulation and TSH binding analysis. To determine inositol phosphate (IP) formation, cells were transiently transfected in 12-well plates (1×10^5 cells/well) with 1 μg of DNA per well using the FuGENE 6 reagent (Roche Applied Science, Indianapolis, IN).

Radioligand Binding Assay. Competitive binding studies were performed as described previously (Wonerow et al., 1998). To determine the TSH radioligand binding at a similar level of TSHR cell-surface expression, we balanced the effect of SIAT1 cotransfection on TSHR expression by cotransfecting equivalent amounts of the human V2 vasopressin receptor (Schoneberg et al., 1996; Neumann et al., 2001). Data were analyzed assuming a one-site binding model using the fitting module of Sigma Plot 2.0 for Windows (Swillens, 1995).

cAMP Accumulation Assay. Measurements of cAMP accumulation were performed 48 h after transfection as described previously (Wonerow et al., 1998).

Stimulation of IP Formation. Transfected COS-7 cells were incubated with 2 μCi/ml [*myo*-^{3}H]inositol (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 8 h. Thereafter, cells were preincubated with serum-free DMEM containing 10 mM $LiCl_2$ for 30 min. Stimulation with bTSH for 1 h was performed with the same medium supplemented with 100 mU/ml bTSH. Evaluation of basal and TSH-induced increases of intracellular IP levels was performed by anion-exchange chromatography as described previously (Berridge, 1983). IP values are expressed as the percentage of radioactivity incorporated from ^{3}H-labeled inositol phosphates over the sum of radioactivity incorporated in IPs and phosphatidylinositols.

FACS Analysis. Forty-eight hours after transfection, cells were detached from the dishes using 1 mM EDTA and 1 mM EGTA in PBS and transferred into Falcon 2054 tubes. Before incubation with the primary antibody, cells were washed once with PBS containing 0.1% bovine serum albumin and 0.1% NaN_3. Afterward, cells were incubated with a mouse anti-human TSHR antibody (2C11, 10 μg/ml; Serotec, Oxford, UK) in the same buffer for 1 h at 4°C. Tubes were washed twice and incubated for 1 h at 4°C in the dark with fluorescein-conjugated $F(ab')_2$ rabbit anti-mouse IgG (Serotec; dilution, 1:1000). Before FACS analysis (FACscan; BD Biosciences, San Jose, CA), cells were washed twice and fixed with 1% paraformaldehyde. Receptor expression was determined by the fluorescence intensity, whereas the percentage of signal positive cells corresponds to the transfection efficiency.

Neuraminidase Treatment. Thirty minutes and 2 and 12 hours before FACS preparation, cells were treated with 0.1 U/ml neuraminidase from *Clostridium perfringens* (Sigma Chemical, St. Louis, MO) in DMEM without fetal calf serum at 37°C and 5% CO_2.

ELISA. The cell-surface expression of the TSHR determined by FACS analysis was confirmed by ELISA as described previously (Wonerow et al., 2000).

Preparation of Solubilized TSHR. COS-7 cells were transiently transfected in 145-cm^2 dishes (2×10^6 cells per dish) with 20 μg of DNA per dish (1:1 pSVL-TSHR-Flag and pcDNA3.1/V5-His-SIAT) using the FuGENE TM6 reagent (Roche). Cells were grown to confluence for 2 days after transfection, and the cells were washed with PBS and scraped into 10 ml of ice-cold buffer A (50 mM NaCl and 10 mM Tris/HCl, pH 7.5, containing 1 mM phenylmethylsulfonyl fluoride). The cells were centrifuged at 1000*g* for 5 min at 4°C, and the pellet was resuspended in 1 ml of buffer A and homogenized with a glass homogenizer on ice. This homogenate was then centrifuged at 12,000*g* for 30 min at 4°C, resuspended in 0.5 ml of ice-cold buffer A containing 1% Triton X-100, homogenized, and centrifuged at 90,000*g* for 2 h at 4°C. The supernatant was aliquoted and stored at −80°C (Oda et al., 1999).

SDS-PAGE Followed by Western Blotting. Aliquots of solubilized material were mixed with an equal volume of SDS-PAGE sample buffer (4% SDS, 20% glycerol, 100 mM Tris-HCl, pH 6.8, and 0.002% bromphenol blue), heated to 37°C for 30 min, electrophoresed on 10% acrylamide gels (SDS-PAGE; Bio-Rad, Hercules, CA) at 140 V for 110 min, and blotted onto nitrocellulose (Whatman Schleicher & Schuell, London, England) at 120 V for 120 min. The membranes were blocked using 5% low-fat milk in TBST, incubated overnight at 4°C with a 1:2000 dilution of anti-FLAG M2 (Sigma), and developed using anti-mouse horseradish peroxidase conjugate (Cell Signaling Technology Inc., Beverly, MA) followed by SuperSignal West Pico chemiluminescence reagents (Pierce Chemical, Rockford, IL).

Immunoprecipitation of TSHR. COS-7 cells were grown and transfected as described above (see *Preparation of Solubilized TSHR*). Cells were scraped into 10 ml ice-cold PBS and centrifuged at 1000*g* for 5 min at 4°C. The pellet was resuspended in 50 μl of lysis buffer (25 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 1 mM NaF, 1 mM Na_3VO_4, 2 mM imidazol, and 1× Complete Protease Inhibitor) and incubated for 30 min at −80°C and then for 1 h on ice with 5× mixing. This homogenate was then centrifuged at 5000 rpm for 5 min at 4°C, and the supernatant was aliquoted and stored at −20°C. Mouse $IgG_1\kappa$ (30 μl; 1 mg/ml; Sigma) was bound to 40 μl of Protein G PLUS/Protein A Agarose (Oncogene Science, Cambridge, MA) in a 1-h incubation at 4°C on a welter blender. After rinsing two times with PBS, 0.4 μl of mouse monoclonal antibody anti-FLAG M2 (4.9 mg/ml; Sigma) diluted in 20 μl of PBS was added followed by a 1-h incubation at 4°C. After rinsing two times with PBS, 25 μl of cell lysate was added and incubated for 3 h at 4°C. Afterward, the pellet was rinsed four times with PBS (Chazenbalk et al., 2002). TSHR was eluted in 20 μl of SDS-PAGE sample buffer and heated to 37°C for 30 min with shaking.

Lectin Analysis. Ten-microliter samples of immunoprecipitated TSHR (described above) were electrophoresed on 10% SDS-PAGE gels and blotted onto nitrocellulose membranes. The membranes were incubated for 1 h with 10 μg/ml biotin-labeled lectin [*Maackia amurensis* lectin 2 (MALII) or *Sambucus nigra* (SNA); Vector Laboratories, Burlingame, CA] in TBST and afterward were washed three times in TBST. Incubation with a 1:2000 dilution of anti-biotin horseradish peroxidase conjugate (Cell Signaling Technology) in TBST was then carried out for 60 min, and the reaction was developed with SuperSignal West Pico chemiluminescence reagents (Pierce).

Lectin Binding ELISA. The lectin binding ELISA was performed on Reacti-Bind Anti-GFP Coated Plates (Pierce). COS-7 cells cotransfected with TSHR-GFP and pcDNA3.1 or SIAT1 were harvested in Nonidet P-40 lysis buffer and added to the wells. After 1-h incubation at room temperature, the plate was washed three times in PBST, incubated for 1 h with 10 μg/ml biotin-labeled lectin (SNA; Vector Laboratories) in PBST, and afterward washed three times in PBST. Incubation with a 1:2000 dilution of anti-biotin horseradish peroxidase conjugate (Cell Signaling Technology) in PBST was then carried out for 60 min, and the reaction was developed with substrate (*o*-phenylendiamine, H_2O_2, citrate buffer, pH 5.2) and measured at 492 nm.

Results

Real-Time RT-PCR. We detected differences in expression of SIAT1, -4a, and -8a in AFTNs and CTNs versus STs (Eszlinger et al., 2004) using light cycler quantification (Fig. 1). The mRNA expression of SIAT1 in AFTNs is increased compared with STs, whereas the expression in CTNs is decreased or shows no difference compared with STs, respectively. In contrast, the expression of SIAT8a mRNA is decreased in AFTNs compared with STs, and the expression in CTNs shows no difference compared with STs. The mRNA expression of SIAT4a in AFTNs and CTNs shows no significant differences compared with STs. The mRNA expression of these three genes (SIAT1, SIAT4a, and SIAT8a) was also studied in primary thyrocyte cultures. Stimulation of primary thyrocyte cultures with TSH increased the SIAT1 and SIAT4a mRNA expression, whereas the mRNA expression of SIAT8a decreased (Fig. 1).

Radioligand Binding Assay. The influence of TSHR-sialylation on its ability to bind TSH was determined by a competitive binding analyses. Cotransfection of SIAT1 markedly increased the binding of ^{125}I-labeled TSH by the receptor (B_{max} = 189.1 ± 13.3%) compared with cells cotransfected with TSHR and empty pcDNA vector (100%). Similar data could be obtained for SIAT4a (B_{max} = 177 ± 19.8%) and SIAT8a (B_{max} = 162.3 ± 14.3%) compared with cotransfection of the empty pcDNA vector (Fig. 2). To determine whether the increased TSH binding after SIAT1 cotransfection can be attributed to the increased cell-surface expression

of the TSHR, we reduced the cell-surface expression of the TSHR in SIAT1 cotransfection experiments by additional cotransfection of the human V2 receptor (Schoneberg et al., 1996), as described previously (Arturi et al., 1998). Thus, the TSHR cell-surface expression was reduced to less than 100% (FACS, 76 ± 6%; ELISA, 85 ± 10%) of the reference experiment (transfection of wt TSHR with an empty pcDNA vector). Concurrent with the reduced cell-surface expression of the TSHR, the binding of labeled TSH by the TSH receptor decreased to B_{max} = 81 ± 5% (data not shown).

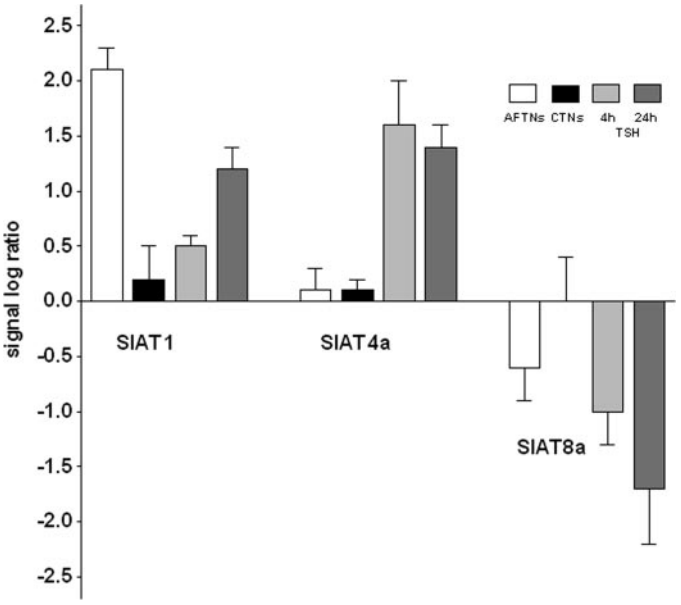

Fig. 1. Comparison of the differential gene expression of SIAT1, -4a, and -8a determined by real-time RT-PCR in AFTNs versus STs (□), CTNs versus STs (■), and in primary thyrocytes after stimulation with TSH versus unstimulated cells (4 h, ▨; 24 h, ▩). The signal log ratio is the logarithm to the basis two of the mean fold change (n = 15) for the expression in AFTNs versus STs.

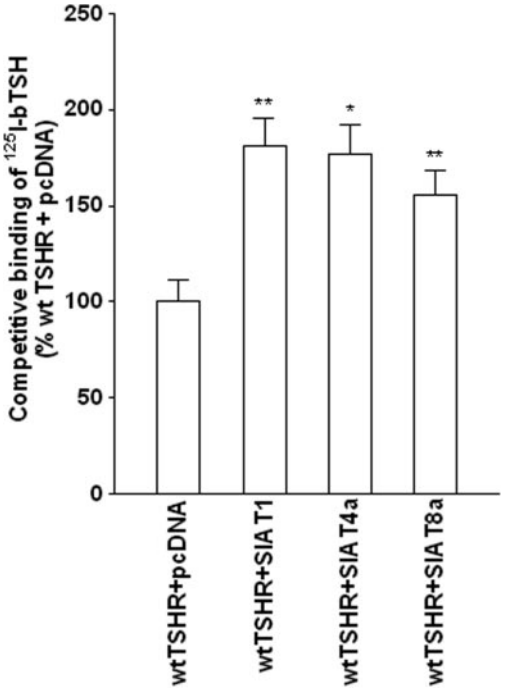

Fig. 2. Competitive binding of [^{125}I]bTSH on COS-7 cells transfected with pSVL-TSHR and pcDNA3.1-SIAT1, -4a, -8a, or empty pcDNA3.1 vector. To determine B_{max} values, transfected cells were subjected to displacement binding using 160,000 to 180,000 cpm [^{125}I]bTSH per well and increasing concentrations of unlabeled bTSH. TSH-binding is significantly higher after coexpression of the TSHR with any of the sialyltransferases compared with coexpression of TSHR with the empty pcDNA3.1 vector (⋆, $p < 0.05$; ⋆⋆, $p < 0.01$). All data are presented as means ± S.E.M. of two independent experiments, each performed in duplicate.

cAMP Accumulation Assay. The consequence of sialylation on the ability of the TSHR to activate adenylatcyclase was determined by measurement of cAMP accumulation. Cotransfection of TSHR and SIAT1 markedly increased the accumulation of cAMP (basal, 27.818 ± 6.941 pmol; stimulated, 592.86 ± 37.5 pmol) compared with cells cotransfected with TSHR and empty pcDNA vector (basal, 14.452 ± 1.69 pmol; stimulated, 397.65 ± 37.18 pmol). Similar data could be obtained for SIAT8a (basal, 32.455 ± 6.983 pmol; stimulated, 431.86 ± 56.67 pmol) compared with cotransfection with the empty pcDNA vector.

Stimulation of IP Formation. In addition to cAMP accumulation, the effect of sialylation on the ability of the TSHR to activate phospholipase C was determined by the measurement of IP formation. Cotransfection of TSHR and SIAT1 markedly increased the formation of IP (basal, 1.6 ± 0.4%; stimulated, 32.7 ± 1.2%) compared with cells cotransfected with TSHR and empty pcDNA vector (basal, 2.4 ± 1.1%; stimulated, 22.8 ± 1.7%). Similar data could be obtained for SIAT8a (basal, 1.3 ± 0%; stimulated, 28.3 ± 1.9%) compared with cotransfection with the empty pcDNA vector. IP values are expressed as the percentage of radioactivity incorporated from [^{3}H]inositol phosphates over the sum of radioactivity incorporated in IPs and phosphatidylinositols.

FACS Analysis. We determined the TSHR expression by the fluorescence intensity and the transfection rate by the relation of signal-positive cells to the total number of cells. Cotransfection of TSHR with SIAT1, -4a, or -8a results in an increased cell-surface expression of the TSHR. Cotransfection with SIATs increased cell-surface expression of the TSHR up to 151.6 ± 4.1% (SIAT1), up to 132 ± 4.3% (SIAT4a), or up to 185.7 ± 4.4% (SIAT8a) (Fig. 3).

Neuraminidase Treatment. FACS analyses after treatment with neuraminidase for 30 min showed a decrease of cell-surface expression of TSHR coexpressed with SIAT8a from 190 to 140% (Fig. 4). For coexpression of the TSHR and SIAT1, reduction of cell-surface expression was most pronounced at 12 h (from 134% to the level of the TSHR cotransfected with empty pcDNA vector) (Fig. 4).

ELISA. The investigation of TSHR cell-surface expression by ELISA confirmed the FACS data. The ELISA revealed an increase of TSHR cell-surface expression to 144 ± 19% after cotransfection with SIAT1 (data not shown).

Western Blot. Western blotting analysis of solubilized TSHR-FLAG from membrane preparations is shown in Fig. 5A. The identification of the human TSHR as two bands of 100 and 120 kDa was carried out using the anti-FLAG-M2 antibody (Sigma). After coexpression of the TSHR and sialyltransferases, especially SIAT8a (lane 3), TSHR protein level is increased in the membrane, indicating a higher TSHR cell-surface expression. In contrast, similar transfection/expression levels of TSHR are shown by Western blotting analysis of total cell lysates from COS-7 cells cotransfected with pSVL-TSHR-FLAG and different SIAT constructs (Fig. 5B).

Lectin Analysis. The investigation of TSHR-sialylation was carried out using biotin-labeled lectins: *Maackia amu-*

rensis lectin 2 (MALII), which is specific for carbohydrate structures containing α(2,3)-linked sialic acids, and *Sambucus nigra* (SNA), which is specific for carbohydrate structures containing α(2,6)-linked sialic acids. For this experiment, we used immunopurified TSHR as described under *Materials and Methods*. Coexpression of TSHR with SIAT1 (lane 2), which specifically transfers carbohydrate structures containing α(2,6)-linked sialic acids, shows a clear reaction of the upper band with SNA, whereas coexpression with SIAT4a (lane 3), which specifically transfers carbohydrate structures containing α(2,3)-linked sialic acids, shows a clear reaction of the upper band with MALII (Fig. 6). We did not study the coexpression of TSHR with SIAT8a because a lectin specific for carbohydrate structures containing α(2,8)-linked sialic acids was not available. The stronger reaction of MALII compared with SNA, after cotransfection with SIAT1 or the empty pcDNA vector, could be caused by the basal expression of SIAT4a in COS-7 cells, which do not show basal expression of SIAT1 (data not shown).

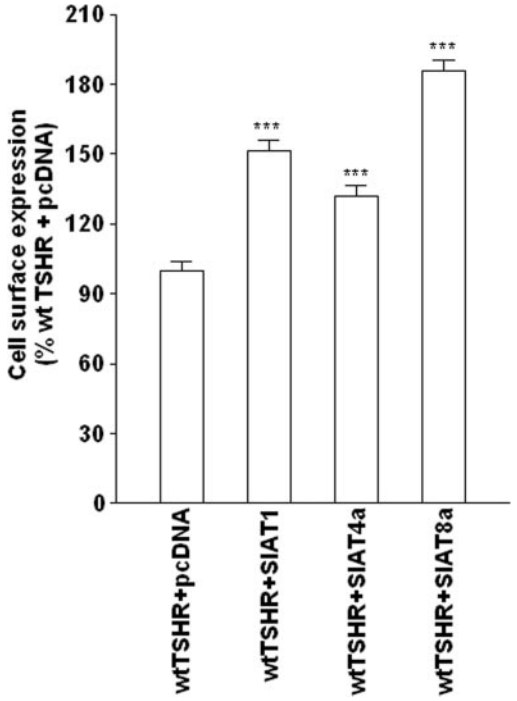

Fig. 3. Cell-surface expression of wt TSHR measured by flow immunocytometry using a mouse anti-human TSHR antibody (2C11, Serotec). pSVL-TSHR was cotransfected with empty pcDNA3.1-vector (lane 1), pcDNA3.1-SIAT1 (lane 2), pcDNA3.1-SIAT4a (lane 3), or pcDNA3.1-SIAT8a (lane 4). Cell-surface expression is significantly higher after coexpression of the TSHR with any of the sialyltransferases compared with coexpression of TSHR with the empty pcDNA3.1 vector (★★★, $p < 0.001$). All data are presented as mean ± S.E.M. of two independent experiments, each performed in duplicate.

Lectin-Binding ELISA. The investigation of TSHR-sialylation by ELISA confirmed the previous data obtained by Western blot. The ELISA revealed an increase of SNA-binding to 146 ± 8.7% after cotransfection with SIAT1 compared with coexpression of TSHR and the empty pcDNA vector (Fig. 6B).

Statistical Analysis. We used one-way analysis of variance to detected significant differences ($p < 0.01$) for FACS analysis and radioligand binding assay between TSHRs cotransfected with different SIATs or with the empty pcDNA vector. In a subsequent Student's t test, we differentiated three levels of significance (*, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$) between experimental groups.

Discussion

Expression of and signaling through the TSHR is an essential part of endocrine regulation that affects thyroid hormone metabolism. The TSHR is subject to a number of regulatory mechanisms that change the efficacy of TSH signaling (e.g., gene and protein expression, receptor phosphorylation, and internalization). Our data add a new aspect of protein modification (i.e., sialylation) that has not been studied for functional consequences so far. Indeed, we demonstrate for the first time that the transfer of sialic acid to carbohydrate residues of the TSHR can improve and prolong cell-surface expression of a transmembrane receptor, thereby regulating the availability of the receptor for ligand signaling.

Motivation for our cotransfection studies came from GeneChip data that show increased SIAT1 mRNA expression in

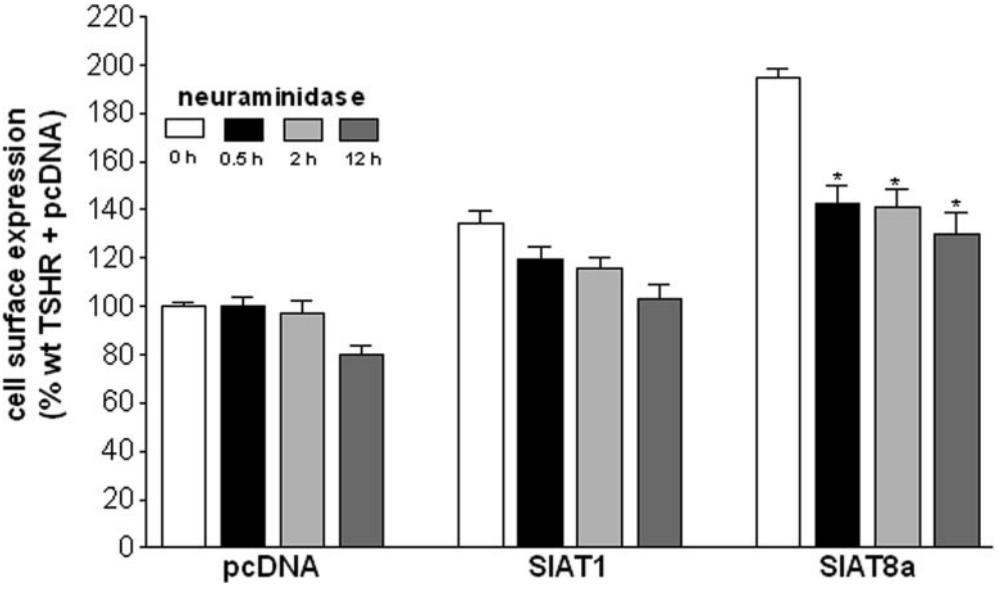

Fig. 4. Cell-surface expression of wt TSHR measured by flow immunocytometry using a mouse anti-human TSHR antibody. pSVL-TSHR cotransfected with empty pcDNA3.1 vector (lanes 1–4), pcDNA3.1-SIAT1 (lanes 5–8), or pcDNA3.1-SIAT8a (lanes 9–12). □, untreated COS-7 cells; shaded bars show neuraminidase-treated COS-7 cells (30 min to 12 h). Neuraminidase treatment significantly reduces cell-surface expression of TSHR cotransfected with pcDNA3.1-SIAT8a compared with untreated cells (★, $p < 0.05$). Details are described under *Materials and Methods*. All data are presented as means ± S.E.M. of two independent experiments, each performed in duplicate.

AFTNs versus surrounding tissue (Eszlinger et al., 2004). To extend this study, we determined SIAT1, -4a, and -8a expression in AFTNs and CTNs, two frequent thyroid pathologies that most likely represent opposite pathophysiological states of thyrocyte activation and differentiation (Fig. 1). Because regulation of sialyltransferase expression was evident in AFTNs but not in CTNs, we performed in vitro investigations with TSH-stimulated primary cultures that very likely model the type of activation seen in AFTNs. Regulation of SIAT1 and SIAT8a in AFTNs is consistent with TSH stimulation in primary thyrocytes. This very likely indicates that functional activation of thyrocytes through signaling downstream of the TSH receptor affects SIAT1 and -8a expression in vivo and in vitro. Because mRNA expression of SIAT1 is up-regulated and mRNA expression of SIAT8a is down-regulated in AFTNs, it is possible that the outcome for the total sialic acid load of the TSHR could be neutral. In contrast, SIAT4a expression is only altered after TSH stimulation of primary thyrocytes but not in thyroid nodules.

Cotransfection of each of the three sialyltransferases (i.e., 1, 4a, and 8a) together with the TSHRs increased cell-surface expression of the TSHR, as shown by FACS analysis, ELISA and Western blotting. Western blotting suggests a considerably higher TSHR cell-surface expression after cotransfection of SIAT8a compared with SIAT1. However, for exact quantification, chemiluminescence detection of Western blots is inferior to FACS analysis. It is therefore more likely that the difference of the effect of SIAT1 and -8a on TSHR cell-surface expression is rather minor. Moreover, no synergistic effect of SIAT1, -4a, and -8a could be detected in FACS analysis and radioligand binding assay (data not shown), which further supports the notion that the type of glycosidic binding plays a minor role compared with the sialic acid load itself. As a practical consequence, we did not use every SIAT isoform in all experiments, also because specific lectins were not available for every isoform. Consequently, increased surface expression of the TSHR induced by sialyltransferases increases the number of binding sites on the cell surface and thereby improves the binding and signaling of TSH. However, we did not detect an increased affinity of the TSHR for TSH, which corresponds to the previous finding that carbohydrates do not directly affect TSH binding (Atger et al., 1999; Nagayama et al., 2000).

To investigate whether increased cAMP and IP accumulation and TSH binding are direct effects of the increased cell-surface expression, we decreased the cell-surface expression of the TSHR by cotransfection with SIAT1 to the level of the TSHR cotransfected with the empty pcDNA vector, using the V2-vasopressin receptor in a triple transfection. Subsequent binding assays normalized for TSHR expression as described above showed no differences between TSH receptors with different amounts of sialic acids. Therefore, the increased B_{max} values after cotransfection of the TSHR with sialyltransferases are more likely a direct effect of the increased cell-surface expression than an effect caused by a higher affinity to TSH.

To investigate whether the improved cell-surface TSHR expression after cotransfection with sialyltransferases is a direct effect of sialylation of the TSHR, we studied Western blots of the TSHR after cotransfection in COS-7 cells. Western blotting of untreated human TSHR showed a characteristic doublet at 100 and 120 kDa (Oda et al., 1999), whereas the peptide chain molecular mass of the TSHR is 84 kDa (Libert et al., 1989; Nagayama et al., 1989; Parmentier et al., 1989; Frazier et al., 1990; Misrahi et al., 1990). Previous reports indicate that glycosylation of the TSHR is responsible for the higher molecular masses observed on Western blotting (Misrahi et al., 1994; Rapoport et al., 1996). In recent studies, Oda et al. (1999) showed that treatment of the receptor with neuraminidase, which removes sialic acids (Ta-

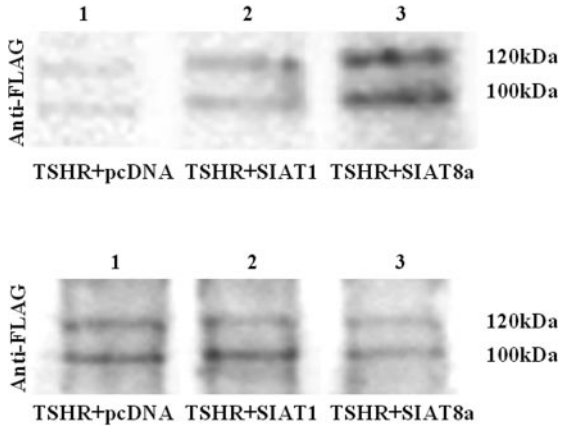

Fig. 5. Western blotting analysis of pSVL-TSHR-FLAG cotransfected with the empty pcDNA3.1 vector (lane 1), pcDNA3.1-SIAT1 (lane 2), or pcDNA3.1-SIAT8a (lane 3) detected with anti-FLAG antibody. Protein extract containing membrane fraction (top) or total cell lysate (bottom). Western blotting analysis has been repeated twice with similar results.

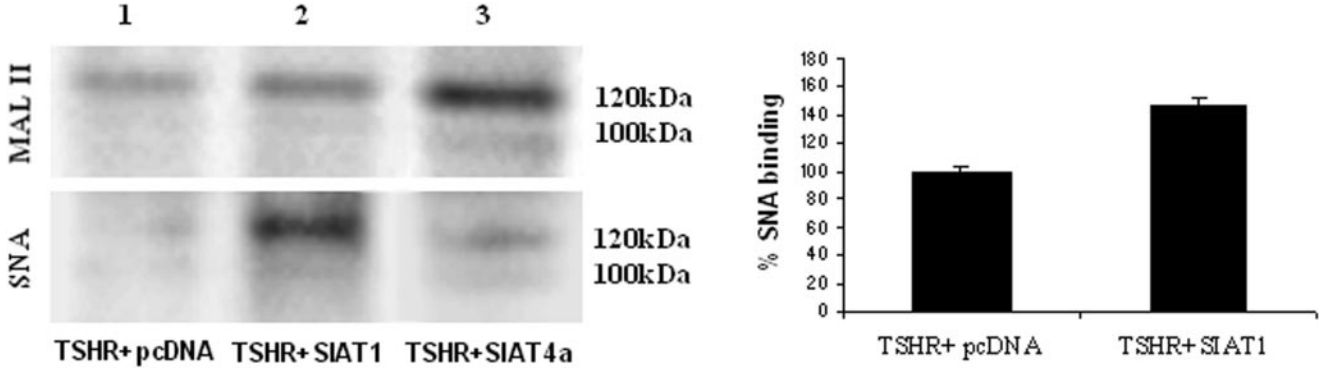

Fig. 6. Left, lectin blotting analysis of pSVL-TSHR-FLAG immunoprecipitate with anti- FLAG antibody bound to protein A/G agarose, cotransfected with the empty pcDNA3.1 vector (lane 1), pcDNA3.1-SIAT1 (lane 2), or pcDNA3.1-SIAT4a (lane 3) detected with MALII (top) or SNA (bottom). Right, lectin binding ELISA of TSHR-GFP cotransfected with the empty pcDNA3.1 vector or with pcDNA3.1-SIAT1 detected with SNA. Western blotting analysis and ELISA were repeated twice with similar results.

rentino et al., 1985), caused the 120-kDa TSHR band to run as a 110-kDa band. However, the 100-kDa band remained unaffected, which suggests that only the 120-kDa band carries sialic acids. Selective binding to the upper TSHR band of the lectin MALII (Fig. 6A) (Oda et al., 1999), which is known to bind to carbohydrate structures containing α(2,3)-linked sialic acids (Wang and Cummings, 1988), further confirms the 120-kDa band as the sialylated TSHR entity.

To clarify in detail which sialyltransferase modifies the TSHR, we also carried out Western blotting using lectins specific for different carbohydrates. After cotransfection of the TSHR with SIAT1, the 120-kDa band of the TSHR was found to react with SNA, which is specific for carbohydrate structures containing α(2,6)-linked sialic acids (Wang and Cummings, 1988). A lectin-binding ELISA was used to confirm this result. Indeed, we detected a stronger SNA signal after cotransfection with SIAT1 compared with the empty pcDNA vector. This result clearly confirmed that the TSHR is a substrate of the SIAT1. We also found that MALII, which is specific for carbohydrate structures containing α(2,3)-linked sialic acids (Wang and Cummings, 1988), binds to the 120-kDa band of TSHR after cotransfection of the TSHR with SIAT4a. This result confirmed that the TSHR is a substrate of both the sialyltransferases, 1 and 4a.

The transfection of sialyltransferases might have some impact on the exit of sialyl-glycoproteins from the trans-Golgi network to plasma membrane. To answer the question of whether sialylation causes a more effective transfer of the TSHR to the cell surface or a longer resting time on the cell surface, we treated TSHR/SIAT1 and TSHR/SIAT8a cotransfected COS-7 cells with neuraminidase to remove sialic acid residues. After the neuraminidase treatment, the cell-surface expression of TSHR decreased. This suggests that sialylation of the TSHR not only improves but also prolongs its resting time on the cell surface.

Our finding of increased SIAT1 mRNA expression in AFTNs and in primary thyrocyte cultures after TSH stimulation, together with an increased cell-surface expression of the TSHR after SIAT1 cotransfection, which in turn increases TSH radioligand binding, underlines the importance of sialylation as one form of post-translational TSHR modifications. Our results suggest that sialylation is likely to be a new mode of regulating the receptor cell-surface presence.

Recent reports controversially discussed the localization of the TSHR to lipid rafts (Latif et al., 2003; Costa et al., 2004; Latrofa et al., 2004). Moreover, a ganglioside component belonging to the G_{M1} family, found in lipid rafts, has been reported to interact with the TSHR α-subunits and is considered to be an integral part of the purified TSHR (Kielczynski et al., 1991). Our finding that sialylation can modulate the surface expression of the TSHR could also impact on its lipid raft localization. Our future studies will therefore also focus on TSHR surface compartmentation as a response to sialylic acid load.

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