Extended Hormone Binding Site of the Human Thyroid Stimulating Hormone Receptor

DISTINCTIVE ACIDIC RESIDUES IN THE HINGE REGION ARE INVOLVED IN BOVINE THYROID STIMULATING HORMONE BINDING AND RECEPTOR ACTIVATION

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The human thyroid stimulating hormone receptor (hTSHR) belongs to the glycoprotein hormone receptors that bind the hormones at their large extracellular domain. The extracellular hinge region of the TSHR connects the N-terminal leucine-rich repeat domain with the membrane-spanning serpentine domain. From previous studies we reasoned that apart from hormone binding at the leucine-rich repeat domain, additional multiple hormone contacts might exist at the hinge region of the TSHR by complementary charge-charge recognition. Here we investigated highly conserved charged residues in the hinge region of the TSHR by site-directed mutagenesis to identify amino acids interacting with bovine TSH (bTSH). Indeed, the residues Glu-297, Glu-303, and Asp-382 in the TSHR hinge region are essential for bTSH binding and partially for signal transduction. Side chain substitutions showed that the negative charge of Glu-297 and Asp-382 is necessary for recognition of bTSH by the hTSHR. Multiple combinations of alanine mutants of the identified positions revealed an increased negative effect on hormone binding. An assembled model suggests that the deciphered acidic residues form negatively charged patches at the hinge region resulting in an extended binding mode for bTSH on the hTSHR. Our data indicate that certain positively charged residues of bTSH might be involved in interaction with the identified negatively charged amino acids of the hTSHR hinge region. We demonstrate that the hinge region represents an extracellular intermediate connector for both hormone binding and signal transduction of the hTSHR.

The glycoprotein hormone receptors (GPHRs)2 thyroid stimulating hormone receptor (TSHR), follicle stimulating hormone receptor (FSHR), and luteinizing hormone/choriogonadotropin (LHCGR) are members of the seven transmembrane-spanning receptor family (1–3). A common structural characteristic of all GPHRs is the large N-terminal extracellular domain, which is responsible for hormone recognition and binding (4, 5). The N-terminal terminal extracellular domain of the TSHR (amino acids Met-1–Asp-410) can be subdivided into the leucine-rich repeat domain (LRRD) and the hinge region. The hinge region can be further subdivided into the N-terminal cysteine-box 2, the cysteine-box 2/3 linker, and the C-terminal cysteine-box 3 located close to transmembrane helix 1 (TMH1) (6).

The hinge region links the extracellular hormone binding LRRD of the TSHR with the transmembrane domain. However, this part of the ectodomain lacks experimental structural data. Recently, the binding arrangements between the human FSH and the hormone binding LRRD of the FSHR have been identified by x-ray analysis (7). However, the hinge region is not included in this crystal structure (7). Moreover, a crystal structure of the corresponding LRRD of the TSHR (amino acids 30–260) in complex with a thyroid stimulating autoantibody also provides only structural information for the particular LRRD portion and not for the entire extracellular domain of the receptor (8). Recent studies using monoclonal antibodies, autoantibodies, and synthetic peptides suggest that epitopes within the hinge region are involved in TSH binding (9–15). A chimeric TSH/LHCG receptor containing only the hinge region of the TSH receptor in the ectodomain revealed also the possibility that TSH can bind to the hinge region (16). Costagliola et al. (9) demonstrated the importance of sulfation on a tyrosine in a conserved motif (383YDY387) located downstream of the third C-terminal cysteine cluster. This post-translational modification outside the LRRD segment is mandatory for high affinity TSH binding and for TSHR activation (9). Furthermore, it has been shown that a mutation at position Tyr-385 affects TSH binding (11). The importance of sulfation on homologous tyrosines was also demonstrated for the corresponding hinge region of the FSHR and LHCGR (17). These findings indicate that such an introduction of a negative electrostatic potential by sulfation at corresponding positions in all three GPHRs seems to be essential for hormone recognition and binding.

In line with this interpretation it is well known that additional positive charges at specific residues of the corresponding hormones improve the binding affinity with the receptor (18,
that applies to bovine TSH (bTSH), which contains more positively charged residues and shows higher affinity to the hTSHR compared with human TSH (hTSH). Moreover, superactive TSH and CG analogs are characterized by additional positively charged amino acid side chains (18). The crystal structure of the human FSHR LRRD-human FSH complex (7) and the expected homologies in other GPHR-GPH complexes suggest that the additional positively charged residues are located in the peripheral loops of the α and β subunits of bTSH and superagonists and would not participate in the LRRD-hormone complex. They rather seem to interact elsewhere, most likely with the hinge region. Consequently, in addition to the bTSH binding at the LRRD, there are very likely simultaneous additional multiple hormone contacts by complementary charge recognitions also to the hinge region of the TSHR.

The goal of our study was to clarify essential contact points for initial TSHR activation by bTSH; in particular, to identify charged determinants for hormone binding and receptor activation at the TSHR hinge region. Twelve charged residues of the hinge region, except the cleavable C-peptide region Ala317-Gly367 (20, 21), were substituted by alanine. Indeed, we identified three distinct mutants at negatively charged residues with a cell surface expression comparable with the wt but with a strong decrease in bTSH binding. Moreover, the functional characterization of further substitutions at these residues showed that the negative charges of Glu-297 and Asp-382 are crucial for bTSH binding and that both positions are involved in cAMP signaling.

Taken together, our data show that apart from amino acids of the LRR domain, distinctive positions at the hinge region are also important for hormone binding. This finding demonstrates that the hinge region of the TSHR represents both a TSH binding site and an important extracellular signal transduction interface for the forwarding of the intramolecular activation signal.

**EXPERIMENTAL PROCEDURES**

**Site-directed Mutagenesis**—Mutations were introduced into the human TSHR via site-directed mutagenesis using PfuTurbo DNA-polymerase (Stratagene, Amsterdam, The Netherlands), whereas the human TSHR-pSVL (22) was used as template. PCR products were transformed into competent DH5α cells. Plasmid preparation was done using a commercial kit (Qiagen). Mutated TSHR sequences were verified by dyeoxy sequencing of the complete TSHR with dRhodamine Terminator Cycle Sequencing chemistry (ABI Advanced Biotechnologies, Inc., Columbia, MD). Sequencing reactions were analyzed on a Genetic analyzer ABI 31000 (ABI Advanced Biotechnologies).

**Cell Culture and Transfection**—For 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. GeneJammer transfection reagent (Stratagene) was used for transfection of COS-7 cells, whereas a transfection efficiency of 60–70% of viable cells could be achieved for each experiment. Cells were transiently transfected in 12-well plates (10 × 104 cells per well) with 1.0 µg of DNA/well for FACS analysis, in 24-well plates (5 × 104 cells/well) with 0.5 µg of DNA/well for determination of cAMP and EC50 values, and in 48-well plates (2.5 × 104 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 simultaneously with a FACS control from the same transfection. Transfection efficiency was kept constant (~60–70% of viable cells) for each mutant in each experiment.

**FACS Analysis**—48 h after transfection cells were detached from the dishes with phosphate-buffered saline and transferred in Falcon 2054 tubes. After washing with phosphate-buffered saline containing 0.1% bovine serum albumin and 0.1% NaN3, cells were incubated for 1 h at 4 °C with a 1:200 dilution of a mouse anti-human TSHR monoclonal antibody 2C11 (MAK1281, Linaris, Wertheim-Bettingen; 10 µg/ml) in the same buffer. Afterward, cells were washed twice and incubated at 4 °C for 1 h in the dark with a 1:200 dilution of fluorescein-conjugated F(ab’)2 rabbit anti-mouse IgG (Serotec, Oxford, UK). Before fixation of cells with 1% paraformaldehyde, they were washed twice. Receptor expression was determined by the mean fluorescence intensity using the FACScan (BD Biosciences). The wt TSHR was set at 100%, and mutant receptor expression was calculated according to this. The percentage of signal-positive cells corresponds to transfection efficiency.

**Radioligand Binding Assay**—To investigate TSH binding properties, competitive binding studies were performed 48 h after transfection as previously described (23, 24). Transfected COS-7 cells were incubated in modified Hanks’ 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, 2.5% milk powder in the presence of 160,000–200,000 cpm of 125I-labeled bTSH (Brahms, Hennigsdorf; specific activity, ~50 µCi/µg) with increasing concentrations of nonlabeled bTSH (0, 0.01, 0.1, 1, 10, 100 milliunits/ml) at 4 °C for 4 h. Thereafter, cells were washed with the same ice-cold buffer and solubilized with 1 N NaOH, and radioactivity was measured in a gamma-counter. The empty pSVL vector was used as control. The kinetic parameters maximal TSH binding capacity and IC50 in nM (concentration of cold bTSH necessary to displace half of bound 125I-labeled TSH) were calculated by nonlinear regression of competition binding curves using Graph Pad Prism 4.0 for Windows assuming a one-site binding model (25). Maximal bTSH binding of wt TSHR was set at 100%, and the bTSH binding of all mutants was calculated according to this.

**cAMP Accumulation Assay**—48 h after transfection measurement of cAMP accumulation was performed. Cells were incubated with serum free Dulbecco’s modified Eagle’s medium without antibiotics containing 1 mM 3-isobutyl-1-methyloxanthine (Sigma) for 1 h at 37 °C in a humidified 5% CO2 incubator. For TSHR stimulation the medium was supplemented with 100 milliunits/ml bTSH (Sigma). For determination of EC50 values, cells were stimulated with 0.03, 0.1, 0.3, 1, 3, 10, and 30, 100 milliunits/ml bTSH. After 1 h the reaction was terminated by aspiration of the medium. Cells were washed once with ice-cold phosphate-buffered saline and then lysed by incubation with 0.1 N HCl for 30 min. Supernatants were collected and dried at 54 °C. The cAMP contents of the cell extracts were determined...
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using the cAMP AlphaScreen™ assay (PerkinElmer Life Sciences) according to the manufacturer’s instructions. EC_{50}/IC_{50} ratio of the mutants were calculated from the respective EC_{50} and IC_{50} values, both in nM.

Statistics—Statistical analysis was carried out by t test using GraphPad Prism 4.03 for Windows.

Molecular Modeling—The x-ray crystal structure of the human FSHR LRRD-human FSH complex (7) (PDB entry code 1XWD) was used as structural template for a complex model for the N-terminal hTSHR-LRRD ectodomain with bTSH. The bTSH model is based on homology to disulfide bridges at human FSH which were incorporated: α-subunit, Cys-31/Cys-55, Cys-34/Cys-84, Cys-52/Cys-106, Cys-56/Cys-108, Cys-83/Cys-111; β-subunit, Cys-22/Cys-72, Cys-36/Cys-87, Cys-39/Cys-125, Cys-47/Cys-103, Cys-51/Cys-105, and Cys-108/Cys-115. The hTSHR LRRD structure is C-terminal-restricted at amino acid Ala-254. The assembled TSHR LRRD-TSH model was soaked with water in a periodic boundary box. Initially the LRRD atoms were kept fixed to relax the water during minimization. Later on the entire system was considered. Reported low root mean square values between backbones of the used FSHR LRRD template structure and the recently presented TSHR-LRRD x-ray structure (8) basically support the reliability of our bTSH/TSHR-LRRD model.

The homology model of the transmembrane domain of hTSHR was generated based on the x-ray structure of bovine rhodopsin (PDB entry codes 1F88 (26), 2135 (27), 2J4Y (28). Reported low root mean square values between backbones of the transmembrane helices of the used rhodopsin structure and the recently solved x-ray structures of the β2-adrenergic receptor (PDB entry codes 2R4R (29) and 2RH1 (30)) support the reliability of our TSHR-TMH model. The major structural difference concerns ECL2. Due to (i) the higher similarity in length and amino acid sequence of ECL2 between rhodopsin and TSHR (36.0%) versus β2-adrenergic receptor (β2-AR) and TSHR (27.6%) and (ii) a β2-AR-specific additional internal cysteine bridge which stabilizes a helical fold in the ECL2 of the β2-AR, we kept the rhodopsin like β-hairpin structure and location of ECL2 also for the TSHR model. Additionally this rhodopsin-like ECL2 conformation is consistent with results of diverse studies at other GPCRs (31, 32) and TSHR (33). Several TSH receptor-specific corrections were made, such as regular helix extensions in TMH2 and TMH5 of TSHR instead of structural bulges in the two helices of rhodopsin, caused there specifically by side chains that are not present in TSHR (two consecutive threonines in TMH2 and a proline in TMH5, respectively). Loops were added by best fit and homology to fragments of other proteins (from the Protein Data Bank). Gaps of missing residues in the loops of the template structure were closed by the Loop Search tool implemented in Sybyl 7.3 (Tripos Inc., St. Louis, MO).

Conjugate gradient minimizations for all models were performed until converging at a termination gradient of 0.05 kcal/(mol·Å); the AMBER 7.0 force field (34) was used. Quality and stability of the model were validated by checking the geometry by PROCHECK (35) during a molecular dynamics simulation of 2 ns (overall backbone root mean square 1.8 Å).

TABLE 1

| Transfected construct | Cell surface expression % of wt TSHR | bTSH binding % of wt TSHR | bTSH binding IC_{50} nm |
|------------------------|--------------------------------------|--------------------------|------------------------|
| pSVL                   | 93.3 ± 0.1                           | 100                      | ND                     |
| TSHR wt                | 100                                  | 100                      | 83.2 ± 24.5            |
| R293A                  | 82.6 ± 5.3a                          | 90.3 ± 1.1a              | 68.2 ± 21.5            |
| E297A                  | 93.9 ± 5.4a                          | 39.6 ± 6.5a              | 37.8 ± 7.9a            |
| E303A                  | 90.3 ± 5.1a                          | 59.9 ± 2.9a              | 17.4 ± 16.8            |
| R312A                  | 94.5 ± 5.5a                          | 93.0 ± 3.7a              | 97.3 ± 24.3            |
| K313A                  | 80.8 ± 2.8a                          | 89.1 ± 5.4a              | 66.8 ± 5.3             |
| E369A                  | 64.8 ± 3.1a                          | 78.6 ± 1.5a              | 57.1 ± 2.9             |
| K371A                  | 94.9 ± 4.7                           | 97.4 ± 4.2               | 68.5 ± 5.8             |
| E375A                  | 83.6 ± 6.3a                          | 70.9 ± 2.3a              | 69.9 ± 19.3            |
| E376A                  | 85.7 ± 5.3a                          | 82.6 ± 4.4a              | 67.3 ± 14.2            |
| E382A                  | 96.5 ± 6.2                           | 50.8 ± 3.3a              | 42.6 ± 7.8a            |
| D394A                  | 92.7 ± 6.2                           | 70.7 ± 4.9               | 62.0 ± 20.0            |
| D1095A                 | 83.4 ± 5.8a                          | 69.8 ± 1.3a              | 100.6 ± 5.6            |

| p | < 0.001, extremely significant. |
| p | 0.001–0.01, very significant. |
| p | 0.01–0.05, significant. |
| p | > 0.05, not significant. |

RESULTS

Residues Glu-297, Glu-303, and Asp-382 in the TSHR Hinge Region Are Sensitive for Hormone Binding—Alanine mutations of eight negatively and four positively charged residues of the hinge region were analyzed by determination of cell surface expression using FACS analysis and maximal bTSH binding (Table 1). For all mutants the FACS analysis showed a cell surface expression of 80–97% of the wt (wt set at 100%), except E69A, with only 65%. Most of the 12 alanine mutants exhibited a bTSH binding similar to the respective FACS result. However, three alanine mutations of the negatively charged residues Glu-297, Glu-303, and Asp-382 revealed a significant decrease in the bTSH binding capability (Table 1; Fig. 1A). The detected bTSH binding was reduced to 60% for E303A, 51% for D382A, and 40% for E297A compared with the wt (Table 1).

Glu-297 and Asp-382 Are Necessary as Multiple Contact Points for bTSH Binding—To investigate the decreased hormone binding capability of E297A, E303A, and D382A in more detail and to answer the question if multiple substitutions at these three positions leads to a further decrease of bTSH binding, we generated and functionally characterized all possible combinations (E297A/E303A, E297A/D382A, E303A/D382A, and E297A/E303A/D382A) of these mutants. All combinations showed a cell surface expression level like the wt (Table 1). However, in comparison to the single mutants, the double mutant E297A/D382A and the triple mutant E297A/E303A/D382A revealed a strongly decreased bTSH binding (Fig. 1B; Table 2). In contrast, bTSH binding for the combinations E297A/E303A and E303A/D382A were similar to the level of the single mutants E297A and D382A (~50%) and did not show a further decrease in TSH binding (Fig. 1B, Table 1). Based on the observed increased negative effect on TSH binding for multiple substitutions containing D382A or simultaneously D382A and E297A we conclude that amino acids Glu-297 and Asp-382 are most essential for bTSH binding and that these residues
most likely act simultaneously during the hormone binding process.

Negative Charges at Positions Glu-297 and Asp-382 Are Essential for bTSH Binding—To investigate whether the negative charge at positions Glu-297, Glu-303, and Asp-382 are necessary for hormone binding, we introduced in a first-step hydrophilic side chain of comparable size but lacking the charge such as glutamine or asparagine residues. E297Q, E303Q, and D382N revealed a cell surface expression measured by FACS comparable with wt (Table 2). E297Q and D382N showed a significantly decreased hormone binding capability (Table 1). In contrast, E303Q exhibited only a slightly decreased bTSH binding compared with the respective FACS results (Table 2). To characterize the side chain specificity on the effect of the negative charge at positions Glu-297 and Asp-382, we replaced in a second step the natural amino acid with a charge-keeping residue but altered size and with a residue of an opposite site charge characteristic. E297D, D382E and even E297K, D382K showed a cell surface expression determined by FACS analysis of 92–102%. Introduction of a positively charged amino acid revealed a strong decreased bTSH binding of 14 or 17% for E297K and D382K, respectively (Fig. 1C). In contrast, the alteration of side chain size while maintaining the negative charge as E297D and D382E exhibited a bTSH binding capability...
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ity of 91–92% of the wt (Fig. 1C; Table 2). These data indicate the necessity of negative charges at TSHR positions Glu-297 and Asp-382 for recognition and binding of bTSH. IC_{50} values are shown in Tables 1 and 2.

Residues Glu-297 and Asp-382 Are Involved in cAMP Signaling—All characterized mutants did not show an elevated basal cAMP signal (Table 2). After treatment with bTSH, E303A showed a slightly and D382A a strongly decreased cAMP response, whereas E297A exhibited with 95% efficacy a result like the wt TSHR. In contrast, the double mutants E297A/E303A, E297A/D382A, and E303A/D382A and the triple mutant E297A/E303A/D382A revealed a significantly decreased cAMP production (Table 2). E297Q, E297D, E303Q, D382N, and D382E showed a slightly decreased maximal cAMP formation. E297K and D382K revealed a decreased cAMP response to 55 or 65% (Table 2). The wt TSHR showed an EC_{50} value of 0.7 milliunits of TSH/ml (Table 2). Stimulation curves revealed for mutants E297A/D382A, E297A, and E297A/E303A/D382A a 2.4–4.8-fold increase of the EC_{50} (Fig. 2), whereas mutations E303A, D382A, E297A/E303A, and E303A/ D382A did not alter the EC_{50} value (Table 2). Stimulation curves showed an increased EC_{50} value for E297K and D382K (Fig. 2). E297Q, E297D, E303Q, D382N, and D382E exhibited a 1.3–2.1-fold EC_{50} value (Table 2). EC_{50}/IC_{50} ratios are shown in Table 2. Taken together, mutations at positions Glu-297 and Asp-382 showed significant effects on the cAMP signaling, whereas mutations at position Glu-303 showed only weak or no influence on the maximal cAMP stimulation and EC_{50} values.

Assembled Model of the Extended Binding Site for bTSH at the Hinge Region of the hTSHR—Established disulfide bridges between cysteine-box 2 and cysteine-box 3 constrain the N- and C-terminal portions of the hinge region in close spatial proximity to each other. Consequently, acidic residues Glu-297 and Glu-303 at the N-terminal portion are probably arranged in close proximity to residue Asp-382 and the sulfated Tyr-385 at the C-terminal portion (Fig. 3). Joining our data with currently available structural information led to a schematic model of the extended bTSH binding site at the hTSHR hinge region. The interaction scheme is composed of (i) a molecular model of bTSH, (ii) a boxed scheme of the hinge region, and (iii) a molecular model of the serpentine domain (Fig. 4). As one conceivable option, the bTSH is, thus, arranged the additional basic residues in the α1 loop of bTSH particularly point toward the acidic residues at the surface of the hinge region. Thereby, the extension of the hormone binding site is created by negatively charged patches most likely clustered in close proximity.

DISCUSSION

In this study we set out to identify and characterize further potential charged determinants in the hTSHR hinge region for bTSH binding and receptor activation by an alanine scan of conserved residues in this region.

N-terminal Portion of the Hinge Region; Glu-297 and Glu-303 Are Relevant for Hormone Binding—The results of our study indicate, that apart from amino acids of the LRR domain the three acidic residues Glu-297 and Glu-303 in the N-terminal, and Asp-382 in the C-terminal portion of the hinge region are indeed involved in bTSH binding. The amino acids Glu-297 and Glu-303 are located in close neighborhood in the N-terminal portion of the hinge region (Fig. 3). The particular TSHR positions Glu-297 and Glu-303 characterized in this paper as significantly influencing the hormone binding of the TSHR turned out to be the essential constituents of previously reported epitopes for TSH binding (11, 13, 14). In our study the side chain variations at Glu-297 revealed a binding capability in the order wt ~ Asp >> Gln ~ Ala >> Lys, supporting the crucial role of a negative charge for bTSH binding at this position. The positively charged lysine led to a loss of ligand binding. This might be explained by repulsion of identical charges instead of negative-charge attraction between the hTSHR and bTSH. With respect to cAMP signaling, the increased EC_{50} value for Ala and Lys substitutions indicate that Glu-297 is also partially involved in intramolecular signal transmission. How-
however, the fact that the cAMP characteristic of the glutamine substitution is comparable with the wt indicates that the negative charge of position Glu-297 is not necessary for signaling. In contrast, position Glu-303 appears to be a solely charge-independent recognition site for bTSH binding and apparently plays no explicit role in signaling. Only the alanine substitution shows an effect on binding, whereas the glutamine substitution is tolerated. This indicates that the interaction partner at the hormone is, rather, a hydrophilic but uncharged amino acid. Position Glu-303 is located next to the potential glycosylation site Asn-302. However, substitution of the asparagine at position Asn-302 did not appreciably affect the affinity of TSH binding (36), indicating that recognition of Glu-297 and Glu-303 are a part of a hormone binding site.

Our results are in line with findings about the missing 27 amino acids in the N-terminal part of the LHCGR hinge region of the New World monkey Callithrix jacchus as well as in a pathogenic deletion mutation of the human LHCGR (37, 38), which cause differences in luteinizing hormone- and choriogonadotropin-induced signaling. This LHCGR portion encoded by exon 10 corresponds to the TSHR region, which includes the binding relevant positions corresponding to Glu-297 and Glu-303. These findings further support the interpretation that residues Glu-297 and Glu-303 are a part of a hormone binding site.

In the particular case of this study the mutant IC50 results and consequently also the EC50/IC50 ratios are difficult to interpret due to the strong decrease of the maximal hormone binding of several mutants. For comparison of IC50 or Kd values, it would be necessary to express all receptor constructs at the same maximal bTSH binding level. However, this reasoning does not affect our main finding that three specific residues in the hinge region affect TSH-TSHR interaction as this finding is primarily based on the decrease of the maximal TSH binding capacity of these mutants.

C-terminal Portion of the Hinge region; Asp-382 in Close Proximity to the Sulfated Tyr-385 Is Relevant for Both Hormone Binding and Signaling—Our studies revealed that the particular residue Asp-382 and, even more prominently, the negative charge at this position is essential for hormone binding and cAMP signaling. A relevance of Asp-382 on hormone binding and cAMP signaling was suggested by recent studies using different approaches like monoclonal antibodies, autoantibodies, or synthetic peptides (9, 10, 12, 14, 15).

It was previously demonstrated that neighboring tyrosine 385 (Tyr-385) of the hinge region of the hTSHR and the corresponding tyrosines in the LHCGR and the FSHR are sulfated and mandatory for high affinity binding of hormones and for receptor activation (9, 17). Peptide studies with the peptide hormone gastrin also suggested that tyrosine sulfation itself is supported by the presence of an acidic residue at least in direct the N-terminal neighborhood to position −1 of the tyrosine.

FIGURE 4. Assembled model of a binding site for bTSH at the hinge region of hTSHR supporting a complementary charge interaction. The model is fragmentary and composed of (i) a molecular model of the bTSH-LRRD complex (red, TSH α subunit: magenta, β subunit: orange, LRRD), (ii) a boxed scheme of the hinge region (beige dashed line), and (iii) a model of the serpentine domain. All positive charged residues localized on the surface of bTSH that are not involved in LRRD binding are visualized. Although the complex model restricts the number of spatial orientations of bTSH, only an approximate adjustment (open black arrows) of basic residues of bTSH toward the identified sensitive acidic patches at the hinge region can be illustrated due to the remaining number of possibilities. One likely orientation of bTSH where the additional basic residues at the qL1 loop of bTSH (green, compared with hTSH) point toward the identified sensitive acidic residues (red) at the surface of the hinge region is shown. The structural proportions clearly indicate that the hormone very likely protrudes beyond the receptor serpentine domain and has at one-side free capabilities for further interactions with its peripheral loops. Ctr, C-terminal; Ntr, N-terminal.
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whereas a positively charged residue at this location abolishes sulfation (39). In addition, for this particular example it was suggested that the degree of sulfation might also be influenced by the flanking acidic residues in the N-terminal positions −2 and −3 of the sulfated tyrosine. However, basic residues in other positions apart from −1 did not reduce sulfation (39). Our finding of a drastic loss of hormone binding by introduction of a positive charge at Asp-382 (mutation D382K; position −3) might, therefore, rather be a hint for a direct charge-charge interaction of Asp-382 with the hormone than only suggesting an involvement in the sulfation process. This is also supported by x-ray structure studies of tyrosine O-sulfation at other proteins demonstrating the interaction of residues that flank sulfated tyrosines (40). Relating to hormone binding at the TSHR, these and our data support a direct charge-charge interaction of Asp-382 with the hormone. Moreover, we suggest that the negative electrostatic potentials of Asp-382 and of the sulfated Tyr-385 are located in close spatial vicinity and contribute together to a bTSH binding site at the hinge region (Fig. 4).

Cooperative Effects on bTSH Binding by Multiple Mutations of Glu-297, Glu-303, and Asp-382—Our finding that the negative charges of Glu-297 and Asp-382 at the TSHR are essential for bTSH binding is most probably related to corresponding observations for the ligands (18). It was previously reported that positively charged residues in the a and β subunits of GPHs are necessary for binding and activation of their receptors, suggesting that interactions of the hormone with negatively charged amino acids of the receptor have been important in the evolution of GPHRs (18). Moreover, specifically introduced additional positively charged residues in TSH or choriodonadotropin (supercactive GPH analogs) improve receptor activation (18). Introduction of additional positively charged residues in different loops in the hTSH led to the engineering of several superactive analogs, and it was shown that combinations of such additional positively charged residues in the hTSH resulted in a positive cooperative effect of the individual substitutions in double and triple mutants of hTSH regarding receptor binding and activation (41, 42).

In our study we identified increased negative (cooperative) effects at the counterpart of the ligand by the simultaneous removal of multiple negative charges directly at the hTSHR. This finding is complementary to observed positive cooperative effects at superagonistic TSH analogs (18, 42). We showed that combining the single mutants to the double E297A/D382A and triple mutant E297A/E303A/D382A led to a further decrease of the bTSH binding capability compared with the single alanine mutants. Cooperative negative effects caused by these mutations are also reflected by the right shift of the cAMP stimulation curves (Fig. 2).

The observed decline is most likely a cooperative negative effect by the mutants on bTSH binding and cAMP signaling and can be explained in two ways. The interaction of bTSH with the hinge region of the hTSHR is driven by (i) a multiple charge-charge recognition and (ii) a distinctive distribution of pattern of complementary electrostatic potentials. The additional positively charged residues of bTSH spatially match the binding-sensitive residues that are most likely arranged in clusters of negative electrostatic potentials at the surface of the hinge region of the TSHR (Fig. 4). Our schematic monomer model allows only a rough adjustment of particular basic residues of bTSH toward acidic residues of the receptor.

Taken together, our data indicate the following. 1) Apart from the amino acids of the LRRD, also positions Glu-297 and Glu-303 in the N-terminal and Asp-382 in the C-terminal portion of the hinge region are involved in hormone/receptor interaction. 2) Moreover, we show that the negative charge of Glu-297 and Asp-382 is necessary for hormone binding. 3) We demonstrate that the three acidic residues Glu-297, Glu-303, and Asp-382 most likely contribute together with the sulfated Tyr-385 to a bTSH binding site at the hinge region. 4) These findings substantiate a scenario where recognition and binding is driven by the attraction of complementary charges between the hormone (positive charges) and the extracellular hinge region (negative charges). 5) Subsequently, our data open a way for narrowing down approximate spatial orientations of the extracellular TSHR domains. Our findings, therefore, demonstrate that the hinge region of TSHR represents an important extracellular intermediate connector for both hormone binding and transduction of the activation signal toward the transmembrane region.

Our findings about a likely charge-charge interaction between the bovine TSH (high affinity) and the hinge region of the human TSHR raise the question of whether human TSH (containing less positive charged residues and providing less affinity) also interacts with the extended hormone binding site at the hinge region in charge-charge mode of action.

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