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The first cytoplasmic loop of the thyrotropin receptor is important for phosphoinositide signaling but not for agonist-induced adenylate cyclase activation

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Abstract

We investigated the role of the 1st cytoplasmic loop of the thyrotropin receptor (TSHR) on signal transduction using mutants as we did the 2nd and 3rd cytoplasmic loops [Kosugi S. et al. (1994) Mol. Endocrinol., in press; and 7, 1009–1020]. Five substitution mutants involving the first cytoplasmic loop showed a TSH- or Graves' IgG-stimulated cAMP response despite the low TSH binding B_{max}. All the mutants completely lost or markedly decreased the TSH- or Graves' IgG-stimulated inositol phosphate increase. These findings suggest that the 1st cytoplasmic loop of the TSHR does not play a crucial role in agonist-induced adenylate cyclase activation but that it is important for phosphoinositide signaling.

Key words: Dual signaling; cAMP signal; PIP_2 signal; G protein; Graves' disease

1. Introduction

The thyrotropin receptor (TSHR) activates phosphatidylinositol bisphosphate (PIP_2) as well as cAMP signals when stimulated by TSH or TSHR auto-antibodies from patients with Graves' disease [1–4]. Both signals are involved in growth and various cellular function in thyroid cells [5]. Localization of sites in TSHR involved in each signal transduction is important for understanding the mechanism of receptor activation. We have investigated the 3rd [2,4] and 2nd [6] cytoplasmic loops of the TSHR by substituting the corresponding sequence from adrenergic receptors (ARs) and have found that: (i) the middle portion of the 2nd loop is important for agonist-induced cAMP production; (ii) widely distributed portions of the both loops are important for PIP_2 signaling; and (iii) both loops are important for regulating basal cAMP levels.

To accomplish characterization of the roles of the cytoplasmic loops in signal transduction activities, we have extended the substitution/mutagenesis study to the 1st cytoplasmic loop.

2. Materials and methods

2.1. Mutagenesis

Oligonucleotide-mediated, site-directed mutagenesis was used to create the mutants as previously described [2,4,6].

2.2. Transfection

Cos-7 cells were transfected with mutant DNA by electroporation [2,4,6]; 25 μg purified plasmid DNA was used in each. To evaluate the transfection efficiency, pSVGH was co-transfected with mutant or wild-type (WT) TSHR or pSG5 vector cDNA. Aliquots of the same batch of transfected cells were plated for Northern blots, Western blots, TSH binding and cAMP/inositol phosphate assays. The medium was inositol free in the latter and supplemented with 2.5 μCi/ml myo-[2-^{3}H(N)]inositol (DuPont-NEN, Boston, MA).

2.3. Assays

All assays were simultaneously initiated 48 h after transfection [2,4,6] and after washing with assay buffer: NaCl-free, Hanks' Balanced Salt Solution containing 0.5% BSA, 222 mM sucrose, 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 assay buffer containing [^{125}I]TSH and 0 to 10^{-7} M unlabeled TSH. Specific binding was obtained by subtracting the values obtained in the presence of 10^{-7} M unlabeled TSH. Total cAMP and inositol phosphate 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, 0.5 or 5.0 mg/ml Graves' or normal IgG, or 10 μM ATP. Total cAMP was measured by radioimmunoassay and the inositol phosphate formation was determined using anion exchange columns.

All assays were performed in duplicate, on at least 3 separate occasions with different batches of cells, and with simultaneously run positive and negative controls, namely cells transfected with WT TSHR or pSG5 vector alone respectively. Values in each well were corrected for amount of cell protein. The program LIGAND [7] and a single high affinity site model were used to calculate the K_d values for TSH binding and EC_{50} values for TSH-increased inositol phosphate and the cAMP levels.

The GH concentration in the cultured media of cells used for the

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assays were determined by RIA. RNA from transfected Cos-7 cells was Northern blotted as previously described [2,4,6] using total RNA and a WT, full-length rat TSHR cDNA probe.

2.4. Western blot

Western blots of membrane proteins derived from an aliquot of Cos-7 cells transfected with WT or mutant receptor cDNA and used for the assays described above, were performed as described [8].

2.5. Statistics

All values in Table 1 are presented as means ± S.E.M. from multiple experiments as noted (Table 1). Student's *t*-test was used for statistical analyses. Statistical differences in K_d and EC_{50} are calculated using log-transformed values.

3. Results

The 1st cytoplasmic loops of the α_1-AR and β_2-AR are identical in length to that of the TSHR, as they are in most receptors with 7 transmembrane domains [9]. Thr-447 in the TSHR is conserved in α_1- and β_2-ARs. We therefore created a series of mutants dividing the 1st cytoplasmic loop of the TSHR into 2 segments, residues 441–446 and 448–450, and substituting counterpart residues from the ARs (Fig. 1). These constructs are denoted as A or B for α_1-AR and β_2-AR substitutions, respectively, and by the initial residues substituted. For example, A441 substitutes α_1-AR residues in the 441–446 positions of TSHR. We additionally created a mutant with the same substitution in the 1st cytoplasmic loop as described by Chazenbalk et al. [10]. This is termed R441 and involves residues 441–450. Further, we created a

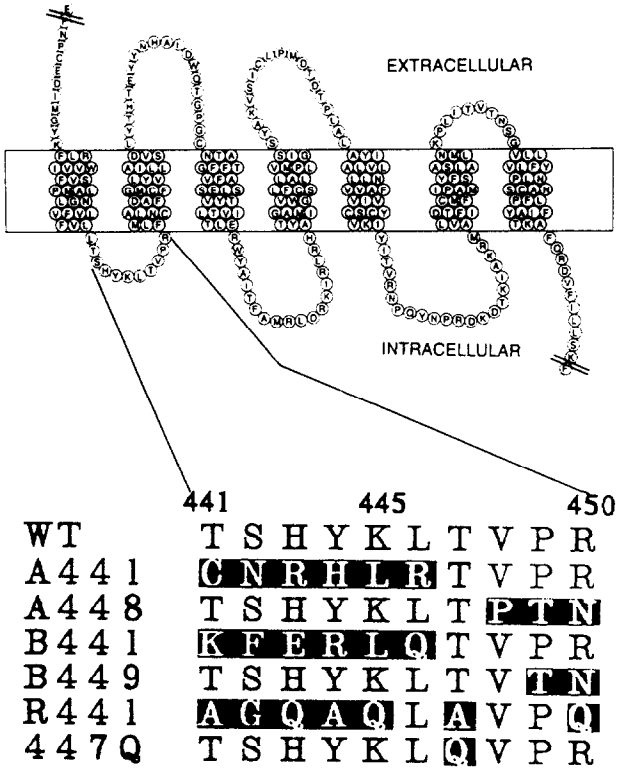

Fig. 1. Putative membrane topology of the transmembrane region of the rat TSH receptor (TSHR) and the amino acid sequence of its 1st cytoplasmic loop between residues 441 and 450. The boxed residues detail the sequence change in each TSHR mutant. The R441 mutant is the same as that previously described (MUT1-TSH-R in [10]).

Table 1
Summary of mutant activities

Mutant	pSG5	WT	A441	B441	A448	B449	R441	448Q
TSH binding								
K_d (pM)	–	213 ± 23	17 ± 5**	9 ± 3**	35 ± 9**	106 ± 31	10 ± 3**	ND#
$\%B_{max}$ (/WT)	–	100	7 ± 3**	6 ± 2**	7 ± 1**	33 ± 2**	6 ± 1**	ND
cAMP increase								
EC_{50} (pM)	–	125 ± 18	150 ± 82	343 ± 167	343 ± 271	275 ± 141	181 ± 90	NR##
% Basal (/pSG5)	100	294 ± 26	104 ± 5**	91 ± 14**	104 ± 15**	121 ± 6**	106 ± 8**	102 ± 7**
Max Resp (/pSG5)+	–	17.8 ± 1.2	7.5 ± 1.1*	3.7 ± 0.3**	5.7 ± 1.1**	10.7 ± 0.3*	4.2 ± 0.5**	–
Max resp (/basal)++	–	6.0 ± 0.7	7.2 ± 1.2	4.1 ± 0.4*	5.5 ± 1.1	8.8 ± 0.3*	4.0 ± 0.4*	–
IPn increase								
EC_{50} (pM)	–	571 ± 62	NR	NR	2100 ± 221*	1920 ± 165*	NR	NR
% Basal (/pSG5)	100	96 ± 2	95 ± 1	101 ± 3	99 ± 3	98 ± +1	102 ± 4	95 ± 4
Max Resp (/pSG5)+	–	3.3 ± 0.2	–	–	1.1 ± 0.1**	1.3 ± 0.1**	–	–
Number of Experiments	9	9	3	3	3	3	3	3

Values are expressed as means ± S.E.M. of all experiments whose numbers are shown in the bottom line.
#Not detectable.
##Non-responsive.
**Statistically significant difference from WT ($P < 0.01$).
*Statistically significant difference from WT ($P < 0.05$).
+Maximal response compared with basal of pSG5 transfectant (-fold).
++Maximal response compared with basal of each mutant transfectant (-fold).

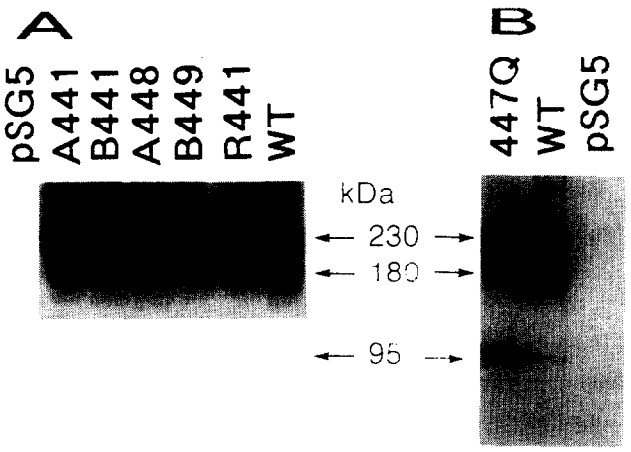

Fig. 2. Western blot of TSHR forms in membranes from Cos-7 cells transfected with 25 μg wild-type and mutant plasmid or the pSG5 vector.

point mutation at the conserved Thr-447 which is replaced by Glu and termed 447Q. The same designation is used to describe the mutant cDNA itself and the Cos-7 cells transfected with the mutant cDNA.

To accurately compare functional activities among transfectants, cells were transfected by electroporation in batches, and aliquots from one transfectant were used for GH measurement, Northern blots, Western blots, TSH binding and cAMP/inositol phosphate assays simultaneously. Transfection efficiency, determined by co-transfecting 0.1 μg pSVGH and measuring the GH concentrations in the culture media, was always within ± 15% of the mean. The mRNA in Cos-7 cells transfected with the mutants, estimated by Northern analysis of the total RNA, was identical in size and similar in amount (within ± 20% of the mean) to that from cells transfected with full-length WT TSHR cDNA. All the mutants exhibited the same pattern and a similar amount of TSHR forms on Western blots of membrane preparations from the cells (Fig. 2). Thus, similar amounts of the three major TSHR forms, 230, 180 and 95 kDa, were identified in three independent experiments using different batches of transfected cells.

Ten μM ATP increased inositol phosphate levels 3.7 ± 0.4-fold (mean ± S.E.M.) in Cos-7 cells transfected with WT. Every mutant transfectant, exhibited essentially the same response to ATP (data not shown), indicating the abnormal inositol phosphate response to TSH or Graves' IgG is TSHR specific.

3.1. Functional activities of mutant receptors

As shown in Fig. 3A and Table 1, A441 and B441 exhibited a markedly increased binding affinity to TSH compared with WT. There was, however, a similar decrease in B_{max}. The EC_{50} for TSH in cAMP assays was not statistically different from that of WT. However, the maximal response relative to the level in pSG5 control transfectants was markedly decreased because the basal cAMP levels of these mutant transfectants were decreased to the pSG5 level (Fig. 3B). Thus, WT increased the basal cAMP level compared with the pSG5 control transfectant as previously described [2]. The maximal response compared with the basal levels of the same transfectant in the absence of TSH, was slightly but significantly decreased in B441 (Table 1). In contrast, that was rather increased in A441. On the other hand, the increase in inositol phosphate levels induced by TSH was completely abolished in both A441 and B441.

Mutants involving the carboxyl-terminal of the 1st cytoplasmic loop, A448 and B449, which replace only 3 or 2 amino acids, respectively, also had marked effects on functional activities (Fig. 4 and Table 1). Both exhibited a significant decrease in TSH binding B_{max} and the affinity of A448 was significantly increased. The EC_{50} in the TSH-cAMP response was not significantly different from the WT and the basal cAMP levels were markedly decreased, like those of A441 and B441. The maximal responses were also significantly decreased compared with WT when expressed relative to the pSG5 control transfectant level. The maximal response relative to the

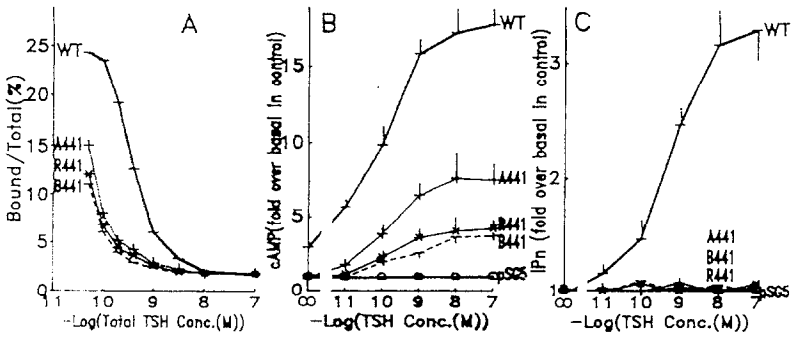

Fig. 3. Displacement of [^{125}I]TSH binding by unlabeled TSH (A) and the ability of TSH to increase cAMP (B) and inositol phosphate formation (C) in Cos-7 cells transfected with wild-type (WT) and mutants involving the 1st cytoplasmic loop (A441, B441 and R441) (Fig. 1). The data in A are from a single experiment performed in duplicate. Essentially identical results were obtained in at least 3 separate experiments. The K_d and B_{max} values determined by the program LIGAND [7] are noted in Table 1 and are the means (± S.E.M.) of all experiments. In B and C, each point is the mean ± S.E.M. of all experiments, the number of which is noted in Table 1. In each case, data are from cells transfected with 25 μg cDNA, including cells with the pSG5 vector alone. TSH had no effect on cAMP or inositol phosphate formation in control cells transfected with pSG5.

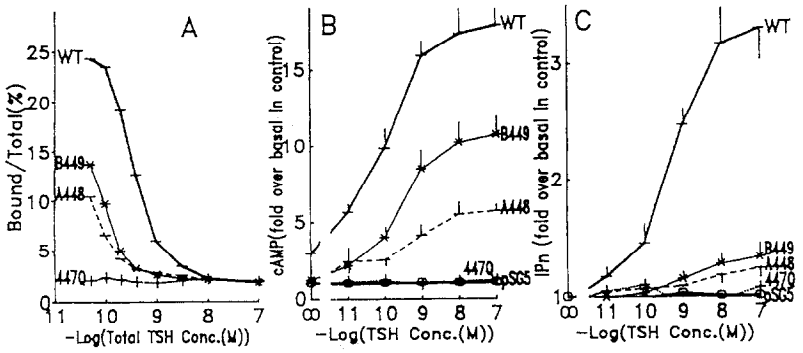

Fig. 4. Displacement of [^{125}I]TSH binding by unlabeled TSH (A) and the ability of TSH to increase cAMP (B) and inositol phosphate formation (C) in Cos-7 cells transfected with WT and mutants involving the 1st cytoplasmic loop (A448, B449 and 447Q) (Fig. 1). The conditions and expression of the data are exactly the same as those in Fig. 3.

basal level without TSH in its own transfectant was actually better in B449. In TSH-inositol phosphate assays, both mutants showed markedly decreased responses in terms of both EC_{50} and maximal responses but they were still detectable, unlike A441 and B441. The only difference between A448 and B449 is residue 448. Val-448 is replaced by Pro in A448. This might be attributed to a more profound loss in B_{max} and the maximal responses of cAMP/inositol phosphates to TSH in A448 by comparison with B449.

Chazenbalk et al. [10] have reported that a non-homologous substitution mutant involving the 1st cytoplasmic loop lost the cAMP response to TSH completely when transfected into Chinese hamster ovary cells. They concluded that the 1st cytoplasmic loop of the TSHR was also important for G_s activation. Because there was no AR substitution mutant involving the 1st cytoplasmic loop that had lost the agonist-induced cAMP response, we created a mutant identical to that described by them [10] and tested it under the same conditions as the AR substitution mutants. As shown in Fig. 3A and Table 1, R441 exhibited higher affinity and lower TSH binding B_{max} compared with WT. Although the maximal response relative to both the pSG5 control level and the basal level in the same transfectant in the absence of TSH were significantly lower than those of WT, the EC_{50} in the TSH-cAMP response was not significantly different from that of WT (Fig. 3B and Table 1). The inositol phosphate response induced by TSH was completely abolished like that of A441 and B441 (Fig. 3C).

Thr-447 is conserved among most receptors with 7 transmembrane domains [9]. To evaluate its importance, we created a point mutation at this residue replacing it with Gln. Unexpectedly, this mutant neither bound TSH nor gave a cAMP/inositol phosphate response to TSH (Fig. 4 and Table 1) or Graves' IgGs (Table 2), providing no informative evidence. This mutant has the smallest change in amino acid sequence among all the mutants created in this series. R441 also have Thr-447 replaced with Ala, but did not completely abolish TSH binding or signal transduction activities.

As shown in Table 2, Graves' IgG_1 which was used in our previous studies [2,4,6] stimulated the cAMP response in all the transfectants except 447Q. The cAMP

Table 2
cAMP and inositol phospate levels stimulated by Graves' IgG#

Mutant	pSG5	WT	A441	B441	A448	B449	R441	447Q
cAMP (/pSG5)+								
IgG# 0.5 mg/ml	1.0	9.7	3.1	1.6	2.1	3.0	1.9	1.0
5.0 mg/ml	1.0	14.0	5.1	2.2	3.1	5.9	2.8	1.0
cAMP (/normal IgG)++								
IgG# 0.5 mg/ml	1.0	3.3	3.3	1.8	2.0	2.5	1.8	1.0
5.0 mg/ml	1.0	4.8	4.9	2.4	3.0	4.9	2.6	1.0
IPn (/pSG5)+								
IgG# 0.5 mg/ml	1.0	1.5	1.0	1.0	1.0	1.0	1.0	1.0
5.0 mg/ml	1.0	2.5	1.0	1.0	1.1	1.1	1.0	1.0

Values are expressed as means of 3 independent experiments performed in duplicate. Variances of values are within 20%.

#Standard Graves's IgG_1 used in previous reports [2,4,6].
+-Fold increase from basal activity in control pSG5 transfectant.
++Fold increase from basal activity with 5.0 mg/ml normal IgG in each transfectant.

responses induced by Graves' IgG in the mutants when compared with the pSG5 control transfectant level were markedly lower than those of WT because only WT increased the basal cAMP level. However, when compared with the basal level of each transfectant in the absence of TSH, A441 and B449 responded to Graves' IgG similarly to WT. B441 and R441 responded poorly as was the case with maximal response to TSH. In contrast, Graves' IgG failed to increase inositol phosphates in any of the mutant transfectants. Generally, the cAMP and inositol phosphate responses induced by Graves' IgG approximated the responses by TSH.

4. Discussion

All the AR substitutions and R441 involving the 1st cytoplasmic loop reasonably retained the agonist-induced cAMP response but decreased the basal cAMP level and almost completely lost the inositol phosphate response. They (except B449) had a markedly decreased TSH binding B_{max} and significantly increased binding affinity despite of a similar amount of the completely processed 95 kDa TSHR form on Western blots.

These results lead to the conclusion that the entire portion of the first cytoplasmic loop of the TSHR is important for agonist-induced PIP_2 signaling and the basal cAMP level, but that it is not crucial for the agonist-induced cAMP response. Furthermore, the present results together with those of other mutants involving the cytoplasmic loops of the TSHR such as mutations of Ala-623 in the 3rd cytoplasmic loop [2] suggest that mutants which couple with G_s but not with G_q are shifted to a direction to higher affinity and lower capacity. In contrast, B525 [6] which involves the 2nd loop and couples only with G_q, shifts the equilibrium in the other direction, lower affinity and higher capacity.

The evidence obtained in these studies seem to be applicable to gonadotropin receptors which are quite homologous to TSHR in their transmembrane region, especially in the first cytoplasmic loop. The differences in the 1st loop among them are as follows. In the FSHR, the counterpart residue of His-443 in the TSHR is replaced by Gln [11]. In the rat, human and mouse LH/CGR, it is replaced by Arg, but in the porcine LH/CGR it remains His [12]. Thr-447 is replaced by Asn in human TSHR [13] but conserved among all other glycoprotein hormone receptors thus far sequenced [11–15]. This and the results of 447Q might indicate the importance of Thr-447 in the functional expression of the receptor, such as proper protein folding.

Finally, the data obtained from all substitution mutants involving one of the three cytoplasmic loops of the TSHR ([2,4,6] and present report) showed that: (i) the middle portion of the 2nd cytoplasmic loop of the TSHR is crucial for agonist-induced cAMP response; (ii) widely distributed portions of the three loops, namely the entire portion of the 1st cytoplasmic loop, the amino-terminal and middle portions of the 2nd cytoplasmic loop, and amino- and carboxyl-terminals of the 3rd cytoplasmic loop, are important for PIP_2 signaling; (iii) those portions are also important for increasing basal cAMP level; and (iv) the middle portion of the 3rd cytoplasmic loop is important for preventing the TSHR from activating signal transduction in the absence of agonist.

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