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Constitutive activation of cyclic AMP but not phosphatidylinositol signaling caused by four mutations in the 6th transmembrane helix of the human thyrotropin receptor

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Abstract Four different somatic mutations (F631C, T632I, D633E, and D633Y) in the putative 6th transmembrane helix of the human thyrotropin receptor (TSHR) were recently described in hyperfunctioning thyroid adenomas [Porcellini et al. (1994) J. Clin. Endocrinol. Metab. 79, 657–661]. We transiently expressed these mutant receptors in Cos-7 cells and measured [^{125}I]TSH binding, basal and TSH-stimulated cAMP production, and phosphatidylinositol hydrolysis. The concentration of receptors expressed at the cell surface was lower for the mutants than for the wild type (WT) TSHR. Compared to the WT, all four mutant receptors caused a marked increase in basal cAMP levels, but did not increase basal production of inositol phosphates. This suggests that autonomous thyroid function and adenoma formation may be related to constitutive activation of the cAMP pathway alone. A cluster of conserved residues at the base of the 6th transmembrane helix of the TSHR and other glycoprotein hormone receptors appears important for maintaining an inactive receptor conformation.

Key words: Dual signaling; G protein; Receptor activation mechanism; Glycoprotein hormone receptor

1. Introduction

Members of the G protein-coupled receptor family share a similar predicted topology, consisting of seven transmembrane-spanning α-helical domains. The ability of certain amino acid substitutions in the C-terminal portion of the third intracellular loop to cause agonist-independent activation was first demonstrated in vitro for the adrenergic receptors [1–3], and subsequently for the thyrotropin receptor (TSHR) [4]. Naturally occurring, constitutively activating mutations of glycoprotein hormone receptor genes can serve as the basis of human disease: somatic and germline mutations of the TSHR are found in hyperfunctioning thyroid adenomas [5]) and autosomal dominant thyroid hyperplasia [6], respectively, and inherited mutations of the lutropin receptor (LHR) are the cause of familial male precocious puberty (FMPP) [7,8]. In these cases, autonomous endocrine activity has been attributed to hormone-independent activation of the cAMP signaling pathway [5–7].

Porcellini and coworkers [9] recently reported 4 new missense mutations of the TSHR (F631C, T632I, D633E, and D633Y) in hyperfunctioning thyroid adenomas. The three affected residues are located in the 6th transmembrane helix, and two of them (T632 and D633) correspond to LHR residues which were previously found to be mutated in FMPP [7,8,10] (Fig. 1). Glycoprotein hormone receptors not only mediate cAMP production, but are also capable of stimulating the phosphatidylinositol (PI) hydrolysis cascade that leads to production of diacylglycerol and inositol phosphates (IP) [11,12]. In order to better understand the role of the 6th transmembrane domain in the activation mechanism of glycoprotein hormone receptors, we transiently expressed the 4 new mutant TSHR in Cos-7 cells and measured [^{125}I]TSH binding, basal and TSH-stimulated cAMP production, and PI hydrolysis.

2. Materials and methods

2.1. Mutagenesis

Human TSHR cDNA [13] was inserted into the *Eco*RI site of M13mp18 vector and oligonucleotide-mediated, site-directed mutagenesis was used to generate clones encoding the desired mutation as previously described [4,14,15]. Residue numbers are determined by counting from the methionine start site. WT and mutant clones were inserted into the *Eco*RI site of the pSG5 expression vector. Mutations were confirmed by DNA sequencing of the final construct and plasmid DNA was purified by CsCl gradient ultracentrifugation.

2.2. Transfection

Transfection of Cos-7 cells with mutant or WT human TSHR or pSG5 vector DNA was performed by electroporation [4,14–17]; 25 μg purified plasmid DNA was used in each. Aliquots of the same batch of transfected cells were plated for TSH binding and assays of cAMP and IP production; medium was inositol free in the latter assays and supplemented with 2.5 μCi/ml myo-[2-^{3}H(N)]inositol (DuPont-NEN, Boston, MA).

2.3. Assays

All the assays were initiated simultaneously 48 h after transfection and after washing with assay buffer: Hanks' Balanced Salt Solution containing 0.5% crystalline BSA, and 20 mM HEPES at pH 7.4. [^{125}I]TSH binding was measured after incubation for 2 h at 22°C in 1 ml of NaCl-free assay buffer containing 222 mM sucrose, ~100,000 cpm [^{125}I]TSH (~40 μCi/μg) and 0 to 10^{-7} M unlabeled TSH. Total cAMP and IP levels were measured in the same wells after incubation for 1 h at 37°C with 0.2 ml assay buffer containing 10 mM LiCl, 0.5 mM 3-isobutyl-1-methylxanthine, and, as noted, 10^{-11} to 10^{-7} M TSH. Total cAMP was measured by radioimmunoassay and IP formation was determined using anion exchange columns [4,16].

All assays were performed at least in triplicate, on at least 3 separate occasions with different batches of cells, and always included control cells transfected with WT DNA or pSG5 vector alone. The program LIGAND [18] was used to calculate K_d and B_{max} values for TSH binding.

K_d data were log-transformed, averaged, and reconverted to calculate geometric mean. 95% confidence limit of K_d was obtained by log-transformation, calculating mean − 1.96 S.D. and mean + 1.96 S.D.,

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and reconversion (Table 1). cAMP and PI data are expressed as fold increase over basal in cells transfected with WT TSHR DNA in Table 1, and as fold-increase over basal in cells transfected with the pSG5 vector alone in Fig. 2.

3. Results

Binding experiments (Table 1) showed that the four mutant receptors were expressed on the cell surface at significantly lower concentrations than the WT TSHR (B_{max} values 36–59% of WT B_{max}). The affinity of the mutant receptors for TSH was slightly increased, but the difference reached statistical significance only for D633E.

In the absence of TSH, cells transfected with the WT TSHR had 2.5-fold higher basal cAMP accumulation than cells transfected with pSG5 vector alone (Fig. 2A). The agonist TSH produced a concentration-dependent increase in cAMP production in cells expressing the WT receptor, with an EC_{50} of 3×10^{-11} M and maximal stimulation of 6-fold over basal (Fig. 2A, Table 1).

Cells transfected with each of the four mutant constructs had a 3- to 4-fold increase in basal cAMP accumulation compared to cells transfected with the WT TSHR, indicating that they were constitutively activated (Fig. 2A, Table 1). Mutant receptors were also capable of responding to increasing concentrations of TSH with additional cAMP production. Maximal TSH-stimulated cAMP production in mutant-transfected cells was similar or somewhat lower than that in WT-transfected cells, and the EC_{50} values were comparable (Fig. 2A, Table 1).

Cells expressing WT TSHR did not show increased accumulation of IP in the absence of agonist (Fig. 2B). TSH produced a concentration-dependent increase in IP production, but at least 10^{-10} M TSH was required to elicit a response. The maximum concentration of TSH tested (10^{-7} M) produced an 8-fold stimulation of basal. Unlike the findings with cAMP production, none of the mutant TSHR elicited a significant increase in IP production in the absence of TSH (Fig. 2B, Table 1). IP production in mutant-transfected cells exposed to 10^{-7} M TSH was lower than that in WT-transfected cells (Fig. 2B, Table 1).

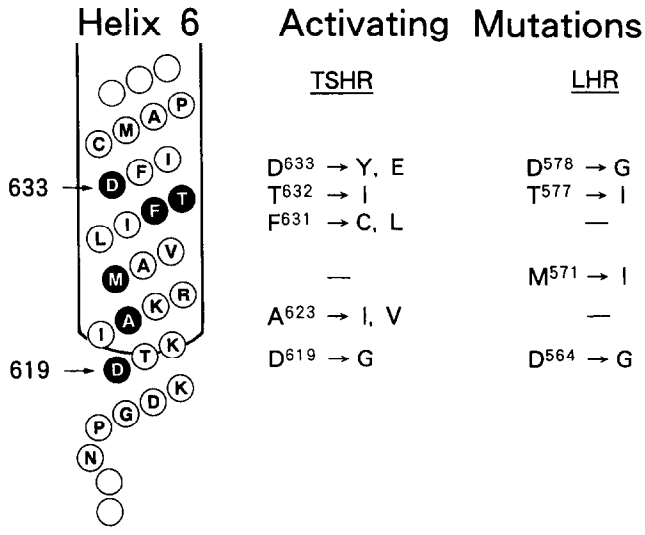

Fig. 1. Schematic view of the putative 6th transmembrane helix and C-terminal portion of the 3rd intracellular loop of the human TSHR. Naturally-occurring activating mutations in this region of the TSHR and LHR [5,7–10,30–32] are shown. Residues equivalent to TSHR 633, 626, 623, and 619 may be located on the surface of the α-helix that is oriented toward the receptor cleft [26,27].

4. Discussion

The glycoprotein hormone receptors are characterized by several common features, including their large extracellular, N-terminal hormone binding domains, a conserved pattern of residues in their transmembrane domains, and their ability to couple dual, G protein-mediated signaling pathways [11,12]. The TSHR and LHR are coupled less efficiently to the PI hydrolysis cascade, which is presumably mediated by a member of the G_q family, than to the G_s-mediated cAMP pathway [11,12,19,20]. The recent observation that identical disease-causing missense mutations occur in the TSHR and LHR (Fig. 1) suggests that these receptors may share a common molecular activation mechanism.

Table 1
Characterization of mutant TSH receptors

	WT	F631C	T632I	D633Y	D633E
TSH binding					
K_d (pM)*	455 (289–714)	103 (28–379)	72 (16–331)	157 (66–374)	144 (133–155)
% B_{max} (/WT**)	100	44.7 ± 7.4	36.1 ± 4.1	58.6 ± 3.2	58.6 ± 0.6
Number of experiments	4	4	3	3	4
cAMP increase					
%Basal (/WT basal)	100	302 ± 4	369 ± 18	405 ± 8	389 ± 13
Max Resp (/WT basal)***	631 ± 22	628 ± 52	518 ± 37	556 ± 17	632 ± 31
IP increase					
%Basal (/WT basal)	100	100 ± 4	128 ± 10	127 ± 11	125 ± 11
Max Resp (/WT basal)***	757 ± 191	646 ± 141	523 ± 133	582 ± 124	425 ± 22
Number of experiments	5	5	4	5	5

*Geometric mean. Values in parentheses are 95% confidence limits.
**WT B_{max} averaged 92.8 ± 14.7 fmol/well.
***Maximal response at 10^{-7}M TSH.
The mutant B_{max}, cAMP, and PI data (mean ± S.E.) are expressed as a percentage of WT TSHR values.

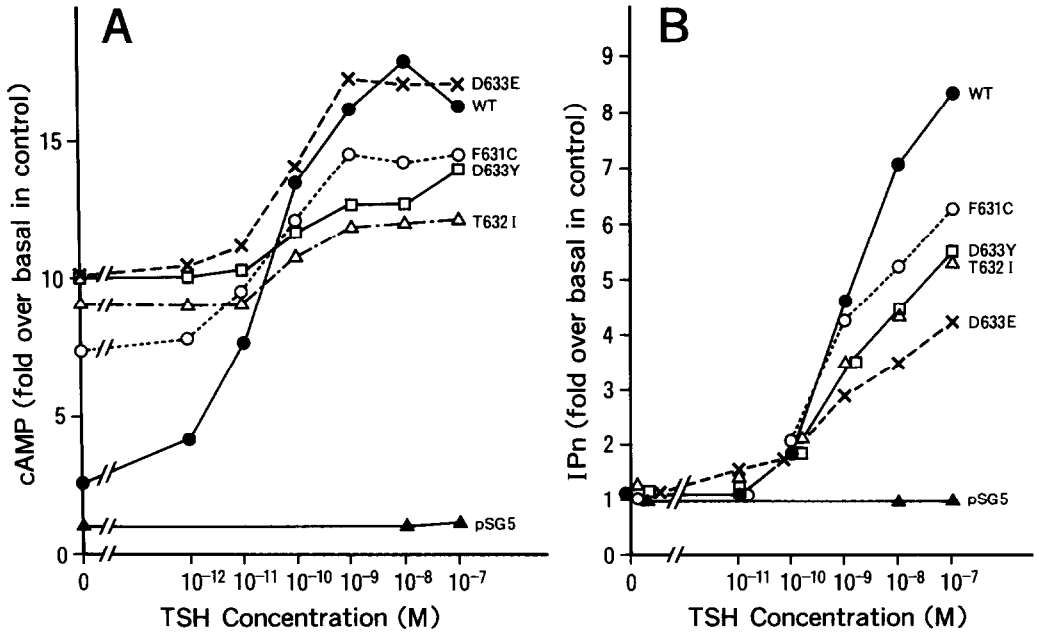

Fig. 2. Basal and TSH-stimulated accumulation of cAMP (A) and inositol phosphates (IPn) (B) in Cos-7 cells transfected 48 h earlier with control vector (pSG5), wild type human TSHR DNA (WT), or TSHR DNA encoding F631C, T632I, D633E and D633Y mutations. Data are means of 4–5 experiments, and are expressed as fold-increase over basal in cells transfected with the pSG5 vector alone.

Characterization of the first four naturally-occurring TSHR gene mutations indicated that the encoded substitutions were capable of causing agonist-independent production of cAMP, but not IP, compared to the WT receptor [5,6]. Two (F631C and T632I) of the four new TSHR mutations were shown to cause constitutive activation of cAMP-dependent transcription when transfected into mouse fibroblasts, but the ability of these mutant receptors to bind TSH and activate PI hydrolysis was not studied [9]. We now show that the activating effect of the four new mutations, like that of the earlier mutations, is restricted to the cAMP pathway. The fact that the B_{max} values of the mutant receptors are decreased compared to WT B_{max} indicate that increased basal cAMP accumulation is not simply due to receptor overexpression, and may explain why maximal TSH-stimulated IP production is decreased in the mutants.

Inactive receptors are thought to exist in a constrained conformation that is relieved by the binding of agonist. The resulting conformational change allows cytoplasmic domains of the receptor, including portions of the third intracellular loop, to interact productively with G proteins. In theory, activating amino acid substitutions may mimic agonist occupancy by increasing the proportion of receptors that are in the active conformation, or by increasing the affinity of the activated receptor for G protein [2].

Fig. 2 shows that the WT human TSHR has the capacity to activate cAMP production (but not IP production) in the absence of agonist. This property has been noted previously with the human TSHR [5,6,21], as well as with the rat TSHR [4,16,17] and the β_2-adrenergic receptor [2]. The amount of cAMP stimulation has been shown to be related to the level of receptor expression [2,6,16]. Substitutions of certain residues in the third cytoplasmic loop of the rat TSHR are capable of eliminating spontaneous receptor activity [4].

Unlike the WT TSHR, the closely related LHR is 'silent', and fails to stimulate basal cAMP production, even when studied under the same assay conditions [7,21]. Thus, normal human TSHR and LHR appear to have an intrinsic difference in their tendency to undergo isomerization and activate G_s in the absence of agonist [2]. Both the physiological significance and the structural basis of this difference remain to be defined.

Why are the activating effects of the TSHR mutations restricted to the cAMP pathway? The coupling of glycoprotein hormone receptors to the PI hydrolysis pathway is known to be inherently less efficient than coupling to the G_s-mediated cAMP pathway, perhaps because of differences in kinetics, or in the nature of the signaling components involved [20]. Relatively high concentrations of agonist and/or receptor are required to elicit stimulation of PI hydrolysis. As noted above, the WT TSHR does not stimulate basal IP production. Study of a series of different mutant rat TSHR constructs showed that receptors that caused constitutive cAMP production (275% over basal) were only capable of producing a small increase (30–50%) in basal IP levels, while mutant receptors with diminished basal cAMP production also lost the ability to mediate even TSH-stimulated PI hydrolysis [4].

We speculate that the concentration of mutant TSH receptors that spontaneously assume an activated conformation in Cos-7 cells may be adequate to drive cAMP production, but inadequate to stimulate a significant increase in PI hydrolysis. Regardless of the mechanism, the finding that naturally-occurring TSHR mutations lead to constitutive activation of cAMP signaling, but not PI hydrolysis, suggests that autonomous thyroid function and adenoma formation is primarily related to activation of the cAMP pathway.

Activating mutations in adrenergic receptors not only lead to agonist-independent signaling, but also cause dramatic increases in agonist binding affinity and agonist potency, and decreased receptor expression levels [1–3]. Activating muta-

tions in the glycoprotein hormone receptors are associated with decreased B_{max} values but little or no increase in agonist affinities (Table 1, unpublished results). This discrepancy may be due to the fact that, unlike the adrenergic receptors, the major determinants of agonist affinity in glycoprotein hormone receptors are residues in the N-terminal domain [11,12], and hormone binding by these two classes of receptors is therefore regulated differently [22].

The localization and characterization of mutations that mimic agonist occupancy may provide insight into the normal mechanism of receptor activation. Residues at the base of helix 6 and in the adjacent C-terminal portion of the 3rd intracellular loop are thought to play an important role in receptor-G protein coupling. Naturally-occurring TSHR mutations in this region occur at residues (D619, A623, F631, T632, D633) that are perfectly conserved among all mammalian glycoprotein hormone receptors and homologous receptors from invertebrates [23,24]. The 3D structure of G protein-coupled receptors is unknown, but models of the possible orientation of the transmembrane helices have been constructed [25–27]. Specific interactions between these helices may be critical for maintaining the inactive receptor conformation [6,7,25].

For example, the effects of amino acid substitutions at D633 in the TSHR, or at the equivalent aspartate in LHR ([7,28] and unpublished results), suggest that the side chain of this residue may normally form a stabilizing interhelical hydrogen bond. It is unclear why substitutions at the adjacent threonine and phenylalanine residues are also constitutively activating, but it is known that threonine has the potential to participate in stabilizing intrahelical hydrogen bonds [29]. Additional study of the activating substitutions found in this and other regions [6,30–32] may shed light on the mechanism by which the TSHR and other glycoprotein hormone receptors normally undergo conformational change.

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