The Superagonistic Activity of Bovine Thyroid-stimulating Hormone (TSH) and the Human TR1401 TSH Analog Is Determined by Specific Amino Acids in the Hinge Region of the Human TSH Receptor*

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Bovine TSH (bTSH) has a higher affinity to the human TSHR (hTSHR) and a higher signaling activity than human TSH (hTSH). The molecular reasons for these phenomena are unknown. Distinct negatively charged residues (Glu297, Glu303, and Asp382) in the hinge region of the hTSHR are known to be important for bTSH binding and signaling. To investigate the potential relevance of these positions for differences between bTSH and hTSH in the interaction to the hTSHR, we determined bTSH- and hTSH-mediated cAMP production of several substitutions at these three hinge residues. To examine specific variations of hTSH, we also investigated the superagonistic hTSH analog TR1401 (TR1401), whose sequence differs from hTSH by four additional positively charged amino acids that are also present in bTSH. To characterize possible interactions between the acidic hTSHR positions Glu297, Glu303, or Asp382 and the additional basic residues of TR1401, we investigated TR1401 binding and signaling properties. Our data reveal increased cAMP signaling of the hTSHR using TR1401 and bTSH compared with hTSH. Whereas Asp382 seems to be important for bTSH- and TR1401-mediated but not for hTSH-mediated signaling, the substitution E297K exhibits a decreased signaling for all three TSH variants. Interestingly, bTSH and TR1401 showed only a slightly different binding pattern. These observations imply that specific residues of the hinge region are mediators of the superagonistic activity of bTSH and TR1401 in contrast to hTSH. Moreover, the simultaneous localization of binding components in the glycoprotein hormone molecule and the receptor hinge region permits important reevaluation of interacting hormone receptor domains.

It is well known that bovine TSH (bTSH)2 has a higher affinity to the human TSHR (hTSHR) and a 6–10-fold higher intrinsic signaling activity than human TSH (hTSH) (1–5). Human TSH and bTSH share high amino acid sequence identity in the α-subunit (74.1%) and β-subunit (88.4%) (6). Studies involving fusion of hTSH and bTSH α- and β-subunits indicate that the higher affinity and the superagonistic cAMP activity of bTSH at the hTSHR depend primarily on amino acid sequences of the β-subunit (6). The most noticeable sequence differences between bovine and human TSH consist of four positively charged residues located in the surface-exposed loops of the α-subunit and one positively charged residue in the β-subunit of bTSH (Fig. 1). Moreover, it has previously been shown that positively charged α loop 1 (α-L1) residues are important for the high bioactivity of bTSH, and they have been implicated in receptor binding. These specific characteristics led to the generation of superagonistic hTSH analogs (6). The human TSH analog TR1401 and bTSH differ from hTSH most importantly by four additional positively charged amino acids located in close spatial proximity at the α-L1, of which three are located at identical positions in bTSH and TR1401 (Fig. 1).

TSH binds to the large extracellular region of its receptor. The extracellular region of the TSHR consists of the leucine-rich repeat domain (LRRD), which is linked with the membrane-spanning serpentine domain by the hinge region. Recently, the binding arrangements between the homologous FSH and a part of the FSH receptor ectodomain including the LRRD (FSH receptor amino acids Cys18–Ala246) have been identified (7). However, the hinge region is not contained in this x-ray structure (7).

In vitro data provide convincing evidence for the functional importance of the hinge region for receptor activation and TSH binding (8–22). Recently, we specified positions Glu297 and Glu303 in the N-terminal portion and Asp382 in the C-terminal portion of the hTSHR hinge region as important for bTSH binding, suggesting that in the process of bTSH binding an extended hormone-binding site is obviously essential (18). The negative charge of positions Glu297 and Asp382 likely interact with positively charged residues of bTSH by complementary charge-charge interaction (18).

To elucidate whether these hinge residues of the hTSHR are specific for interaction with bTSH, we investigated the functional characteristics of the hTSHR analog TR1401 and the native ligand hTSH. For the comparison of these two TSH vari-

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Hinge Region Distinguishes between hTSH and bTSH

A

|    | hTSH | bTSH |
|----|------|------|
| 10 | MDYRKAYAIIFVTLSVFLHVHSAP | MTALFLMSMLFGLTCQAAIIFVTLSVFLHVHSAP |
| 20 | VDCPECFLTCEFSFGAGPLQCMCCFS | CTRDINGKLFLPKYALSDQ |
| 30 |  |  |
| 40 |  |  |
| 50 |  |  |
| 60 |  |  |

B

i

β-L3

ii

β-L3

α-L1

iii

β-L3

α-L1
Hinge Region Distinguishes between hTSH and bTSH

First we characterized the cAMP signaling of the triple hinge variants with bTSH, we used several mutations and alanine combinations at the signaling and bTSH binding-sensitive hTSHR hinge positions Glu257, Glu303, and Asp382. Our data indicate that the higher bioactivity of the TSH variants TR1401 and bTSH are mediated by specific charged residues of the hormone and the hinge region of the hTSHR. Our findings also support the concept that the hinge region of the TSHR is a modulator of TSH potency and efficacy.

EXPERIMENTAL PROCEDURES

Materials—human TSH was obtained from the National Hormone and Pituitary Program (NIDDK, National Institutes of Health) and Dr. A. F. Parlow (University of California, Los Angeles, CA). For bTSH binding analysis 125I-bTSH was ordered from BRAHMS (specific activity, 50 μCi/μg). Cold bTSH was purchased from Sigma-Aldrich. The human TSH analog TR1401 was provided by Trophogen Inc. (Rockville, MD).

Trophogen TR1401 is a highly purified recombinant human TSH analog with four substitutions (Q13K/E14K/P16K/Q20K; numbering without signal peptide) in the α-subunit (6). TR1401 was produced in Chinese hamster ovary cells, purified by a combination of dye, ion exchange, and gel filtration HPLC. The final TR1401 product is more than 98% pure based on SDS-PAGE and reverse phase HPLC. This human TSH analog was 125I-labeled (specific activity, 50 μCi/μg) by GE Healthcare.

Site-directed Mutagenesis—Mutations were introduced into the human TSHR as previously described (18).

Cell Culture and Transfection—COS-7 cells grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen) at 37 °C in a humidified 5% CO2 incubator were used for transfection. For transfection of COS-7 cells, the GeneJammer® transfection reagent (Stratagene, Amsterdam, The Netherlands) was used. For FACS analysis cells were transiently transfected in 12-well plates (10×10^4 cells/well) with 1.0 μg of DNA/well, and for determination of EC50 values, cells were transiently transfected in 24-well plates (5×10^4 cells/well) with 0.5 μg of DNA/well and in 48-well plates (2.5×10^4 cells/well) with 0.25 μg of DNA/well for TSH binding analysis. Controls included transfection of the wild type human TSHR and the pSVL vector alone. Each cAMP or binding experiment was done with a simultaneous FACS control from the same transfection. Transfection efficiency was kept constant (~60–70% of viable cells) for each mutant in each experiment.

FACS Analysis—For determination of cell surface expression and transfection efficiency, FACS analysis was done as previously described (18).

Radioiodinated Binding Assay—48 h after transfection, competitive binding studies were performed as previously described (18, 23) to investigate TSH binding properties. Transfected COS-7 cells were incubated in modified Hank’s buffer (5.36 mM KCl, 0.44 mM KH2PO4, 0.41 mM MgSO4, 0.33 mM Na2HPO4, 5.55 mM glucose) supplemented with 1.3 mM CaCl2, 280 mM sucrose, 0.2% bovine serum albumin, and 2.5% milk powder in the presence of 160,000–200,000 cpm of 125I-TSH (125I-bTSH or 125I-hTSH analog TR1401) with increasing concentrations (0–1.85 μM) of nonlabeled TSH (bTSH or hTSH analog TR1401) at 4 °C for 4 h. The empty pSVL vector was used as control. Afterward cells were washed with the same ice-cold buffer and solubilized with 1 N NaOH, and radioactivity was measured in a γ-counter. The kinetic parameter maximal TSH binding capacity and IC50 in nM (concentration of cold TSH necessary to displace half of bound 125I-TSH) were calculated by nonlinear regression of competition binding curves using Graph Pad Prism 4.0 for Windows assuming a one-site binding model (24). The maximal binding capacity of wt hTSHR was set at 100%, and the maximal TSH binding of all mutants was calculated according to this.

cAMP Accumulation Assay—Measurement of cAMP accumulation was performed 48 h after transfection. The cells were incubated with serum free Dulbecco’s modified Eagle’s medium without antibiotics containing 1 mM 3-isobutyl-1-methylxanthine (Sigma) for 1 h at 37 °C in a humidified 5% CO2 incubator. For stimulation curves the medium was supplemented with increasing concentrations (0, 0.185, 0.555, 1.85, 5.55, 18.5, 55.5, 185.0, and 555.0 nM) of bTSH, hTSH, or hTSH analog TR1401. The reaction was terminated after 1 h by aspiration of the medium. After washing with ice-cold phosphate-buffered saline, the cells were lysed by incubation with 0.1 N HCl for 30 min on ice. The supernatants were collected and dried at 54 °C. The cAMP contents of the cell extracts were determined using the cAMP ALPHAScreenTM assay (PerkinElmer Life Sciences) according to the manufacturer’s instructions.

Statistics—Statistical analysis was carried out by t test using GraphPad Prism 4.0 for Windows (p value of <0.001 extremely significant; p value of 0.001 to 0.01 very significant; p value of 0.01 to 0.05 significant; p value of >0.05 not significant).

RESULTS

To clarify the potential involvement of the three negatively charged amino acids Glu297, Glu303, and Asp382 of the hTSHR hinge region on hTSH compared with bTSH-mediated signaling, we designed single, double, and triple hTSHR hinge mutants and tested these constructs with hTSH, the hTSH analog TR1401 and bTSH.

cAMP Signaling Mediated by Different TSH Variants

Combinations of Mutations at the hTSHR Hinge Region—First we characterized the cAMP signaling of the triple hinge

FIGURE 1. Sequence differences between TSH variants used in the present study. A, alignment of the α- and β-subunit of the hTSH (SwissProt: GLHA_HUMAN P01215, TSHB_HUMAN P01222), bTSH (GLHA_BOVIN P01217, TSHB_BOVIN P01223), and the superagonistic hTSH analog TR1401. The additional positively charged residues at TR1401 and at bTSH compared with wt hTSH are boxed in blue. Sequence numbering for human TSH and human analog TR1401 without signal peptide is shown in blue. B, three-dimensional structural TSH models illustrating the spatial localization of the charge related sequence differences between the TSH variants. The TSH α-subunit is shown in gray, and the β-subunit is in orange. Positively charged residues are highlighted in blue, and the C-α atoms of additional positively charged residues compared with hTSH are highlighted by blue globes. Panel i, bovine TSH, characterized by four additional positively charged residues in the α-L1 (T11K, Q13K, P16K, and Q20K) and one positively charged residue in the β-L3 (L69R); panel ii, human TSH without positively charged residues in the α-L1 and β-L3; and panel iii, the human TSH analog TR1401 is characterized by four additional positively charged residues in the α-L1 (Q13K, E14K, P16K, and Q20K) but shows a lack of the additional positively charged residue in the β-L3.
Hinge Region Distinguishes between hTSH and bTSH

TABLE 1

Functional characterization of mutants at positions Glu297, Glu303, and Asp382 using bovine TSH, human TSH, and the human TSH analog TR1401

The mutated TSH receptors were transiently expressed in COS-7 cells. Characterization of mutants was performed by determination of EC50 and maximal cAMP values via stimulation curves as well as TSH binding (see “Experimental Procedures”). The wt hTSHR and empty pSVL vector were used as controls. The data are given as the means ± S.E. of at least three independent experiments, each carried out in duplicate. For EC50 values wt was set at 1, for maximal cAMP values at 100%, and the mutants were calculated according to this statistical analysis. This statistical analysis was carried out by *t* test using GraphPad Prism 4.0.

| Transfected construct          | Cell surface expression | Bovine TSH | Human TSH | Human TSH analog TR1401 |
|--------------------------------|-------------------------|------------|-----------|-------------------------|
|                                |                        | Binding    | cAMP accumulation | Binding   | cAMP accumulation |
|                                |                        | EC50       | Maximal cAMP    | EC50      | Maximal cAMP    |
|                                |                        | (wt = 1)² | (wt = 100%)     | (wt = 1)² | (wt = 100%)     |
|                                | % of wt                 | % of wt    |            | % of wt            | % of wt |
| pSVL                           | 2.6 ± 0.2              | 100        | 3.7 ± 0.4  | 5.1 ± 2.4          | 5.3 ± 2.5 |
| TSHR wt                        | 100                     |            | 100        | 100                 | 100      |
| **Single mutants**             |                        |            |            |                      |
| E297A                          | 96.2 ± 3.0             | 93.6 ± 6.5²| 2.7 ± 0.7² | 94.4 ± 1.5²        | 1.6 ± 0.0 | 84.1 ± 2.8²  | 41.9 ± 2.3² | 1.6 ± 0.4² | 94.3 ± 2.4² |
| E297Q                          | 92.3 ± 3.2²            | 45.6 ± 3.7²| 3.0 ± 0.8² | 93.4 ± 3.7²        | 1.5 ± 0.1 | 62.6 ± 1.2² | 39.9 ± 4.9² | 0.8 ± 0.3 | 70.1 ± 4.5² |
| E297K                          | 100.6 ± 4.7            | 14.3 ± 2.7²| 9.2 ± 0.3² | 72.4 ± 3.4²        | 1.6 ± 0.8² | 58.7 ± 4.9² | 50.5 ± 0.9² | 9.6 ± 1.4² | 47.7 ± 2.1² |
| E297D                          | 99.7 ± 2.5             | 90.9 ± 4.1²| 2.0 ± 0.2² | 102.0 ± 3.9       | 1.6 ± 0.3 | 88.6 ± 1.0² | 78.8 ± 6.5² | 1.9 ± 0.3² | 96.2 ± 6.6² |
| E303A                          | 102.0 ± 3.2²           | 59.9 ± 2.9²| 0.6 ± 0.3² | 87.0 ± 0.4²        | 1.5 ± 0.2 | 99.0 ± 3.3² | 95.7 ± 2.2² | 0.4 ± 0.1 | 102.7 ± 4.2² |
| E303Q                          | 96.7 ± 2.1             | 88.3 ± 3.9²| 1.1 ± 0.7² | 104.0 ± 3.6       | 0.6 ± 0.1 | 98.3 ± 4.4² | 89.6 ± 6.2² | 1.9 ± 0.4² | 99.4 ± 5.0² |
| E303K                          | 91.7 ± 1.6             | 93.9 ± 8.2²| 1.1 ± 0.3² | 98.0 ± 3.0²        | 0.9 ± 0.3 | 97.0 ± 2.5² | 49.8 ± 4.3² | 1.8 ± 0.5² | 78.5 ± 3.7² |
| E303D                          | 95.9 ± 2.6             | 106.8 ± 8.1| 1.1 ± 0.2² | 98.0 ± 6.3²       | 1.6 ± 0.3 | 89.6 ± 2.3² | 86.2 ± 5.8² | 1.5 ± 0.5 | 84.4 ± 2.6² |
| D382A                          | 93.1 ± 4.0²            | 53.1 ± 4.3²| 0.5 ± 0.1² | 61.4 ± 4.4²       | 1.5 ± 0.5 | 80.4 ± 1.2² | 66.1 ± 6.9² | 0.5 ± 0.2 | 74.2 ± 5.2² |
| D382N                          | 94.0 ± 3.0²            | 56.9 ± 1.8²| 1.5 ± 0.2² | 103.0 ± 5.0       | 1.5 ± 0.2 | 92.2 ± 3.6² | 54.4 ± 3.6² | 2.4 ± 0.6² | 79.3 ± 2.7² |
| D382K                          | 94.0 ± 3.4²            | 16.9 ± 0.3²| 3.6 ± 0.6² | 74.6 ± 1.5²       | 1.4 ± 0.5 | 68.3 ± 5.1² | 9.5 ± 1.1²  | 3.0 ± 1.3² | 65.7 ± 3.8² |
| D382E                          | 91.7 ± 3.7²            | 92.2 ± 4.9²| 1.5 ± 0.5² | 100.7 ± 4.5       | 1.6 ± 0.4 | 100.1 ± 3.7 | 106.1 ± 4.4² | 0.9 ± 0.5 | 92.4 ± 4.5² |
| **Double mutants**             |                        |            |            |                      |
| E297A/E303A                    | 101.2 ± 6.7            | 46.0 ± 6.4²| 1.2 ± 0.2² | 68.5 ± 1.6²        | 1.7 ± 0.2 | 91.8 ± 2.7² | 31.0 ± 1.2² | 1.2 ± 0.7² | 50.8 ± 0.3² |
| E297A/D382A                    | 97.1 ± 3.7             | 12.1 ± 2.2²| 2.2 ± 0.7² | 62.8 ± 2.9²        | 1.6 ± 0.1 | 91.0 ± 1.8² | 6.8 ± 0.2²  | 2.9 ± 0.7² | 68.1 ± 5.1² |
| E303A/D382A                    | 95.9 ± 6.1             | 50.8 ± 5.8²| 0.9 ± 0.4² | 67.9 ± 7.2²       | 1.6 ± 0.1 | 94.7 ± 4.7² | 36.3 ± 4.3² | 1.3 ± 0.3 | 72.9 ± 6.5² |
| **Triple mutant**              |                        |            |            |                      |
| E297A/E303A/D382A              | 95.0 ± 4.0             | 8.6 ± 1.3² | 4.7 ± 0.8² | 62.0 ± 2.1²       | 1.5 ± 0.2 | 94.7 ± 2.6² | 4.6 ± 0.6²  | 5.3 ± 2.4² | 58.5 ± 3.8² |

* p value of 0.01 to 0.05, very significant.

* p value of <0.001, extremely significant.

* p value of 0.01 to 0.05, significant.

* For comparison of TSH binding and EC50 values determined with bTSH, hTSH, and TR1401, previously published data for bTSH (18) are additionally listed in the table.

![Figure 2](image-url)

**FIGURE 2.** cAMP stimulation curves of the hinge mutant E297A/E303A/D382A after treatment with bTSH (A), hTSH without reaching a plateau (B), and the hTSH analog TR1401 (C) by blotting TSH concentration (x axis) versus cAMP accumulation (y axis) using the sigmoidal dose-response function of Graph Pad Prism 4.0 for Windows. Representative examples of three stimulation curves of each TSH variant, each performed in duplicate, are given. The vertical lines indicate the EC50 value and, in the case of hTSH, the estimated EC50 value. The empty pSVL vector was used as control.

mutant E297A/E303A/D382A, which previously showed the strongest decrease of bTSH-mediated signaling and binding (18). This mutant showed a cell surface expression similar to that of the wt (95.0 ± 4.0%; Table 1). After treatment with bTSH, the triple mutant revealed a decrease of the maximal cAMP signal to 60% compared with the wt (set at 100%) and a 4.7-fold increase of the EC50 value (Table 1 and Fig. 2A). In contrast, treatment with hTSH led to a maximal cAMP signal of the triple hinge mutant similar to the wt hTSHR (Fig. 2B, without reaching a plateau). Moreover, E297A/E303A/D382A and the wt hTSHR showed a nonsignificantly different EC50 value (estimated EC50: triple mutant, 38 nM; wt, 26 nM) after stimulation with hTSH. Using hTSH, stimulation curves of the double mutants E297A/E303A, E297A/D382A, and E303A/D382A also exhibit a cAMP signaling comparable with the wt (Table 1).

However, activation of the triple mutant by the hTSH analog TR1401 resulted in a cAMP signaling comparable with the use
of bTSH, which was indicated by a decrease of the maximal cAMP signal. Furthermore, E297A/E303A/D382A revealed a 5.3-fold increased EC50 value compared with the wt with an EC50 of 2.4 nM (Fig. 2C and Table 1).

Single Substitutions at the hTSHR Hinge Region—For more detailed information we analyzed the cAMP signaling of the single mutants at positions Glu297, Glu303, and Asp382 after stimulation with the hTSH analog TR1401, in comparison with the effects of human and bovine TSH. All single and double mutants showed a cell surface expression measured by FACS similar to that of the wt (Table 1).

Using bTSH all hTSHR substitutions at position Glu297 showed an increase of the EC50 value in the range of 2-fold (E297D) to 9.2-fold (E297K). However, among the mutants at position Glu297, only E297Q did not reveal an elevated EC50 value using TR1401. Moreover, after hTSH stimulation only E297K exhibited a right shift of the stimulation curve with a 11.6-fold increase of the EC50 value associated with a decrease of the maximal cAMP response. Substitutions at Glu303 did not show a significant alteration of the EC50 value for bovine and hTSH. Using TR1401, only substitution to lysine and glutamine at Glu303 showed a slight increase of the EC50 value. In contrast, to hTSH, replacement of aspartic acid at Asp382 led to an increased EC50 and decreased maximal cAMP response for D382N using TR1401 and for D382K using both bTSH and TR1401.

**Binding Pattern of TR1401 Versus bTSH**

In a next step we characterized the binding properties of the hTSH analog TR1401 and compared the results to our previous data of bTSH binding (18) and the new data for E303K and E303D. We analyzed all alanine mutants as well as mutants with different side chain characteristics of positions Glu297, Glu303, and Asp382. The results are summarized in Table 1. As a control, cell surface expression of all mutants simultaneously measured by FACS ranged from 95 ± 4.0% for the triple mutant to 102 ± 3.2% for E303A (Table 1). The new mutants E303K and E303D showed a cell surface expression of 92 and 96%, respectively (Table 1). All of the mutants except E297D, E303A, E303Q, E303D, and D382E were characterized by a strong decrease of TR1401 binding compared with the respective FACS value. Substitution to the positively charged lysine at positions Glu297 and Asp382 led to the strongest decrease of the TR1401 binding together with the combination of both positions as mutants E297A/D382A and E297A/E303A/D382A with −5 to −10% of the wt hTSHR binding. In contrast, introduction of a negatively charged residue showed no decrease or only a weak decrease of the TR1401 binding for all three positions. The calculated IC50 values of all mutants using TR1401 were comparable with the wt hTSHR (IC50 = 2.8 ± 1.6 nM), except for mutants E297A/D382A (IC50 = 8.3 ± 2.3 nM), E297A/E303A/D382A (IC50 = 10.5 ± 2.1 nM), and E297K (IC50 = 24.9 ± 2.6 nM) with a significant increase (data not shown). Most of the mutants showed maximal TR1401 binding comparable with the previously described maximal bTSH binding (18) (Table 1). However, the TR1401 binding was more strongly reduced by the positively charged mutants E297K and D382K as well as by all double and triple mutants. Interestingly, mutant E303A showed a TR1401 binding of 96% of the wt, whereas bTSH binding was decreased to 60%. Furthermore, substitution to a positively or negatively charged residue at Glu303 led to bTSH binding like the wt. In contrast, introduction of a positive charge at position Glu303 showed a reduced TR1401 binding to 50% of the wt.

**DISCUSSION**

**Recent Insights into the Function of the Glycoprotein Hormone Receptor Hinge Region**—In the past, functional studies of the glycoprotein hormone receptors (GPHRs) FSH receptor (25), luteinizing hormone/choriogonadotropin receptor (26, 27), and TSHR (28–32) emphasized primarily the importance of the extracellular LRRD and the extracellular loops of the serpentine domain for hormone binding, activation, and/or signal transduction. However, identification of constitutively activating and inactivating mutations underscored the importance of the GPHR hinge region for receptor activation (20–22, 33–38). Moreover, TSHR studies using monoclonal antibodies, autoantibodies, and synthetic peptides indicated that epitopes within the hinge region may be involved in TSH binding (9, 11–13, 15, 16, 19). However, more recent investigations indicated that the GPHR hinge region could be primarily involved in a direct interaction with the hormone, leading to receptor activation (10, 17, 18, 39).

**Different bTSH-, hTSH-, and TR1401-mediated cAMP Signaling**—In this study we set out to investigate whether the potential charge-charge interaction of negatively charged amino acids in the hTSHR hinge region with positive charges of bTSH also occurs with hTSH. Human TSH lacks four positively charged α-L1 amino acids and one β-L3 amino acid, which exists in bTSH. Furthermore, to distinguish between the influence of α-L1 and β-L3 positively charged residues, we used the purified hTSH analog TR1401 (6). This analog differs from hTSH in the four positively charged residues Q13K, E14K, P16K, and Q20K of the α-L1 (Fig. 1; hormone numbering is related to hTSH without signal peptide).

In this study we concentrated on cAMP signaling because the TSHR favors coupling to Gαs over Gαq. At first we compared triple mutant cAMP production and the EC50 value after stimulation with hTSH, bTSH, and TR1401. The increased maximal cAMP signaling of the wt hTSHR induced by TR1401 and bTSH (Fig. 2) reflects their higher bioactivity compared with hTSH (1–6, 43). Interestingly, the right shifted dose-response curve and the reduced cAMP signal of the triple hinge mutant after bTSH and comparably after TR1401 stimulation (Fig. 2, A and C) could not be observed with hTSH (Fig. 2B). The wild type-like behavior of the triple hinge mutant after the addition of hTSH is most likely due to the sequence differences or more precisely to the lack of the additional positive charges most likely in the α-L1 of hTSH in contrast to bTSH and TR1401. Therefore, the TR1401 signaling effect of the triple hinge mutant strongly suggests that the positively charged α-L1 residues of bTSH and TR1401 are most likely involved in the interaction with the previously identified negatively charged residues of the hTSHR hinge region (18).

Furthermore, very similar divergent effects of bTSH or TR1401 and hTSH were also obtained with the double mutant...
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E297A/D382A, whereas combinations E297A/E303A and E303A/D382A did not show alterations of the EC$_{50}$ for all three TSH variants (Table 1). This indicates that mainly positions Glu$_{297}$ and Asp$_{382}$ are responsible for or sufficient to cause the difference between bTSH or TR1401 and hTSH cAMP signaling. In contrast, all substitutions at position Glu$_{303}$ did not show a significant alteration of the cAMP response for bovine as well as hTSH. Therefore, Glu$_{303}$ is most likely not responsible for differences in cAMP signaling between bovine and hTSH. To detect the specific impact of the selected single hinge positions on receptor signaling, we further analyzed the cAMP dose-response curves of several side chain substitutions.

The Lysine Mutant at the hTSHR Hinge Position Glu$_{297}$ Has a General Signaling Effect for All Three TSH Variants—Despite the strong decrease of bTSH or TR1401 binding up to 40% of the wt, some mutants like E297A and E279Q showed a maximal cAMP response or in the case of E297Q even an EC$_{50}$ comparable with the wt (Table 1). This is mainly due to the fact that also a low receptor number on the cell surface or a low TSH binding capability in the range of 40% like in this case is sufficient to obtain the maximal cAMP response. This has previously been described for other mutants like C31S (44), I486F (45), V597F (46), or F631S (47). Position Glu$_{297}$ seems to play an important role for bTSH-mediated signaling because all substitutions, even the charge-maintaining E297D, which showed a wild type-like binding behavior, displayed an elevated EC$_{50}$ value. Glu$_{297}$ also has an influence on TR1401 signaling. However, only E297Q, despite a strong decrease of TR1401 binding, did not show an increased EC$_{50}$. Interestingly, introduction of a positively charged residue at Glu$_{297}$ led to an increased EC$_{50}$ value associated with a decrease of the maximal cAMP signaling not only for bTSH and TR1401 but also for hTSH (Table 1). These findings reveal that specific mutations on position Glu$_{297}$ can have a signaling effect of all three TSH variants, which might also be due to indirect conformational effects in the neighborhood. So far, we are limited to signaling studies with hTSH. This is due to the known relatively low affinity interaction of hTSH binding to the hTSHR (29), which leads to very low detectable $^{125}$I-hTSH binding (48, 49).

Position Asp$_{382}$ Is Important for bTSH- and TR1401- but Not for hTSH-Mediated Signaling—Mutations at Asp$_{382}$ did not show an altered EC$_{50}$ value for both hTSH and TR1401, whereas both bTSH and TR1401 revealed an increased EC$_{50}$ value for D382K and in the case of TR1401 also for D382N (Table 1). These findings suggest that position D382 is important for bTSH- and TR1401-mediated signaling, but not for hTSH. Therefore, position Asp$_{382}$ seems to be one of the crucial hTSHR residues responsible for differences in cAMP signaling of bovine and human TSH. In summary, the observed differences of bTSH- and hTSH-mediated signaling reveal that future functional characterizations should consider both TSH variants to allow a more comprehensive decision regarding the influence of the analyzed hTSHR mutations on receptor activation.

Similarities and Differences in Binding Properties between bTSH and the hTSH Analog TR1401—We analyzed the TR1401 binding of all single substitutions and combined alanine mutants using $^{125}$I-TR1401 and compared the results with bTSH binding. The similar binding pattern of TR1401 and bTSH with all substitutions at Glu$_{297}$ and Asp$_{382}$ as well as the combined alanine mutants (Table 1) indicate that, comparable with bTSH, the negatively charged hinge positions are also involved in TR1401 binding. However, the reduction of TR1401 binding, stronger than bTSH binding with double and triple alanine mutants of the three hinge positions, suggests that TR1401 binding depends more on combined negatively charged components in the hinge region. Analysis of the charge influence on the TR1401 binding pattern with the single hinge positions by introduction of positively or negatively charged amino acids leads to the finding that the negative charge at Glu$_{297}$ and Asp$_{382}$ is not only essential for bTSH but also important for TR1401 binding.

The main common characteristics of the human TSH analog TR1401 and bovine TSH are four basic residues (Fig. 1) in the surface-exposed $\alpha$-L1, indicating that these positive charges are potential direct contact points for the negatively charged hinge residues Glu$_{297}$ and Asp$_{382}$. This supports the previous finding that alanine substitutions of corresponding residues Gln$_{13}$, Pro$_{16}$, and Gln$_{20}$ in the hTSH $\alpha$-L1 did not affect receptor binding or bioactivity. This indicates that a weak hydrogen-type interaction between hTSH and the receptor is very likely replaced by a strong electrostatic interaction of basic TSH amino acids with acidic residues of the TSHR in the case of bTSH and TR1401 (6, 31). However, mutations at position Glu$_{303}$ showed a slightly divergent binding pattern for bTSH and TR1401 (Table 1). E303A maintained the wt level for TR1401 binding but showed a reduced binding for the introduction of a positive charge. This effect was reciprocal for bTSH. These data suggest that an additional positive charge in the hinge region leads to a strong repulsion of the TR1401, whereas a change of the side chain size does not alter TR1401 binding. In contrast, because only the alanine substitution led to a decreased binding, bTSH interaction at Glu$_{303}$ seems to depend on the size of the side chain. Such relatively minor discrepancies between the properties of TR1401 and bTSH are quite surprising taking into consideration almost 30% nonidentical residues in their $\alpha$-subunits, four additional N-terminal amino acid residues (GEFT) in the bTSH $\alpha$-subunit (Fig. 1A), and differences in their respective carbohydrate structures. However, the divergent binding patterns for bTSH and TR1401 at position Glu$_{303}$ is likely due to the fact that TR1401 lacks the positive charge at position Leu$_{69}$ in the $\beta$-L3 and that only three of the four additional positively charged residues (Q13K, P16K, and Q20K) are at identical positions in the $\alpha$-L1 of bTSH and TR1401, whereas T11K does not exist in TR1401, and E14K does not exist in bTSH (Fig. 1).

Positively Charged $\alpha$-L1 Residues of bTSH and TR1401 Most Likely Interact with the hTSHR Hinge Region—Despite the identified discrepancies both TR1401 and bTSH seem to require for their better binding and their higher agonistic activity the same residues Glu$_{297}$ and Asp$_{382}$. The negative charge at position Glu$_{297}$ is present in the majority of homologous TSHR sequences from different species, except ruminants such as cow and sheep. At Asp$_{382}$ the negative charge is strictly conserved in all known vertebrate and primate TSHR sequences. These two residues are located outside of a 50-amino acid "insertion" (50) that is not present in other glycoprotein hormone receptors and...
are included in the Lys^{287}–His^{384} deletion, resulting in the total loss of TSHR binding and TSH-induced activation (17). In fact, TSHR chimeras containing substitutions to luteinizing hormone/choriogonadotropin receptor sequence 377–384, which among 8 amino acid substitutions also includes D382G, resulted in a major loss in bTSH-induced cAMP response. This observation is highly compatible with our findings of the role of Asp^{382} for bTSH and TR1401 but not for hTSH-induced signaling (51).

Because the hTSH analog TR1401 shows a similar binding pattern and superagonistic activity like bTSH, the relatively low affinity interaction of hTSH with the hTSHR (29) is most likely due to the lack of the four positively charged α-L1 residues in hTSH (bTSH: T11K, Q13K, P16K, and Q20K; TR1401: Q13K, E14K, P16K, and Q20K). However, the similar binding results of bTSH and the hTSH analog indicate that hTSH and bTSH have a similar binding pattern to the hTSHR with regard to the analyzed hinge positions. It remains to be clarified whether further residues of the hinge region are involved in both bTSH and TR1401 binding. More data are necessary to clarify whether the hinge region is necessary for hTSH binding.

The present data provide a novel mechanistic explanation for several previous findings, including the activation of the TSHR by TSH α-subunit analogs (52). Monomeric and fused homodimeric human α-subunit analogs, but not the wt human α-subunit, have been found to act as weak agonists based on cAMP production. These findings together with the present data challenge the dominant concept that the hormone subunits may activate the TSHR after interaction only with the LRRD. Indeed, based on recent studies the hinge region of glycoprotein hormone receptors has been called the signaling and specificity domain (17, 53). Such specificity will likely include not only hormone, but also species specificity, as illustrated here by differential properties of hTSH, bTSH, and the human TR1401 analog. Because the cluster of charged residues in the α-L1 of TSH is present in all vertebrate species except hominoids (31), the present findings suggest that specific residues identified in the TSHR hinge region were important in the evolutionarily driven decrease of TSH bioactivity in selected vertebrates (TSHR: E297Q in ruminants) and higher primates (elimination of TSH α-L1 charge cluster in apes and human).

Taken together our study provides evidence for (i) potential electrostatic interactions between negatively charged residues (Glu^{297}, Gln^{303}, and Asp^{382}) of the hinge region and positively charged residues of bTSH and the human TSH analog TR1401 (18, 41, 54); (ii) these interactions are the molecular cause for the higher potency and efficacy of the tested TSH variants in comparison with hTSH; (iii) together with the different binding characteristics of bTSH and TR1401 at Gln^{303}, the interactions provide new detailed information for the orientation of TSH toward the hinge region; (iv) these TSH species-specific interactions support a novel concept of selected hinge region residues as evolutionary important modulators of TSH potency and efficacy; and (v) hormone analogs with minimal modifications of the common α-subunit as in the case of TR1401 can be used to probe selected GPHR domains. In addition, the loss of recombinant hormone super activity is an important additional approach for the probing of glycoprotein hormone-receptor interactions.

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