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The third exoplasmic loop of the thyrotropin receptor is partially involved in signal transduction

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Abstract

We characterized the role of the three exoplasmic loops in the transmembrane region of the thyrotropin receptor (TSHR) by substituting each loop with the counterpart of β_2-adrenergic receptor amino acid sequence. Mutant E13 with a substitution of the 3rd exoplasmic loop showed significant decrease in the maximal level and sensitivity of TSH-stimulated cAMP response despite good retention of TSH binding. These findings suggest that the third exoplasmic loop of the TSH receptor is partially involved in the signal transduction mechanism.

Key words: cAMP signal; PIP_2 signal; G protein-coupled receptor; Dual signaling

1. Introduction

The thyrotropin receptor (TSHR) as well as gonadotropin receptors have a long amino-terminal extracellular domain which is postulated to confer ligand binding specificity, and a carboxyl-terminal transmembrane domain with 7 transmembrane spanning helices which directly regulates signal transduction activity [1–8]. How such receptors are activated by a ligand which initially binds to the extracellular domain is of interest in terms of the structural characteristics of this class of receptors. This is because 7 transmembrane receptors for small ligands such as amines, short peptides, chemical odorants and light photons, have been known to bind the ligands on the outer surface of their transmembrane region to directly change their conformation and activate guanine nucleotide binding (G) proteins [9]. There is a speculation that ligand/extracellular domain complex or the extracellular domain which has been conformationally changed by ligand binding, contacts the outer surface of the transmembrane domain to activate the receptor [4]. To clarify this possibility and to examine the roles of the exoplasmic loops, we made substitution mutants involving individual exoplasmic loops of the TSH receptor, transfected the mutants into Cos-7 cells and measured TSH binding and cAMP/inositol phosphate responses.

2. Materials and methods

2.1. Mutagenesis

Oligonucleotide-mediated, site-directed mutagenesis was used to create the mutants as previously described [10,11].

2.2. Transfection

Transfection of Cos-7 cells with mutant or wild type (WT) TSHR or pSG5 vector DNA was performed by electroporation [10,11]; 25 μg purified plasmid DNA was used in each. To evaluate transfection efficiency, pSVGH was co-transfected with mutant or WT TSHR or pSG5 vector cDNA. Aliquots of the same batch of transfected cells were plated for Northern blot, Western blot, TSH binding and cAMP/inositol phosphate assay; 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 [10,11] 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 of assay buffer containing [^{125}I]TSH and 0 to 10^{-7} M unlabeled TSH. Specific binding was obtained by subtracting 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, or 10 μM ATP. Total cAMP was measured by radioimmunoassay and inositol phosphate formation was determined using anion exchange columns.

All assays were performed in duplicate, on at least 3 separate occasions with different baths of cells, and with simultaneously run positive and negative controls: cells transfected with WT or pSG5 vector alone. Values in each well were corrected for cell protein. The program LIGAND [12] and a single high affinity site model were used to calculate Kd values for TSH binding and EC_{50} values for TSH-increased inositol phosphate and cAMP levels.

GH concentration of cultured media of cells used for the assays was determined by RIA. Northern blot of RNA from transfected Cos-7 cells was done as previously described [10,11] using total RNA and 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 [13].

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. As for K_d and EC_{50}, statistical differences are calculated using log-transformed values.

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3. Results

We created mutants involving one of the three exoplasmic loops in the transmembrane region of the rat TSHR by substituting with the counterpart residues from human β_2-adrenergic receptor (AR) (Fig. 1) [14]. The longest exoplasmic loop, the 2nd loop, is divided into amino- and carboxyl-segments and the β_2-AR sequence was substituted in each segment; E12N-1 and E12C-1. E12N-2 and E12C-2 were obtained by chance during mutagenesis and tested as well. The mutants are designed not to change the cysteine residues (Cys-494 in the 1st exoplasmic loop and Cys-569 in the 2nd loop; residue numbers are counted from methionine start site) conserved among most receptors with 7 transmembrane helices [9]. These cysteines are postulated to be involved in the three-dimensional structure such as disulfide bond formation because the point mutations of the cysteines lost all functional activities [15]. Substitution mutants involving the 1st or 3rd exoplasmic loop are designated as E11 and E13, respectively. 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 and Western blots, TSH binding and cAMP/inositol phosphate assays simultaneously. The transfection efficiency, determined by co-transfecting 0.1 μg pSVGH and measuring the GH concentrations in the culture media, was always within ± 14% 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 ± 22% of the mean) to that from cells transfected with full-length WT TSHR cDNA. All the mutants exhibited the same profile and a similar amount of TSHR forms on Western blot 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 that the inositol phosphate response to TSH is TSHR specific.

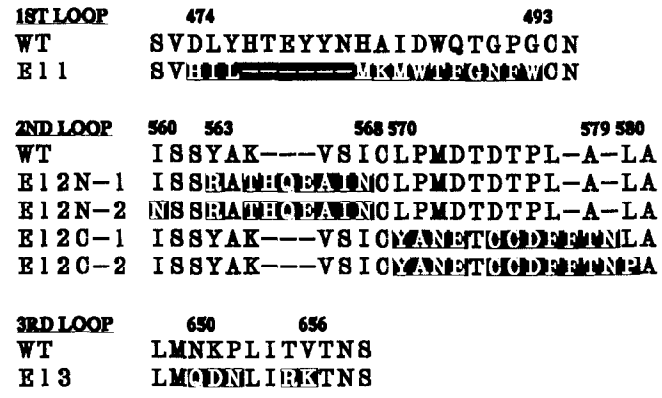

Fig. 1. Amino acid sequence of each substitution mutant involving one of exoplasmic loop of the rat TSH receptor (TSHR). The boxed residues detail the sequence change; these are from human β_2-adrenergic receptor amino acid sequence (14). (–) indicates no corresponding amino acid.

Table 1
Summary of mutant activities

Mutant	pSG5	WT	E11	E12N-1	E12N-2	E12C-1	E12C-2	E13
TSH binding								
K_d (pM)	–	213 ± 23	ND†	11 ± 1**	ND	ND	ND	23 ± 6**
$\%B_{max}$ (/WT)	–	100	ND	9 ± 1**	ND	ND	ND	28 ± 3**
cAMP increase								
EC_{50} (pM)	–	125 ± 18	NR#	54 ± 16	NR	NR	NR	435 ± 17*
% Basal (/pSG5)	100	294 ± 26	153 ± 25**	126 ± 9**	120 ± 20**	84 ± 12	107 ± 10**	213 ± 51
Max Resp (/pSG5)*	–	17.8 ± 1.2	–	12.4 ± 2.3	–	–	–	7.4 ± 0.4**
Max Resp (/basal)‡	–	6.0 ± 0.7	–	9.8 ± 1.1*	–	–	–	3.4 ± 0.4**
IPn increase								
EC_{50} (pM)	–	571 ± 62	NR	921 ± 198	NR	NR	NR	1100 ± 290
% Basal (/pSG5)	100	96 ± 2	111 ± 9	100 ± 8	99 ± 7	102 ± 3	90 ± 6	92 ± 4
Max Resp (/pSG5)*	–	3.3 ± 0.2	–	1.8 ± 0.2*	–	–	–	1.5 ± 0.2**
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 the basal level of pSG5 transfectant (fold).
‡ Maximal response compared with the basal level of each mutant transfectant (fold).

3.1. Functional activities of mutant receptors

As shown in Table 1, El1, El2N-2, El2C-1 and El2C-2 did not show any measurable TSH binding despite of similar profile in Western blot to that of WT. These mutant transfectants exhibited no TSH-stimulated cAMP or inositol phosphate increase. Graves' IgGs did not stimulate cAMP or inositol phosphate increase either (data not shown). Basal cAMP and inositol phosphate levels were also similar to pSG5 control transfectant level.

El2N-1 and El3 exhibited a significantly decreased B_{max} for TSH binding compared with WT. There was however, a significant increase in the affinity.

In El2N-1, the EC_{50} for TSH-cAMP assays was not statistically different from that of WT. The maximal response relative to the level in the pSG5 control transfectant was not different significantly from that of WT. When compared with the basal cAMP level of the same transfectant, the maximal response was rather better than that of WT, because the basal cAMP level of El2N-1 was decreased to the pSG5 level (Fig. 3B). Thus, WT increased the basal cAMP level compared with the pSG5 control transfectant as previously described [10]. On the other hand, the maximal TSH-induced inositol phosphate response was significantly decreased, although the EC_{50} was not significantly different from that of WT (Fig. 3C and Table 1).

In El3, the EC_{50} in the TSH-stimulated cAMP response was slightly (3.5-fold) but significantly increased. Further, the maximal TSH-cAMP response whether compared with the pSG5 control level or with the basal level of the same transfectant, was significantly decreased. The basal cAMP level without agonist stimulation was not significantly decreased from WT in contrast to El2N-1. Although the EC_{50} in the TSH-stimulated inositol phosphate increase was not significantly different from that of WT, the maximal response was significantly decreased.

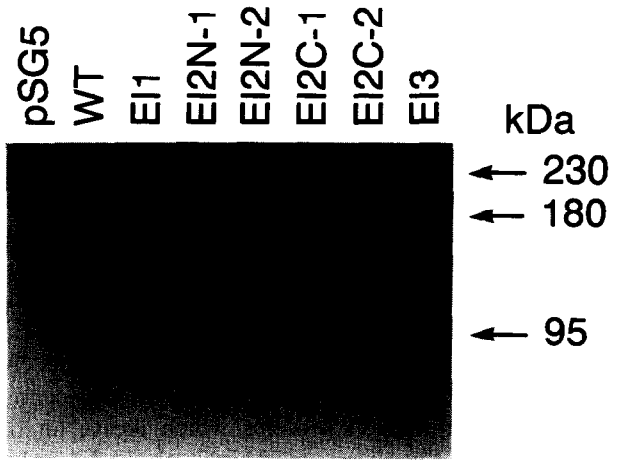

Fig. 2. Western blot of TSHR forms in membranes from Cos-7 cells transfected with 25 μg wild-type or mutant TSHR or pSG5 vector DNA.

4. Discussion

Only 2 out of the 6 mutants created in this series had specific TSH binding. Although El2N-1 exhibited a very low (1/11 of WT) TSH binding B_{max}, the cAMP response was fairly well retained. It should be noted that WT transfectant with 1/8 B_{max} of usual transfectant by decreasing the amount of DNA for transfection only showed a 6.8-fold maximal cAMP increase from the pSG5 basal level [10]. Thus, the effects of the El2N-1 mutation, including those upon the inositol phosphate

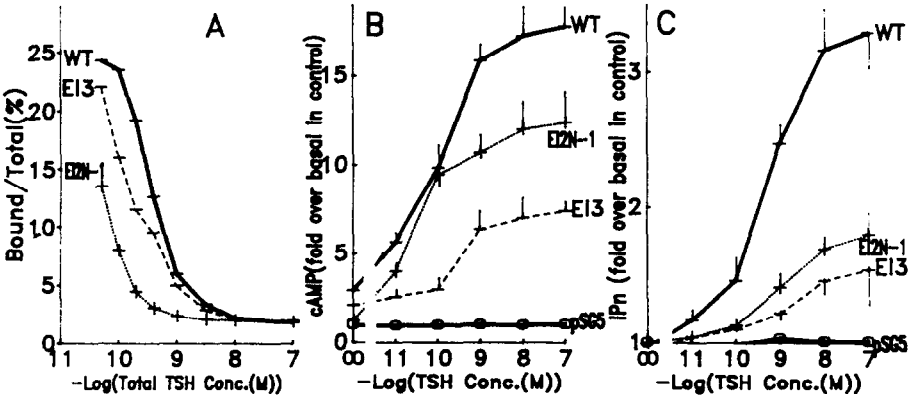

Fig. 3. Displacement of [^{125}I]TSH binding by unlabeled TSH (A) and the ability of TSH to increase cAMP (B) and inositol phosphate formation (C) in Cos-7 cells transfected with wild-type (WT) and mutants involving the exoplasmic loop (El2N-1 and El3) (Fig. 1). In A, data are from a single experiment 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 harboring the pSG5 vector alone. TSH had no effect on cAMP or inositol phosphate formation in control cells transfected with pSG5.

response are attributed to the effect of the decreased B_{max}, and a direct effect of the 2nd exoplasmic loop of the TSHR on signal transduction seems less likely.

In contrast, El3 with a substitution of the third exoplasmic loop, had a 3-times larger B_{max} than El2N-1, but an apparently lower maximal response and a higher EC_{50} in the TSH-cAMP assay and a smaller maximal inositol phosphate response. These results indicate that the third exoplasmic loop is important, at least in part, for cAMP and inositol phosphate responses. It should be noted that El3 has only 5 amino acids substituted and is the least different from WT among all the mutants created in this series. Because the exoplasmic loops cannot directly interact with G proteins which locate on the inner surface of the plasma membrane, the 3rd exoplasmic loop might intact with ligand/extracellular domain complex.

However, 4 other mutants did not give us any information. They totally lost TSH binding as well as cAMP/inositol responses although the Western blots showed these mutants were fully glycosylated. Similar TSHR mutations made by others which had lost all functional activities, were not able to provide any informative evidence about signal transduction either [16]. These mutations probably confer deleterious effects on the entire three-dimensional structure of the TSHR molecule.

The extracellular domain of the TSHR is the primary binding site of TSH [17,18], as it is with gonadotropins and gonadotropin receptors. Mutant lutropin/choriogonadotropin receptors (LH/CHR) without entire extracellular domain bound hCG with low affinity [19]. It is not clear whether TSH binds to the transmembrane region of the receptor with low affinity, because TSH also binds to the cells without TSHR with low affinity ($K_d = \sim 10^{-7}$ M) [20]. However, this does not exclude the possibility that there are two kinds of TSH binding sites (high affinity/low affinity). How does TSH bind to the extracellular domain activate the receptor and promote G protein coupling? One possibility is that a conformational change of the extracellular domain caused by the binding of TSH is directly transmitted to the transmembrane region which interacts with G proteins. Another is that some contact between TSH/extracellular domain complex and the outer surface of the transmembrane domain is necessary to cause a conformational change of the transmembrane domain.

Our present findings on the mutant involving the 3rd exoplasmic loop suggest that the latter is more likely because the former notion, direct transmission without contact between TSH/extracellular domain complex and the transmembrane domain fails to explain the present results.

Ji et al. have reported that mutations of Asp-397 in the first exoplasmic loop of the LH/CGR totally impair the cAMP response without affecting human CG binding [21,22]. Because this Asp is conserved among all glycoprotein hormone receptors [9], not only the third loop but also the first exoplasmic loop of this class of receptors seems to play an important role in signal transduction.

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