

Constitutive activation of the TSH receptor by spontaneous mutations affecting the N-terminal extracellular domain

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Abstract Activating mutations of the TSH receptor gene have been found in toxic adenomas and hereditary toxic thyroid hyperplasia. Up to now, all mutations have been located in the serpentine portion of the receptor. We now describe two additional mutations affecting Ser-281 (Ser-281-Thr and Ser-281-Asn) in the ectodomain of the receptor. After transfection in COS cells, both mutants displayed increased constitutive activity for cAMP generation despite expression at a lower level than the wild type. The mutants were responsive to TSH. The present results are compatible with a model in which the activity of the unliganded receptor is kept at a low level by an inhibitory interaction between the N-terminal domain and the serpentine portion of the receptor.

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Key words: TSH receptor; Activating mutation; Extracellular domain; Model of activation

1. Introduction

G protein-coupled receptors (GPCRs) belonging to the opsin subgroup constitute the largest family of membrane receptors, with more than 500 members expected [1,2]. Their sequence similarity and probable common evolutionary origin suggest that these receptors share common mechanisms of action. According to a current model for GPCR activation, the receptor would exist under at least two interconverting conformations: R (silent conformation) and R* (the active form). Binding of the ligand, to the slit between the transmembrane segments (for biogenic amines) [1] and/or residues of the N-terminal segment or extracellular loops (for neuropeptides) [3], is believed to stabilize the R* conformation [4]. The resulting change in the relative position of transmembrane helices translates into conformational changes of the cytoplasmic domains interacting with G proteins.

A variety of amino-acid substitutions at different positions of GPCR can lead to their constitutive activation [5–7]: the silent form of the receptors (R) would be submitted to a structural constraint which could be released by a number of different mutations [4,5]. The thyrotropin receptor is an interesting GPCR in this respect: (1) with the other glycoprotein hormone receptors (LH/CG α and FSH α), it is character-

ized by a structural and functional dichotomy between ligand binding, to the long N-terminal domain typical of these receptors, and activation of G α_s by the serpentine portion; (2) the wild-type receptor displays readily measurable constitutive activity upon transfection in COS cells [8,9]; and (3) amino-acid substitutions at as many as 19 different residues of the receptor increase its constitutive activity [9–11], present and submitted data).

All spontaneous activating mutations have been found in the serpentine portion of the receptor encoded by exon 10 [9,10,12,13]. Analysis of the functional characteristics of a series of constitutively active TSH receptor mutants [13] and the observation that low concentration of trypsin activates the receptor [14] led us to hypothesize that the unliganded N-terminal extracellular domain might contribute in keeping the serpentine portion of the receptor in the inactive (R) conformation. If correct, this model predicts the existence of activating mutations, not only in the exoloops of the serpentine domain (where they have been found [13]) but also in the N-terminal domain. In both cases, destruction of the interaction or emergence of new repulsive interactions between the extracellular and the serpentine domains would result in receptor activation.

2. Materials and methods

2.1. PCR, sequence and cloning

DNA was extracted from nodular tissue, juxtanodular quiescent tissue and peripheral blood leukocytes. PCR for exons 1–9 were performed using intronic primers [15] as described. PCR products were sequenced on both strands. After cloning, constructs were verified by sequencing on both strands.

2.2. Transfection and functional tests

COS-7 cells were used for transient expression allowing functional assays. They were transfected by the DEAE-dextran method followed by a dimethylsulfoxide shock [16].

Two days after transfection, cells were used for flow immuno-cytofluorometry, cAMP or inositol phosphate determinations and ¹²⁵I-TSH-binding studies. Triplicate dishes were used for each assay. Each experiment was repeated at least twice. Ser-281-Asn and Ser-281-Thr constructs were compared with the wild-type receptor. Cells transfected with pSVL alone were always run as controls.

(1) cAMP determination, inositol phosphate determination and binding assays were performed as previously described [17–20].

(2) Flow immuno-cytofluorometry: cells were prepared as described [14]. They were incubated with PBS-BSA 0.1% containing either the 2C11 monoclonal antibody (10 μ g/ml) against TSH α , kindly provided by Dr. A.P. Johnstone [21], or a new monoclonal antibody, BA8, obtained from genetic immunization with the wild-type TSH receptor cDNA (S. Costagliola et al., to be published). 2C11 recognizes a linear epitope (354–359; VFFEEQ) [14] whereas BA8 recognizes only the native receptor as expressed at the surface of cells (S. Costagliola, to be published).

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2.3. Computation of transfection efficiency and specific constitutive activity

Fold constitutivity above COS cells was determined for each construct using the following formula:

$$\{C_e = [A_n - Z(1-e)]/Ze\}$$

where A_n is the basal cAMP value as experimentally measured, Z is the basal cAMP value for mock-transfected cells (pSVL) and e is the transfection efficiency defined as the fraction of cells having taken and amplified the pSVL-based constructs. e was measured, with identical results, from FACS analyses using the wild-type TSH receptor construct and the 2C11 or BA8 monoclonal antibodies, or pSVL constructs harboring the green fluorescent protein. Transfection efficiency was consistent within a given experiment but varied significantly between different experiments. In preliminary experiments, it was checked that cAMP levels in untransfected COS-7 cells were not different from those in cells transfected with the empty vector. C_e is thus the ratio between the cAMP level in cells expressing the receptor constructs, over the 'house-keeping' cAMP in the same number of cells transfected with the empty pSVL vector. $\{C_e/R_e\}$ normalizes C_e for the level of expression of each construct (see below). A measure of the relative constitutivity of the mutant receptor over the wild type is given by $\{F = C_e \text{ mutant}/C_e \text{ wild type}\}$. This value does not account for the differences in expression of the mutants at the cell surface. It gives a measure of relative constitutivity at the actual level of expression achieved by individual receptor mutants. An estimation of 'specific constitutivity' (i.e. constitutivity normalized to equal level of expression) is given by $\{SCA = F/R_e\}$ where R_e is a measure of the expression of the mutant receptors relative to the wild type with $\{R_e = (\text{Mut-pSVL})/(\text{WT-pSVL})\}$, the ratio between the fluorescence (expressed in arbitrary units) of cells transfected with the mutant receptors (Mut) and the wild type (WT), after subtraction of the background fluorescence displayed by mock-transfected cells (pSVL). The comparison of SCA calculated for each construct determines the fold increase in constitutivity of each mutant over the wild type if their levels of expression were identical. In more recent experiments, R_e was determined with both 2C11 and BA8 monoclonal antibodies: the results were identical.

2.4. Statistics

The level of significance of the differences observed between the various constructs was evaluated by a two-tailed Student's *t*-test, two-tailed Wilcoxon's test or two-tailed Mann-Whitney's test as indicated in the text and in the legends to figures. $P < 0.05$ was considered significant.

3. Results

We analyzed exons 1–9 of the TSH receptor gene from a series of 9 autonomous nodules for which no mutation had been found in the serpentine portion of the receptor encoded by exon 10 or in $G_s\alpha$. Exons 1–9 were amplified by PCR on DNA extracted from individual adenomas, adjacent tissue and peripheral white blood cells. Direct sequencing of the PCR products on both strands identified two different mutations affecting the same Ser-281 residue in three adenomas: substitution of asparagine for serine (AGC→AAC; Ser-281-Asn) in one nodule (Fig. 1a) and threonine for serine (AGC→ACC; Ser-281-Thr) in two others (Fig. 1b). In the three cases, the mutations were in the heterozygous state and confined to the adenoma. Direct sequencing indicated a ratio of the mutant vs. the wild-type allele of < 1 (Fig. 1a,b) suggesting that the nodular tissues were heterogenous or contaminated by normal cells harboring the WT genotype (fibroblasts, endothelial cells, blood cells, etc.).

The functional characteristics of the Mut receptors were studied by transient expression in COS cells. The WT receptor and the empty pSVL vector were used as controls. Levels of expression at the cell surface were evaluated by flow cytometry using two monoclonal antibodies recognizing different

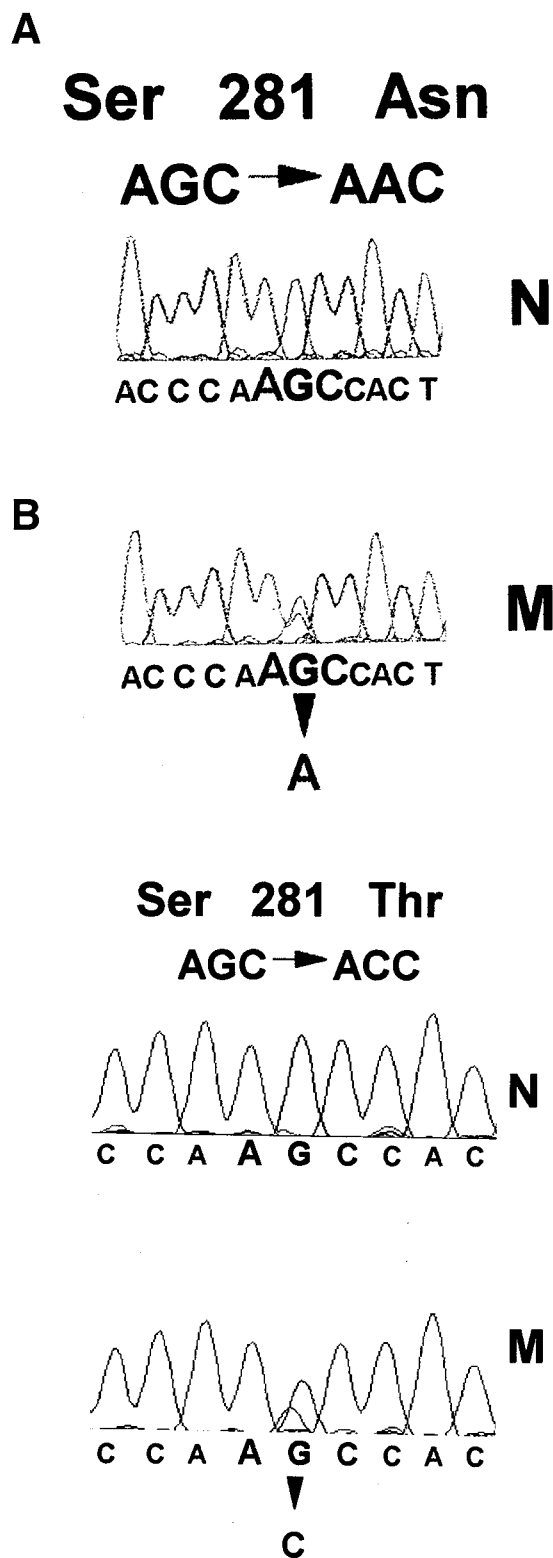


Fig. 1. Sequencing results: Direct sequencing of PCR products from a nodule shows a G to A transition leading to a substitution of asparagine for serine at position 281 (AGC→AAC) (a). In two different nodules, a G to C transversion led to a substitution of threonine for serine at the same position (AGC→ACC) (b). Sequencing was performed on both strands. Coding strands are shown for quiescent tissue harboring the normal allele (panel N) and for a nodule harboring the mutated allele (panel M).

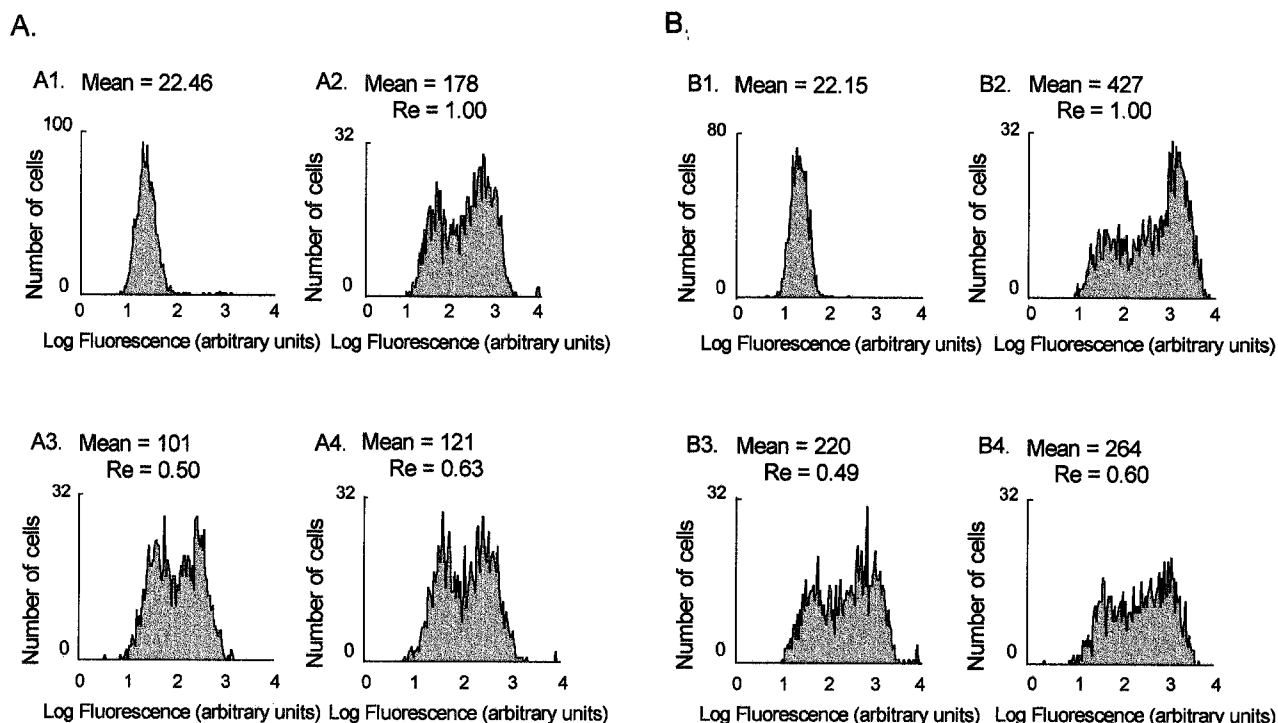


Fig. 2. Expression of the Mut receptors: Analysis by flow cytometry of the expression of the mutants. Graphics show the fluorescence intensity expressed in arbitrary units (log scale, abscissa) vs. cell number (in ordinates) (see Section 2). Panel A, the 2C11 monoclonal antibody was used; A1, mock-transfected cells (pSVL alone); A2, cells transfected with the WT receptor construct; A3, cells transfected with the Mut receptor Ser-281-Thr; A4, cells transfected with the Mut receptor Ser-281-Asn. Panels B1–4, same as A1–4, except that the BA8 monoclonal antibody was used. The mean fluorescence (mean) of the cells and the level of Mut receptor expression relative to the wild type (R_e , see Section 2) are indicated.

Table 1
Characteristics of the Ser-281-Thr and Ser-281-Asn mutants

	Expt.	cAMP base (pm/dish)	Ce	F	Re	Ce/Re	SCA
WT TSHr	1	6.60 ± 0.90	15.60	1.00	1.00	15.60	1.00
	2	22.30 ± 2.30	75.60	1.00	1.00	75.60	1.00
	3	9.75 ± 1.10	16.80	1.00	1.00	16.80	1.00
	4	18.70 ± 2.10	46.30	1.00	1.00	46.30	1.00
	5	5.90 ± 0.20	31.40	1.00	1.00	31.40	1.00
	6	8.84 ± 3.60	20.70	1.00	1.00	20.70	1.00
	7	69.47 ± 7.95	13.73	1.00	1.00	13.73	1.00
	8	22.29 ± 1.48	5.54	1.00	1.00	5.54	1.00
S281T	1	17.80 ± 3.90	45.90	2.94	0.71	64.65	4.06
	2	53.70 ± 9.00	187.80	2.48	0.90	208.67	2.75
	3	24.15 ± 7.30	46.60	2.77	0.46	101.30	6.08
	4	56.60 ± 1.90	151.60	3.27	0.64	236.88	5.10
	5	19.25 ± 0.20	107.25	3.42	0.29	369.83	11.56
	6	30.90 ± 3.90	79.00	3.82	0.53	149.06	7.14
	7	141.90 ± 14.55	28.49	2.07	0.63	45.22	3.29
	8	97.62 ± 6.07	19.46	3.50	0.72	27.03	4.88
		* $P = 0.0078$	* $P = 0.0078$	3.03 ± 0.58		* $P = 0.0047$	5.61 ± 2.79
S281N	1	22.20 ± 4.20	57.80	3.71	1.12	51.61	3.30
	2	44.20 ± 7.50	153.80	2.03	0.60	256.33	3.30
	3	23.90	47.40	2.82	0.66	71.82	4.20
	4	46.00 ± 3.30	122.10	2.64	0.55	222.00	4.80
	5	19.70 ± 0.14	109.80	3.50	0.43	255.35	8.05
	6	39.60 ± 1.00	102.00	4.93	0.51	200.00	9.50
	7	188.5 ± 0.71	37.99	2.77	0.50	75.98	5.53
	8	125.63 ± 2.27	25.17	4.50	0.50	50.34	9.08
		* $P = 0.0078$	* $P = 0.0078$	3.36 ± 0.99		* $P = 0.0011$	5.97 ± 2.55
			** $P = 1$			* $P = 0.7984$	

See Section 2 for the definition and computation of the C_e , F , R_e and SCA parameters. Results from 8 independent experiments performed in triplicate are shown. According to this parameter, Ser-281-Thr and Ser-281-Asn mutants are 6.10 ± 3.10- and 5.50 ± 2.60-fold more constitutive than the WT receptor, respectively. The differences between the mutants and the wild type were significant (* and ° P values) but the values obtained for the two mutants were not significantly different from each other (** and °° P values) (*two-tailed Wilcoxon's test, °two-tailed Mann-Whitney's test).

epitopes in the extracellular domain of the receptor [14,21] (see Section 2) (Fig. 2). Results indicate a slightly lower expression for both mutants (Fig. 2, Table 1). Although the mutations affect the extracellular domain, the apparent affinity of the mutant for bovine TSH was not decreased. On the contrary, and as previously observed for other TSHr mutants [9], the Mut receptors had a lower apparent dissociation constant (K_d) as compared to wild type: the K_d values for wild type, Ser-281-Thr and Ser-281-Asn mutants were 1.24 ± 0.71 , 0.58 ± 0.1 and 0.49 ± 0.11 mU/ml, respectively (mean \pm S.D., $n=4$). The difference between the K_d of the mutants and the K_d of the wild type was significant ($*P=0.0286$) although the difference between the mutants was not ($P=0.2$) (two-tailed Mann-Whitney's test).

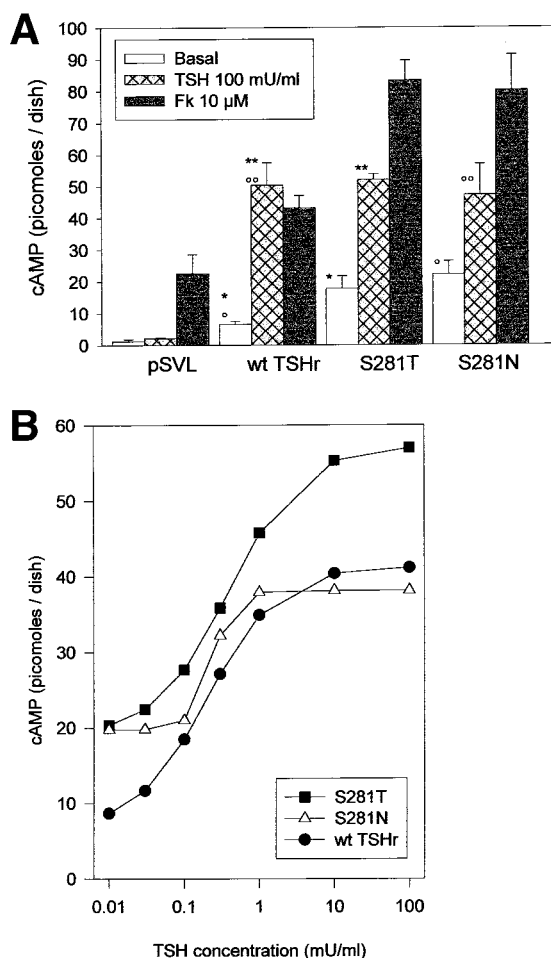


Fig. 3. cAMP determination: (a) Mut receptors (Ser-281-Asn and Ser-281-Thr), WT receptor (wt TSHr) and pSVL alone were transfected in COS cells (150 000 cells/dish). Accumulation of cAMP was determined under basal conditions (white columns), stimulation by TSH 100 mU/ml (crossed columns) or forskolin 10 μ M (gray columns). Values are mean \pm S.D. from a representative experiment out of 6 where determination was performed in triplicate. The significance of the differences between the mutants and the wild type was established after performing an unpaired two-tailed Student's *t*-test: $*P=0.0084$; $^{\circ}P=0.0033$; $**P=0.7073$; $^{\circ\circ}P=0.6735$. The difference between the basal values of the two mutants was not significant ($P=0.2544$). (b) Accumulation of cAMP was determined after stimulation of transfected cells by various TSH concentrations. The difference between the EC_{50} values obtained for the three constructs was not significant: 0.2 mU/ml for wt TSHr, 0.39 mU/ml for Ser-281-Thr ($P=0.9746$) and 0.24 mU/ml for Ser-281-Asn ($P=0.3703$) (unpaired two-tailed Student's *t*-test).

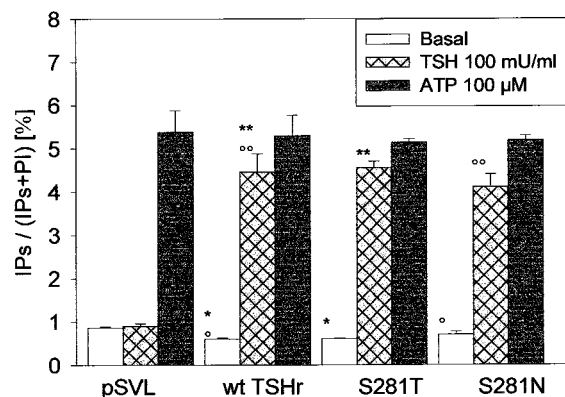


Fig. 4. IP determination: Mut receptors (Ser-281-Asn, Ser-281-Thr), WT receptor (wt TSHr) and pSVL alone were transfected in COS cells (150 000 cells/dish). Accumulation of IP was determined under basal conditions (white columns), stimulation by TSH 100 mU/ml (crossed columns) or ATP 100 μ M (gray columns). Results are expressed as the percentage of radioactivity incorporated in inositol-phosphates over the sum of radioactivity in inositolphosphates and phosphatidylinositols ($(IP_1+IP_2+IP_3)/(IP+PI) \times 100$). Values are mean \pm S.D. from a representative experiment out of two where determination was performed in triplicate. The differences between the mutants and the wild type were not significant: $*P=1$; $^{\circ}P=0.6681$; $**P=0.5292$; $^{\circ\circ}P=0.1913$ (unpaired two-tailed Student's *t*-test).

Cells transfected with the Mut receptors display a higher basal cAMP level than those transfected with the wild type (Fig. 3a). As described previously [17,22], there is synergism between stimulation by forskolin and the constitutive activity of the receptors (Fig. 3a). The data from flow immuno-cytofluorometry allow measurement of efficiency of transfection and computation of the increase in cAMP within the effectively transfected cells (see Section 2). It demonstrates a 3.03 ± 0.58 - and 3.36 ± 0.99 -fold increase in cAMP in cells transfected with the Ser-281-Thr and Ser-281-Asn Mut constructs over cells transfected with the WT construct, respectively (Table 1). Assuming that the mutations do not affect recognition by the monoclonal antibodies, these data allow also normalization of the increase in cAMP accumulation to the amount of receptor expressed at the cell surface, yielding an estimation of 'specific constitutive activity' (see Section 2): the Ser-281-Thr and Ser-281-Asn mutants were 5.61 ± 2.79 and 5.97 ± 2.55 more active than the wild type, respectively (Table 1).

When stimulated by TSH (Fig. 3b), the WT and Mut receptors displayed similar EC_{50} (≈ 0.2 mU/ml) and maximally active TSH concentration (≈ 10 mU/ml) for cAMP generation.

Basal inositol phosphate accumulation was identical in COS cells transfected with both Mut or WT receptor constructs. Under stimulation by 100 mU/ml TSH, the response was the same for the three constructs (Fig. 4).

4. Discussion

Mutations leading to an increase in the constitutive activity of the TSH receptor have been described in the second, third, sixth and seventh transmembrane segments, in the first and second exoloops and in the third cytoplasmic loop [9,10,13,17,18,23–31]. The majority of these Mut receptors share similar functional characteristics. The interest of the two new mutants described here, Ser-281-Thr and Ser-281-

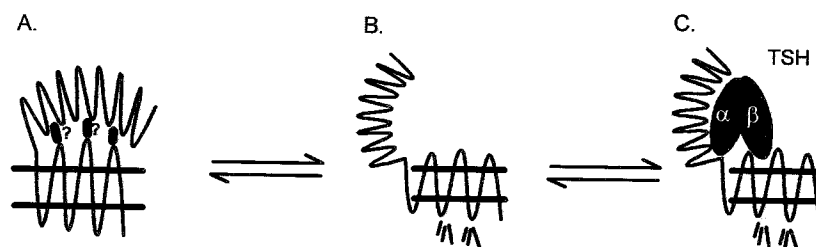


Fig. 5. Model for receptor activation: The model postulates that two forms of the unliganded TSH receptor co-exist: the 'closed' or inactive conformation (A), stabilized by putative interactions between the extracellular and the serpentine domains and the 'open' or active conformation where these interactions are released (B). Activation by TSH would result from the stabilization of the 'open' conformation (C).

Asn, resides more in the location of the Mut residue than in their functional characteristics which do not deviate from the general pattern.

Ser-281 is conserved in the extracellular domain of glycoprotein hormone receptors [28], downstream from the leucine-rich domain for which a structural model has been proposed recently [29]. Site-directed mutagenesis and chimeras studies have been performed to probe the structure–function relationships of the extracellular domain of the TSH receptor ([6,8,28] and references therein) Ser-281 belongs to a segment (amino acids 171–418) proposed to play a role in signal transduction [30]. Residues 270–278 and 287–297 would be particularly important in this regard [31] but the various constructs were not explored for their constitutive activity.

A currently favored model for GPCR activation holds it that a structural constraint is responsible for the maintenance of unliganded receptors in the inactive state [4]. This model was elaborated from the observation that a variety of amino-acid substitutions, first in the third intracytoplasmic loop of adrenergic receptors [32], then in transmembrane helices of others [33] could activate them in the absence of agonists. Whereas it is relatively easy to understand how binding of norepinephrine between transmembrane helices would stabilize the active conformation of adrenergic receptors, it is difficult to envision how binding of the bulky TSH to the N-terminal domain would result in the activation of the serpentine portion of the receptor.

A series of experimental observations led us to hypothesize that the extracellular domain of the TSH receptor could contribute in keeping its serpentine portion inactive: (1) amino-acid substitutions in the first (Ile-486) and second extracellular loops (Ile-568) are amongst the strongest activating mutations identified [13]; (2) the TSH receptor can be activated by a limited proteolytic treatment by trypsin which removes an epitope (residues 354–359) of the extracellular domain [14]; and (3) the group of Kosugi has demonstrated significant increase in constitutive activity of a deleted mutant lacking residues 339–367 [34]. These observations are compatible with a model in which the unliganded inactive conformation of the receptor would be stabilized by interactions between the extracellular N-terminus and the extracellular loops. Rupture of these interactions would activate the serpentine portion while increasing the affinity of the extracellular domain for TSH binding. The replacement of Ser-281 with two different amino acids (Asn or Thr), increasing the constitutive activity of the receptor, fits well with such a model. The recent identification of an additional and more drastic amino-acid substitution at the same residue (Ser-281-Ile) strengthen this view (P. Kopp and L. Jameson, personal communication).

According to the model, unliganded receptors would exist as an equilibrium between a 'closed' inactive conformation and an 'open' active conformation lacking the interaction between the loops and the N-terminal domain (Fig. 5). The concentration of the latter would be responsible for the constitutive activity of the WT receptor. Binding of TSH to the extracellular domain would activate the receptor by stabilizing the 'open' conformation. The model does not exclude that an interaction of TSH with the extracellular loops contributes to the stabilization of the active conformation of the serpentine portion as suggested by some experiments with the LH/CG receptor [35–38]. A model for activation of the LH/CG receptor is based on similar premises [39].

Our model is compatible with many experimental observations but not with all: (1) the expression of the serpentine domain alone should cause constitutive adenylyl-cyclase stimulation in transfected cells. This was apparently not the case in a widely quoted experiment performed with the LH/CG receptor [38]. For the TSH receptor, there has been no convincing report of expression at the cell surface of such truncated forms; (2) if stabilization of the 'open' conformation would automatically activate the receptor, all TSH binding molecular species should always be agonists. However, deglycosylated forms of TSH can lose their agonist properties while still capable of binding efficiently [40–42]. It is conceivable, as suggested for the LH/CG receptor [39], that the carbohydrate moiety of TSH contributes to the steric hindrance that follows the binding of the hormone and leads to stabilization of the activated conformation of the receptor. The 'open-closed' model is certainly a gross oversimplification of reality. We believe, nevertheless, that it has heuristic value as it provides an explanation for the activation of the receptor by its normal agonist, by partial proteolysis and suggests an appealing explanation for the activation by autoantibodies in Graves' disease. Similarly, and as evidenced by recent experiments [43], the effects of experimental antibodies with blocking activity (TSBab) could be to stabilize the silent, 'closed' conformation of the receptor thus behaving as inverse agonists.

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