The thyrotropin receptor in thyroid carcinoma

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Chapter 4

Superior Thyrotropin Receptor Binding and Activation of a Novel, Modified, Single Chain Thyroid Stimulating Hormone

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ABSTRACT

We describe the design of a modified single chain TSH molecule which is capable of potently stimulating the TSH-receptor. Furthermore, the enhanced stimulating effect is maintained after the fusion of small proteins to the N-terminus indicating that our modified single chain TSH may be able to function as a carrier of proteins.

TSH subunits α and β were cloned from a human pituitary tumor, amplified with overlapping primers and fused in a second PCR resulting in a β-α orientated TSH with an intact β-chain secretion signal. In order to create a superactive scTSH, we introduced several mutations in the scTSH. We tested the properties of scTSH for binding to and activation of the TSH receptor using a TSHR expressing CRE-Luc modified CHO cell-line and the relevant biological endpoint of iodine uptake by FRTL-5 cells. We subsequently fused a 6xhistidine tag with flexible linker, alone and in combination with a six amino-acid sequence to the N terminus of scTSH. We found that the modified scTSH has superior TSHR binding and activation properties in comparison with wtTSH which lead to an increased uptake of radioiodine by FRTL-5 cells. Addition of a 6xhistidine tag with a flexible linker with and without a small protein extension did not compromise these properties.

A possible application of modified TSH may lie in the specific targeting of metastases derived from differentiated thyroid carcinoma (DTC). The success of conventional radioiodine therapy in metastases of differentiated thyroid carcinoma (DTC) is limited by insufficient uptake of radioiodine. Therefore, new therapeutic strategies are needed. The retained expression of the receptor for thyroid stimulating hormone (TSHR) in most advanced DTC tumors offers a perspective for a targeted approach. The development of this modified TSH may be the first step in the development of a carrier-protein for cytotoxic drugs capable of specifically targeting thyroid tumor cells through the TSHR.

INTRODUCTION

Thyroid stimulating hormone (TSH) is a 28- to 30-kDa member of the glycoprotein hormone family, which also includes chorionic gonadotrophin (CG), luteinising hormone (LH) and follicle stimulating hormone (FSH). These hormones consist of a common α- and a hormone-specific β-chain, which are non-covalently linked (132;137;241). TSH which is synthesized and secreted by the pituitary regulates thyroid hormone production in the thyroid gland and is essential for thyroid hormone homeostasis in target organs by the classical pituitary-thyroid feedback loop. In addition, TSH inhibits its own secretion by an ultra short negative feedback loop within the pituitary gland (132;242). Both the α- and β-chain of TSH are important for binding to, and activation of the TSH receptor (TSHR). The α- chain is identical for all the members of the glycoprotein-hormone family and consists of 92 amino acids whereas the 118 amino acid beta chain is unique to TSH and determines specificity (132;137;138). The TSHR, as well as other proteins involved in iodine metabolism and thyroid hormone production, are largely specific for the thyroid and thyroid derived tumors. For decades, one of these unique features, i.e. the expression of the sodium iodine symporter (NIS), has been successfully used in the treatment of thyroid tumors by using radioactive iodine therapy, since through the presence of NIS, iodine selectively accumulates in thyroid tissue. In part because of the success of radioiodine therapy, thyroid cancer has an excellent prognosis. However, in a subset of patients, undifferentiated thyroid tumors, and more particularly distant metastases, have lost this unique characteristic. In these patients, radioactive iodine therapy is ineffective and new treatment modalities are needed. The specificity of TSH for the TSHR may be used for delivering of drugs specifically to the tumor cells, for example by using TSH as a carrier of fusion proteins or cytotoxic drugs. Although TSHR expression is lost in poorly differentiated thyroid carcinoma, TSHR expression is more persistent than other thyroid specific proteins. This has been demonstrated by the presence of TSHR expression in a large panel of thyroid carcinomas by immunohistochemistry (155;156). Before TSH can be used as a selective carrier of toxic proteins to thyroid carcinomas, various problems need to be solved. Wild type (wt) TSH is produced as separate alpha and beta chains and assembly of these alpha and beta chains is a rate limiting step in TSH formation. This may be a problem in the pharmaceutical production of recombinant TSH. Furthermore, TSH is posttranslationally modified by glycosylation, which is important for both hormone stability and bioactivity. This posttranslational glycosylation is another problem in the production of recombinant fusion proteins (170;171). At least part of these problems can be circumvented by the production of TSH as a single chain hormone (scTSH), in which the beta-chain is fused to one side of the alpha chain. Furthermore, a scTSH has improved hormone stability and increased serum half-life. This stabilizing effect of scTSH also compensated mutagenesis-induced defects in TSH that impaired dimer formation (172). The possibility to use these favourable characteristics of scTSH over wtTSH in creating a carrier of fusion proteins or cytotoxic drugs to thyroid carcinomas has not been explored in much detail.

Much of the success of TSH as a carrier of drugs depends on its specificity for thyroid derived cells and its affinity for the TSHR. The conversion of TSH to a scTSH alone does not result in a more potent TSH (168;243). However, specific mutations in the hairpin loops βL3 and αL3 improve binding dramatically in recombinant TSH consisting of a non-covalently bound α

and β chain (168;243). Whether the incorporation of these mutations in a scTSH also results in improved TSHR binding characteristics is unknown.

MATERIALS AND METHODS

CELL-LINES

We used TSHR expressing JP26-26 cells (151;194), stably transfected with a cAMP responsive element (CRE)-luciferase construct (TSHR-luc cells) as a read out for TSHR activation (244). B1 and CHO cells were routinely cultured in Ham's F12 medium (Gibco BRL, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS; Integro BV, Zaandam, The Netherlands), 100 IU/ml penicilline (Life Technologies, Rockville, USA) and 100 µg/ml streptomycin (Life Technologies, Rockville, USA). TSHR and CREe-luciferase in TSHR-luc cells were maintained by addition of 2µg/ml blasticidin and 400µg/ml geneticin (Life Technologies, Rockville, USA).

RECOMBINANT HUMAN TSH

Recombinant human TSH used as a comparison to our TSH constructs was obtained from Fitzgerald industries (Fitzgerald Industries, Concord, USA)

PITUITARY cDNA

Total RNA was extracted from a pituitary tumor using Trizol LS reagent (Invitrogen, Carlsbad, CA, USA) and treated with RQ-DNAse (Promega, Madison, USA) to remove genomic contamination. RNA was reverse transcribed into cDNA using the Superscript First-Strand Synthesis System for RT-PCR in the presence of oligo$(dT)_{12\text{-}18}$ according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA).

CONSTRUCTION MSC-TSH BY PCR

All PCR reactions were done using the proofreading polymerase Pfu-ultra high fidelity (Stratagene, La Jolla, CA). The TSH α- and β-subunits were amplified by PCR from pituitary cDNA and subsequently fused in a second PCR using overlapping primers at the TSH beta terminus and TSH alpha start site removing the TAA stopcodon of the β-chain and the ATG startcodon of the α-chain. After the introduction of HINDIII and EcoRI restriction sites at the flanking regions (Figure 1) the single chain TSH construct was inserted into the expression vector pcDNA3.1 and sequence verified. Subsequently the mutations N66K (N(AAC) > K(AAG)), I58R (I(ATC)>R(CGC)) and E63R (E(GAA)>R(CGG)) were introduced into the L3 loops of the alpha and beta chain by overlapping primers resulting in a modified single chain TSH (Figure 2).
After the creation of the modified single chain TSH (mscTSH) six histidine residues were placed directly behind the β-chain secretion signal followed by 12 base pares coding for

a flexible linker (GAGG) with and without an additional 27 base pares using overlapping primers.

TABLE 1. Primers used for the conversion of wtTSH into a modified single chain TSH

	primer	primer sequence
Conversion to single chain TSH		
Introduction HindIII restriction site	1 fw	atatatat **aagctt** gccaccatgactgctctctttctgatgtc
Elimination stop codon β-chain	2 rev	cacatcaggagcgacagaaaatcctac
Elimination secretion signal+ stop α-chain	3 fw	ggattttctgtcgctcctgatgtgcag
Introduction EcoRI restriction site	4 rev	atatatat **gaattc** ttaagatttgtgataataacaagtact
Modifications		
I(ATC)> R(CGC)	5 rev	tacagtcctgta **gcg** gaagt
E(GAA)> R(CGG)	6 fw	acaggactgta **cgg** atacca
N(AAC)> K(AAG)	7 rev	ccattactgtgaccct**ctt**atatgatt
N(AAC)> K(AAG)	8 fw	**aag** agggtcacagtaatgggggtttc
Extensions		
His tag	rev	accagcaccatggtgatggtgatgatgagacatcgcttgcccacatg
	fw	caccatcaccatggtgctggtggcttttgtattccaactgagtata
His tag+extension	fw	caccatcaccatggtgctggtggctgttgtcctggctgttgcggtggcggc ttttgtattccaactgagtata

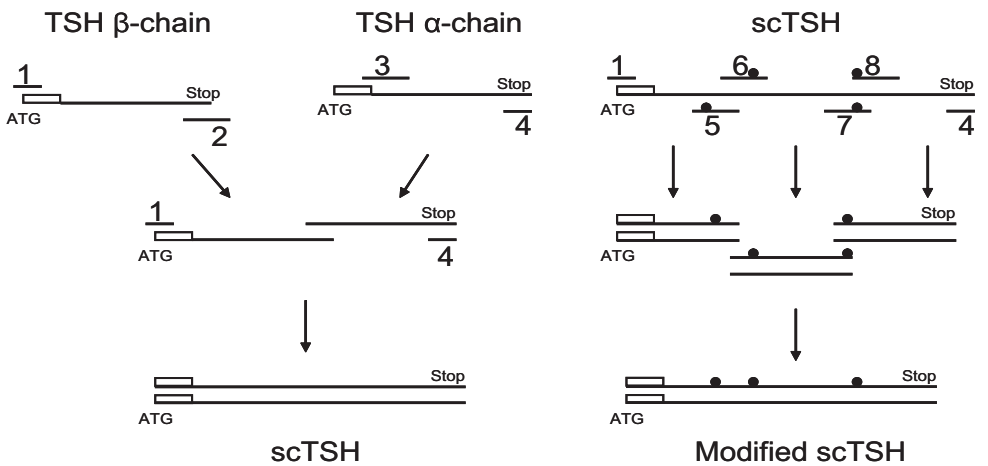

FIGURE 1. PCR scheme of the creation of modified single chain TSH constructs for insertion into the expression vector pcDNA3.1. The primer numbers correspond with primers depicted in Table 1. (A) The β chain with intact secretion signal minus termination signal was directly fused to the α chain removing the secretion signal in a two step PCR. (B) Mutations were introduced by modified primers (Table 1) resulting in three fragments which were subsequently fused in a PCR reaction using overlapping sequences.

STABLE TRANSFECTION OF CHO WITH MSCTSH

CHO cells were seeded at a density of $1,0*10^4$ cells/cm2 in a 6-wells plate and were incubated overnight before transfection. The next day the cells were transfected using Fugene 6 transfection reagent according to the manufacturer's instructions (Roche, Basel, Switzerland). The lipid–DNA complex was prepared by mixing 105 µl α-MEM and 2,5 µl fugene with 1µg pcDNA3.1-mscTSH. The mixture was incubated for 15' at room temperature and the lipid/DNA mix was added to the cells drop by drop. The cells were incubated for 8 h at 37°C/5%CO2 with the lipid–DNA complex, the medium was refreshed and 400µg/ml geneticine (Life Technologies, Rockville, USA) was added. Cells were incubated for 4 days at 37°C/5%CO_2 in an incubator and stably transfected clones were isolated. Conditioned medium which had been in contact with the mscTSH expressing cells for 3 days was tested for TSHR activating properties using the TSHR/CRE-luc transfected cell-line B1. Subsequently TSH levels were measured with the electrochemiluminescence immunoassay (Roche Diagnostics, Indianapolis, USA) on the Modular Analytics E-170 (Roche Diagnostics, Indianapolis, USA).

CLONE SELECTION BY THE LUC BIOASSAY FOR TSH RECEPTOR ACTIVATION

B1 cells were seeded at a density of $2,5*10^4$ cells per well in 24 well plates in normal medium supplemented with blasticidin and incubated at 37˚C/5%CO_2 for 24 h followed by an interval in minimal medium (Ham's F12 medium supplemented with 0,5 % BSA). After 4h B1-cells were stimulated with conditioned medium from mscTSH producing cells. Luminescence was measured after 20h with the Luciferase Reporter assay system (Promega, Madison, USA) according to the protocol. Ten microliter of cell lysate was assayed for firefly luciferase using the Wallac 1450 Microbeta Trilux luminescence counter (Perkin–Elmer, Boston, MA, USA).

PURIFICATION 6XHIS TAGGED MSCTSH BY NICKEL AFFINITY GEL

Purification of his-tagged proteins was done using HIS-Select™ Nickel Affinity Gel according to the manufacturer's protocol (Sigma-Aldrich Biotechnology, Saint Louis, USA)

IODINE UPTAKE

For I-uptake experiments, cells were grown in 12-well plates. Cells were incubated O/N (15h) after addition of 10mU/l of the TSH variants at 37 °C in a humidified atmosphere. Prior to the uptake studies, the cells were washed three times in Hanks' Balanced Salt Solution (HBSS), buffered with 10 mm Hepes (pH 7·5) Thereafter, HBSS containing 20 µm Na125I with a specific activity of 100 mCi/mmol was added to the cells and incubated for 30 min with the radioactive solutions.
The reaction was terminated by aspirating the radioactive mixture and cells were washed three times with ice-cold HBSS. Accumulated 125-I was determined by permeabilizing the cells with 500 µl ethanol for 20 min at 20 °C and measuring the released radioisotope in

a gamma counter. The DNA content of each well was subsequently determined after trichloroacetic acid precipitation by the diphenylamine method (245). Based on the specific activity of the substrates, the efficiency of the gamma counter and the DNA content of each well, iodide uptake was expressed as picomoles of substrate transported per microgram of DNA or as percentage of control conditions.

STABILITY

Recombinant hTSH and the mscTSH constructs were stored in CHO-II-SFM medium for 0, 1, 2 and 4 days at 37- and 56°C. TSHR stimulating activity at these time points was determined with the Luc-bioassay.

COMPETITION ASSAY

To measure binding competition we used a modified version of the Medizym T.R.A. (Medipan, Berlin, Germany) which is a competitive enzyme immunoassay test for autoantibodies to the TSHR. Here, we abandoned the step where patient serum should be added and instead we added our mscTSH directly to the TSH complex for competition. Subsequently, the protocol was followed according to the manufacturer (Medipan, Berlin, Germany). The optical density was measured at 450 nm versus 690 nm within 20 min after adding the stop solution.

STATISTICAL ANALYSIS

Results are expressed as the mean plus or minus the standard error of mean. Student's t-tests were used for all hypotheses testing. All statistical analysis was performed using graphpad prism (Graphpad software, inc., San Diego,USA)

RESULTS

DESIGN AND CONSTRUCTION OF MSCTSH

Total mRNA was isolated from a human pituitary tumor and converted to cDNA. TSH subunits α and β were amplified with overlapping primers and fused in a second PCR resulting in a β-α orientated TSH with an intact β-chain secretion signal. The sequence of the single chain TSH was verified and subsequently mutations were introduced into the alpha and beta chain resulting in a modified single chain TSH. Furthermore the accessible N-terminus of mscTSH was modified by inserting a 6xHis tag and 4-13 aminoacids in between the cleavage signal and the mature protein (Figure 2). Following construction all constructs were sequence verified.

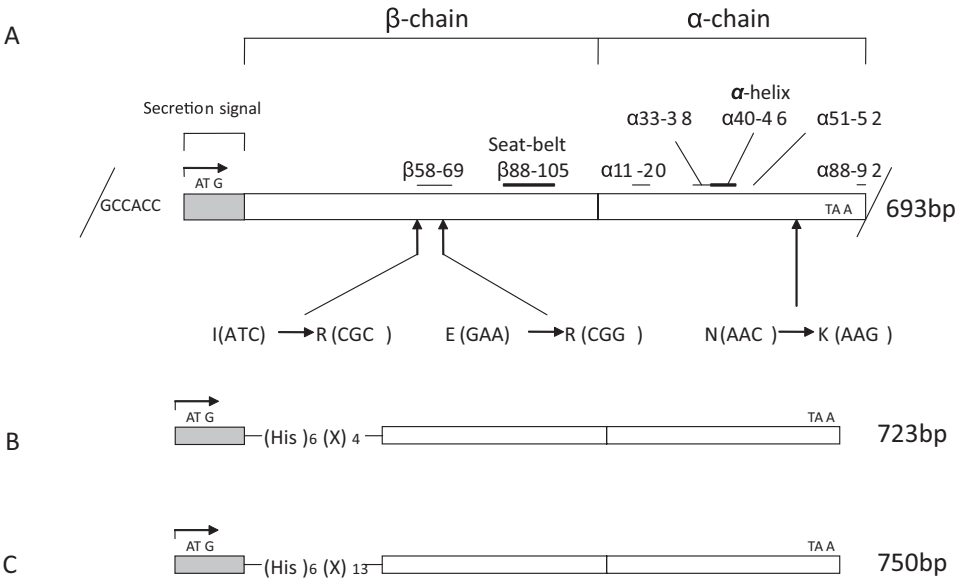

FIGURE 2. Modified single chain TSH constructs for insertion into the expression vector pcDNA3.1. (A) The β chain with intact secretion signal minus termination signal was directly fused to the α chain lacking the secretion signal. Modifications of TSH are depicted by arrows. Furthermore, structural features within TSH which have been shown to be important for binding to and activation of the TSH receptor (168;169) are depicted above the construct. The numbers represent the position of the modified aminoacids. The modified N184 corresponds with N66 in the native α chain. (B) Six histidine residues were placed directly behind the β-chain secretion signal followed by either 4(B) or 13(C) aminoacids coding for a flexible linker (GAGG).

EXPRESSION OF THE CONSTRUCT

scTSH and mscTSH expression constructs were stably transfected in CHO cells. For each construct 8 clones were picked and the presence of TSH was measured in conditioned

medium. In none of the scTSH transfected clones, TSH protein was detected above control levels. In contrast, in conditioned medium of the mTSH transfected clones tested, TSH was detected (Figure 3) suggesting beneficial effects of the point mutations on protein expression and/or secretion into the medium. Subsequent experiments were performed with the mscTSH construct.

Following the creation of a modified scTSH we examined the feasibility of TSH-fusion proteins. Therefore, we generated expression constructs containing extended mscTSH constructs as depicted in Figure 2. mRNA expression levels of the TSH constructs in stably transfected CHO clones were comparable (Figure 4). To optimize production mscTSH transfected cells were grown in CHO-II-SFM resulting in an amount of approximately 4mU/l TSH excreted in the medium.

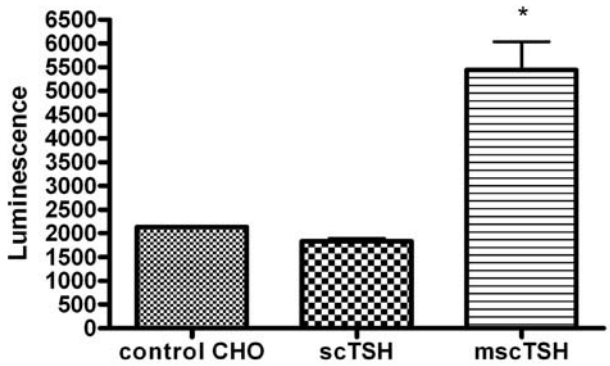

FIGURE 3. TSH-receptor stimulation by conditioned medium of 8 stably transfected CHO cell-lines per construct: 1) non transfected cells, 2) scTSH, 3) mscTSH. TSHReceptor activation increase by mscTSH when compared to control was significant (t-test; p=0,0417)

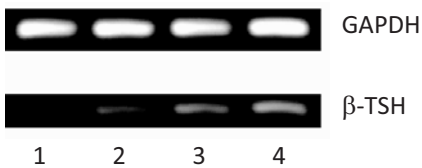

FIGURE 4. mRNA expression levels in stably transfected CHO clones: 1) pcDNA3.1 only, 2) mscTSH, 3) 6xHis-mscTSH and 4) 6xHis-13X-mscTSH.

TSHR ACTIVATION BY THE MODIFIED SINGLE CHAIN TSH CONSTRUCTS

TSHR activation of the modified single chain TSH constructs was compared with commercial recombinant human TSH (Fitzgerald industries, USA) using the TSHR/CRE-luciferase transfected B1 cell-line. All mscTSH constructs had similar activities being 20 fold more potent than commercial recombinant human TSH at 20mU/l (Figure 5).
The Presence and accessibility of the 6xHis tag was confirmed by His-gel purification (Figure 6) showing binding and elution of His tagged TSH constructs whereas mscTSH lacking the 6XHis tag did not bind to the gel.
Binding of mscTSH to the TSHR receptor was tested with a modified version of the Medipan kit and showed a 10fold improved binding to the receptor when compared to rhTSH.
In order to test if modified TSH is still biological active we tested mscTSH for its ability to promote iodine uptake in FRTL-5 cells (Figure 8). mTSH not only was biologically active but showed increased ability to promote I-uptake when compared to rhTSH.

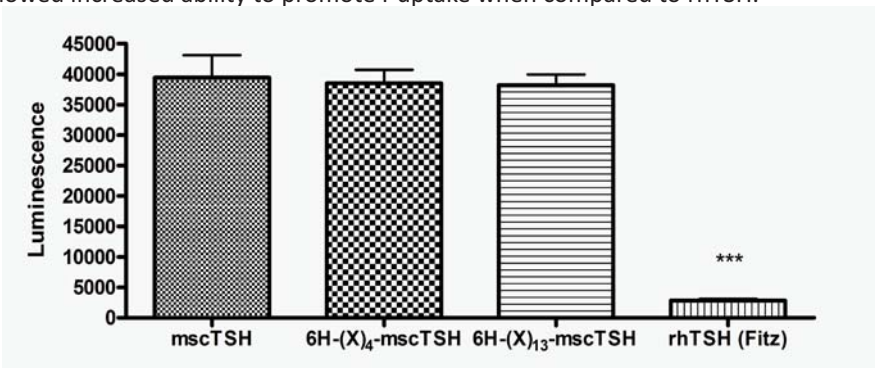

FIGURE 5. TSHR activation by the mscTSH constructs and rhTSH as measured with our TSHR activation assay at a TSH concentration of 20mU/L (Hovens, 2006). T-test (P<0.0001)

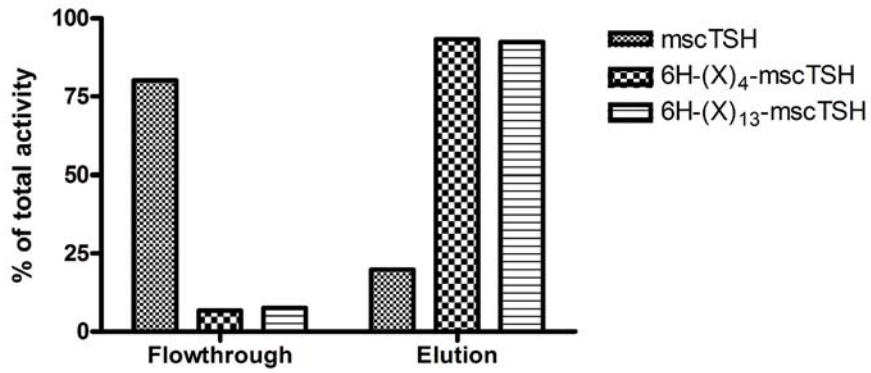

FIGURE 6. TSHR activation by conditioned medium of CHO cells expressing the three TSH constructs as measured with the B1-luc assay 1) mscTSH, 2) 6xHis-mscTSH and 3) 6xHis-13N-mscTSH in non binding-(flowthrough) and binding fractions (after elution) to His tag affinity gel. Results are shown as % of total TSHR activation after addition of medium that had been conditioned for two days.

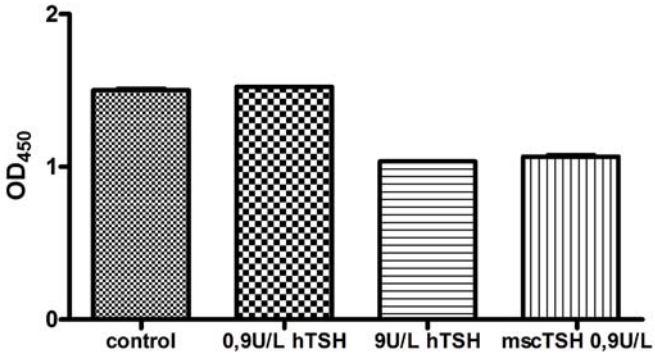

FIGURE 7 Binding of rhTSH and mscTSH to the TSHR as measured with the modified medizym TRA kit. Added TSH competes with TSH conjugate of the kit for binding to the TSHR. Improved binding results in lower levels of bound TSH-conjugate and consequently lower OD_{450}.

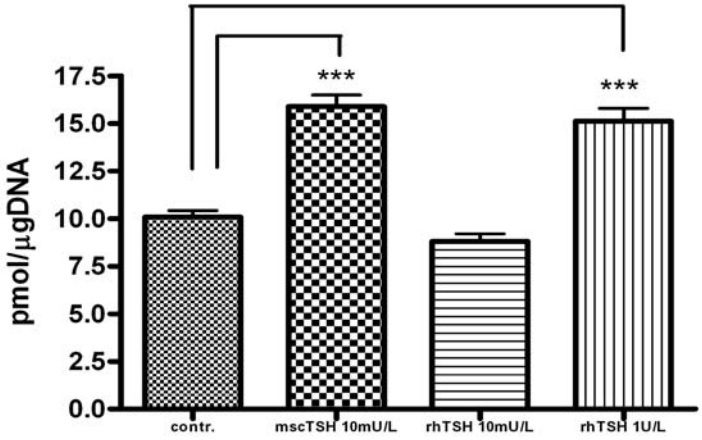

FIGURE 8. Iodine uptake after TSH stimulation was corrected for DNA content and is depicted as pmol/ugDNA. Conditions were as follows: 1) control lacking TSH, 2)mscTSH: modified scTSH at 10mU/L and 3) rhTSH: recombinant human TSH at 10 and 1000mU/L. I-uptake after stimulation with 10mU/L mscTSH significantly increased when compared to control (t-test; p<0,0001) whereas rhTSH did not stimulate I-uptake at 10mU/L. A 100-fold increase of the rhTSH concentration resulted in an I-uptake comparable to mscTSH at 10mU/L (t-test; p=0.0003)

STABILITY OF MSCTSH

Stability of the single chain constructs was tested for up to four days at 37 and 56°C.
Both the rhTSH and single chainTSH variants displayed loss of biological activity at 37ºC (Figure 9A), expressed as luciferase reporter activation in our TSHR activation assay (244). All mscTSH constructs displayed a higher stability than rhTSH as approximately 50% of scTSH and 25% of rhTSH activity remained after 48h. The presence of a His tail with and without a

protein extension did not impair stability of the TSH constructs.
In addition to the effect we found at 37ºC, we saw a sharp decrease of activity at 56ºC with approximately 25% of activity remaining in all TSH constructs after 24h, while activity was almost completely abolished after 48h.

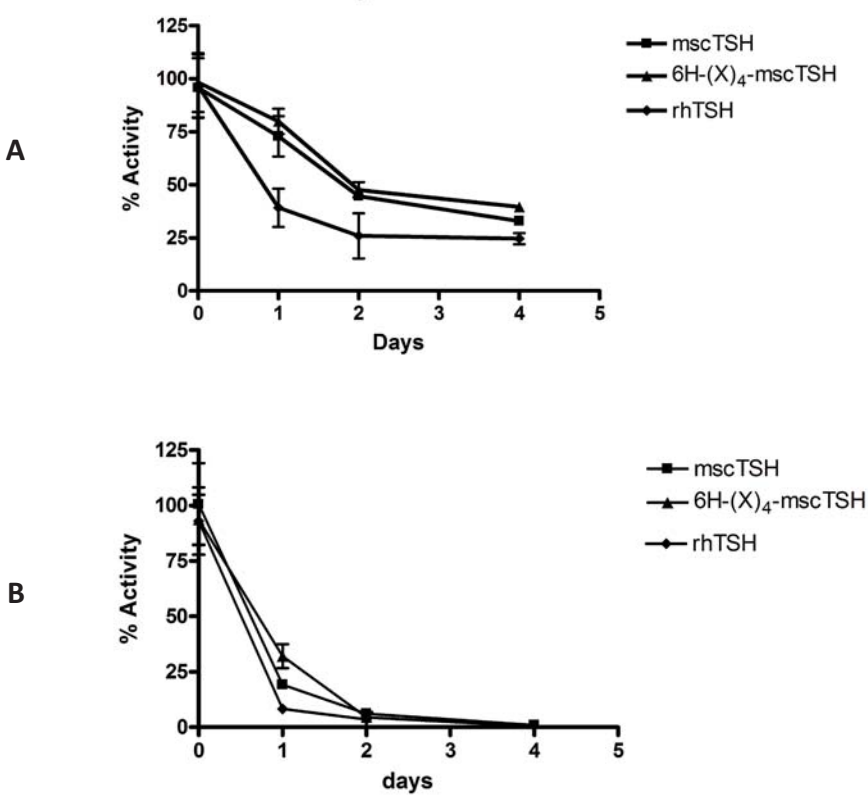

FIGURE 9. Stability of (His)-modified single chain TSH compared to rhTSH at 37°C(A) and 56°C(B). Remaining activity of the TSH variants was measured with the B1Luc-bioassay. Activity is displayed as % of total activity at t=0.

DISCUSSION

In this manuscript we describe the design of a modified single chain TSH molecule, which has powerful TSH stimulating effects. We aimed to develop a protein capable of specifically targeting the thyroid, and, more specifically, thyroid tumor cells. As the TSHR is almost exclusively expressed in thyroid tissue and its expression is maintained in various thyroid tumors (155;156) the TSHR is an interesting target for directing proteins (e.g. toxins) to the

thyroid or thyroid tumor cells. As TSH is the natural ligand of the TSHR, a TSH-fusion product would in theory be able to bind exclusively to TSHR bearing cells. After binding, the TSH fusion protein could be transported into the cell, which is essential for certain applications such as a TSH-toxin fusion protein.

STABILITY

By fusing the beta and alpha chain of TSH we intended to create a more stable protein (168;243). Furthermore, the fusion of the subunits bypasses the rate limiting assembly step, which is essential for secretion, and hormone specific glycosylation of TSH (170;171). Using an immunoassay specific for heterodimeric TSH, Grossmann et al. showed previously that single chain TSH as well as rhTSH were stable at 37C for at least 21 days, while mscTSH was significantly more stable than hTSH at 55C (172). In contrast, we found degradation of both the rhTSH and single chain TSH at 37ºC, when using our TSHR activation assay (244). The mscTSH constructs displayed a higher stability than rhTSH, as approximately 50% of mscTSH and 25% of rhTSH activity remained after 48h. In contrast to the effect we found at 37ºC, there was a sharp decrease of activity at 56ºC with approximately 25% of activity remaining in all TSH constructs after 24h, while activity was almost completely abolished after 48h. These contradictionary effects of temperature on TSH stability between the study of Grossmann et al. and our study may be due to the different methods used for measuring stability. In the study by Grossmann et al. the results were based on an immunoassay specific for heterodimeric TSH rather than on biological activity, whereas our method is based on actual TSHR activating properties. This discrepancy between the two studies suggests that loss of activity may not be directly linked to dissociation of the subunits but may occur prior to this event.

IMPROVING TSH

When using scTSH as a vehicle to guide components to, and into, TSHR bearing cells improved binding to, and activation of, the TSHR is likely to improve specificity and internalization of the scTSH in vivo, as the TSHR internalization rate increases 3-fold after activation (149). In order to create a super-active scTSH we introduced several mutations in our single chain TSH, known to improve rhTSH binding to the TSH-receptor (168;169;172). We tested the properties of our modified scTSH for binding to, and activation of, the receptor and the relevant biological endpoint of iodine uptake. Both binding to, and activating of, the TSH-receptor by mscTSH were improved when compared to commercially available rhTSH, by respectively 10 and 20 fold.

One possible application of super agonistic TSH analogues may lie in improved ^{131}I treatment. Radioiodine ^{131}I is routinely used in the management of thyroid cancer for treatment and diagnostic purposes. As TSH stimulates ^{131}I uptake, patients used to be treated with thyroid withdrawal protocols to increase TSH levels. In recent years recombinant hTSH has become an alternative and phase III trials have demonstrated that rhTSH treatment is nearly or as effective in stimulating ^{131}I uptake as traditional methods (3;246). In FRTL-5 cells our mscTSH was almost twice as effective in inducing ^{131}I uptake compared to rhTSH, making mcTSH a

potential candidate for inducing more efficient ^{131}I uptake also in vivo.
Direct labelling of mscTSH with a radioactive ligand may be another feasible application, especially when distant metastases are involved, which sometimes lose the ability for iodine, and thus ^{131}I, uptake but maintain TSHR expression (155;156).

FUSION PRODUCTS

We wanted to know whether it would be possible to fuse a protein to our hyperactive single chain TSH and still maintain biological activity. As a model for mscTSH fusion proteins, we fused a 6xhistidine tag with flexible linker, alone and in combination with a six amino-acid sequence to the N terminus of mscTSH since the α-carboxy terminus (α 88-92) is unavailable for binding due to its critical role in TSHR binding and activation (138;173). Use of a nickel gel purification step confirmed the presence, and the accessibility, of the 6xHis tag.
We subsequently tested the biologic potential of this 6xHis tagged with and without the small protein extension mscTSH constructs with our bio-luc assay and found that the full TSHR stimulating potential was maintained. Furthermore, the addition of a His tag and the small protein extension to the mscTSH construct did not impair the stability, when compared to the single chain TSH. This suggests, that the conformation of mscTSH was not dramatically influenced by the additional extension on the N-terminus.
The maintained TSHR activating potential of TSH is essential for a formed TSH-TSHR complex to be internalized into the TSHR bearing cell. As the His-mscTSH and His-13X-mscTSH fusion products still possess the full potential of the modified single chain TSH, it is feasible that our mscTSH is able to guide proteins into the thyroid and thyroid tumors in vivo.
After TSHR activation the normal route of TSH leads to the lysosymes. Triggered by the activation of the receptor the TSH-TSHR complex is internalized through clathrin-coated vesicles followed by the recycling of the majority of receptors to the surface and degraded of TSH by lysosomes (149). In theory, this mechanism would enable TSH bound components, e.g. toxins, to enter thyroid (tumor) cells expressing the TSHR, because various toxins of bacterial origin (e.g. pseudomonas exotoxin (PE), Diphteria toxin (DT), Ricin, Shiga toxin use the lysosomal route to kill eukaryotic cells and it is likely that they, when fused to mscTSH, would be able to follow their normal route into the target cell (174;183;247) .
The normal cell binding domain of these toxins can be replaced with a different binding domain and possibly with our mscTSH. Within the group of toxins the ones with a cell binding domain on the carboxy terminal side will be best compatible with our mscTSH as the carboxy terminus (α 88-92) of mscTSH is unavailable due to its crucial role in TSHR binding and activation (241;248).
Other applications of a TSH fusion protein may lie in the field of diagnostics. Our mscTSH may be able to guide markers towards TSHR bearing cells. However, for diagnostic purposes internalization of mscTSH may not be needed, or even be undesired, and a receptor blocking TSH molecule would be more favourable. The introduction of novel mutations, which abolish oligosaccharide chain formation, might be able to achieve this goal (249). In this way it could be possible to attach markers to the surface of TSHR bearing cells without risking degradation.

A limitation of the use of cytotoxic TSH molecules, is the presence of the TSHR in non-thyroid tissues. A number of papers have reported the prevalence of TSHR mRNA and/or protein in non-thyroid tissues such as lymphocytes, thymus, pituitary, testis, kidney, heart and orbital tissues (157;158). Although TSHR appears to reside in non-thyroid tissues, the TSHR in those tissues is only found at very low levels and the relevance of the TSHR in these non-thyroidal tissues remains to be elucidated. Nonetheless, the use of mscTSH-toxin constructs and subsequent destruction of TSHR bearing tissues may cause problems if the TSHR really plays an active role in other tissues. Finally, when using mscTSH constructs for visualization of the thyroid or thyroid derived tumors the presence of the TSHR in other tissues is unlikely to interfere due to the high expression rate of TSHR present in the thyroid when compared to other tissues.

IN SHORT

Compared to rhTSH our mscTSH has higher stability and increased activity, which is potentially very useful for diagnostic purposes and thyroid cancer treatment. Our improved single chain has proven to maintain biologic activity when fused to short extensions. This opens the way to using TSH as a highly specific vehicle to deliver proteins to TSHR bearing cells e.g. toxins to thyroid tumors.