Aspartate-474 in the first exoplasmic loop of the thyrotropin receptor is crucial for receptor activation

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Abstract Asp-474 in the first exoplasmic loop of the thyrotropin receptor (TSHR), which is conserved among all glycoprotein hormone receptors, was mutated to Glu which is similarly charged but is longer by one methylene group and expressed in Cos-7 cells. Cells expressing this mutant receptor showed markedly impaired TSH- and TSAb (thyroid stimulating antibody)-stimulated cAMP responses with no effect on TSH binding affinity when compared with cells expressing a similar number of wild-type receptors. These results suggest the importance of Asp-474 in TSHR in receptor activation as demonstrated for LHR (lutropin receptor), but this, unlike LHR, is not due to the electrostatic interaction of this Asp residue with the α-subunit Lys-91 of the hormone.

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Key words: cAMP signal; G-protein-coupled receptor; Glycoprotein hormone; Receptor activation

1. Introduction

It has been suggested that the long extracellular domain of glycoprotein hormone receptors plays a major role in high-affinity ligand binding [1–5]. However, how conformational changes in the transmembrane domain of the receptor resulting in G-protein coupling occur following ligand binding to the extracellular domain remains to be elucidated.

It has been reported [6] that Asp-397 of the lutropin receptor (LHR) at the junction of the second transmembrane helix and the first exoplasmic loop may interact with Lys-91 of the α-subunit of hCG (human choriogonadotropin) to evoke receptor activation. This hypothesis was suggested by the partial restoration of hormone-induced receptor activation by reciprocal mutagenesis of negatively charged Asp and positively charged Lys. As the α-subunit is common to all glycoprotein hormones and this Asp is uniquely conserved among all glycoprotein receptors, we hypothesized that this phenomenon might be common to all glycoprotein hormones and their receptors.

To investigate the role of the corresponding Asp residue located at the junction of the second transmembrane helix and the first exoplasmic loop in the thyrotropin receptor (TSHR), we made substitution mutants at Asp-474 and functional assays were performed using transfected cells.

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2. Materials and methods

2.1. Site-directed mutagenesis of TSHR

Rat TSHR cDNA [7] was inserted into the *Eco*RI site of the M13mp18 vector and oligonucleotide-mediated site-directed mutagenesis was used to generate clones with the desired mutation (T7GEN kit, US Biochemical, Cleveland, OH) [8,9]. Residue numbers were determined by counting from the methionine start site. Wild-type (WT) and mutant clones were inserted into the *Eco*RI site of the SV-40-driven pSG5 vector (Strategene, La Jolla, CA). Mutations were confirmed by DNA sequencing of the final construct, and plasmid DNA was purified by CsCl-gradient ultracentrifugation.

2.2. Transfection

Cos-7 cells (–10^7 cells) were transfected by electroporation (Bio-Rad, Richmond, CA) with 25 g of purified plasmid DNA containing a mutant or WT TSHR sequence [8,9]. When smaller amounts of TSHR DNA were used, the total amount of DNA per cuvette was kept constant by adding pSG5 vector DNA. To evaluate the transfection efficiency, 0.1 μg pSVGH was co-transfected with mutant or WT TSHR and/or pSG5 vector DNA. After electroporation, each batch of transfected cells was divided into aliquots for binding, cAMP and inositol phosphate assays. Cells intended for binding assays were suspended in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, and transferred to 6-well plates (–5×10^5 cells/well). Cells for cAMP and inositol phosphate assays were suspended in inositol-free medium supplemented with 10% fetal calf serum and 2.5 μCi/ml myo-[2-^{3}H]inositol (DuPont-NEN, Boston, MA) and were transferred to 24-well plates (–10^5 cells/well).

2.3. Assays

Forty-eight hours after transfection, cells were washed with assay buffer (NaCl-free, Hanks' balanced salt solution containing 0.5% (w/v) crystalline bovine serum albumin, 222 mM sucrose and 20 mM HEPES (2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid)-NaOH, pH 7.4) [8]. [^{125}I]TSH binding was measured by incubating cells for 16 h at 4°C in 1 ml of assay buffer containing approximately 80 000 cpm [^{125}I]TSH (30 U/mg, National Hormone and Pituitary Program; labeled to about 50 μCi/μg by Hazelton Washington, Vienna, VA) and 0–10^{-7} M unlabeled TSH. Cyclic AMP and inositol phosphate production were measured concurrently by incubating cells for 1 h at 37°C in 0.2 ml of assay buffer containing 10 mM LiCl, 0.5 mM IBMX (3-isobutyl-1-methylxanthine) and 0–10^{-7} M TSH or TSAb (thyroid-stimulating antibody) IgG from Graves' patients purified by Econo-Pac columns (Bio-Rad). Perchloric acid was added to each well, samples were centrifuged, aliquots of supernatant neutralized with KOH and HEPES, and total cAMP in each aliquot was determined by ^{125}I-radioimmunoassay (Eiken, Tokyo, Japan). Total inositol phosphates were measured by Dowex AG1-X8 anion exchange column chromatography (Bio-Rad). All assays were performed at least in triplicate, on at least three separate occasions with different batches of cells, and always included control cells transfected with WT TSHR and pSG5 vector DNA. Cos-7 cells transfected with pSG5 vector alone were not stimulated by TSH or TSAb, and did not exhibit specific [^{125}I]TSH binding. The program LIGAND [10] was used to calculate K_d and B_{max} values for TSH binding. The GH concentration in the cultured media of cells used for the assays was determined by radioimmunoassay and was always within 15% of the mean. The density of live cells when the assays were performed varied

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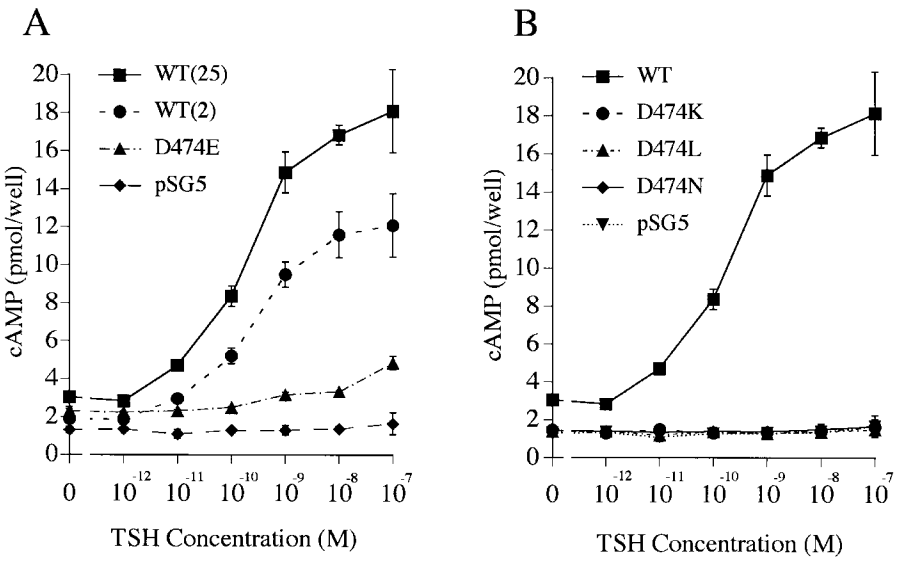

Fig. 1. cAMP accumulation in response to TSH in cells transfected with WT or mutant TSHR, or pSG5 vector DNA. Data are means ± SEM ($n \geq 3$). Transfections were performed with 25 μg of DNA, except as noted for WT(2) in (A).

by < 10% between wells transfected with WT TSHR and those transfected with mutant constructs. Therefore, data were not normalized and were expressed as means ± SEM ($n \geq 3$).

3. Results

Cos-7 cells transfected with WT TSHR DNA (Fig. 1A) responded to TSH with an EC_{50} of about 2×10^{-10} M and maximal response compared with its own basal cAMP level of about 6-fold. These cells showed 2.5-fold increase in basal cAMP level in the absence of agonist stimulation compared with cells transfected with pSG5 vector alone. In contrast, cells transfected with mutant D474E DNA (Fig. 1A) responded to TSH with an EC_{50} larger than 2×10^{-8} M and maximal response at 10^{-7} M TSH compared to its basal level was 2.1-fold. These cells also showed an increased basal cAMP level of about 1.8-fold over that in control cells transfected with pSG5 vector alone. Cos-7 cells transfected with mutant D474K, D474N or D474L DNA (Fig. 1B) did not respond to TSH. These mutant transfected cells showed similar basal cAMP levels to those in control transfectants.

Table 1 shows Graves' IgG-stimulated cAMP responses in Cos-7 cells transfected with WT or mutant TSHR DNA. Cells with WT receptor responded well to Graves' IgG #1; 2.3-fold at 0.5 mg/ml and 4.9-fold at 5 mg/ml. D474E mutant-transfected cells showed a slight increase of 1.4-fold only at high concentration (5 mg/ml) of Graves' IgG #1. This observation was consistent with those obtained with two other Graves' IgG samples. Cells transfected with D474K, D474N or D474L as well as pSG5 vector DNA showed no significant increase in cAMP levels by Graves' IgGs ($P > 0.05$ compared with normal IgG).

The TSHR mediates inositol phosphate signals as well as those of cAMP [8,11]. Inositol phosphate levels were determined in aliquots of samples used for cAMP assays. None of the mutants showed increases in inositol phosphate levels in response to TSH or TSAb, whereas WT-transfected cells did but with less sensitivity (data not shown) as described previously [8,11].

Table 2 summarizes TSH binding parameters of cells transfected with WT or mutant TSHR DNA. No TSH binding was detected in cells transfected with D474K, D474N or D474L DNA. Therefore, the lack of cAMP responses by stimulation with TSH and TSAb in these transfected cells may be attributed to disruption of the 3-dimensional structure or impaired membrane insertion. WT-transfected cells bound TSH with K_d of about 200 pM. D474E-transfected cells bound TSH with similar K_d, but B_{max} of TSH binding was about 40% of that of WT-transfected cells. Decrease in receptor density on cells with mutant D474E receptor might have caused poor TSH- or TSAb-induced cAMP responses. Thus, we reduced the amount of WT DNA used for transfection from 25 to 2 μg (WT(2)). The B_{max} of these cells was decreased to 37% of that of normal cells with WT receptors, a value similar to or less than that of D474E-transfected cells.

However, these cells still responded well to TSH with EC_{50} similar to that of normal WT-transfected cells and a maximal response of 6.4-fold (Fig. 1A, WT(2)). These observations for

Table 1
Cyclic AMP accumulation in response to IgGs from Graves' patients

Mutant	WT	D474E	D474K	D474N	D474L	pSG5
5 mg/ml normal IgG	3.01 ± 0.18	1.95 ± 0.10	1.21 ± 0.08	1.32 ± 0.15	1.39 ± 0.07	1.28 ± 0.10
0.5 mg/ml Graves' IgG #1	6.93 ± 0.02**	1.89 ± 0.09	ND	ND	ND	1.37 ± 0.08
5.0 mg/ml Graves' IgG #1	14.71 ± 0.62**	2.65 ± 0.15*	1.41 ± 0.01	1.35 ± 0.20	1.40 ± 0.13	1.35 ± 0.11
5.0 mg/ml Graves' IgG #2	9.26 ± 0.78**	2.20 ± 0.19*	1.29 ± 0.09	1.29 ± 0.14	1.30 ± 0.12	1.25 ± 0.19
5.0 mg/ml Graves' IgG #3	7.55 ± 0.19**	1.88 ± 0.16	1.34 ± 0.09	1.28 ± 0.10	1.22 ± 0.13	1.23 ± 0.09

cAMP accumulation (pmol/well), mean ± SEM ($n \geq 3$). ND, not done. *$P < 0.05$, compared with normal IgG. **$P < 0.01$, compared with normal IgG.

Table 2
TSH binding parameters of cells expressing mutant TSH receptor

Mutant	WT(25)	WT(2)	D474E	D474K	D474N	D474L
K_d (pM)	231 ± 33	210 ± 16	194 ± 29	UD	UD	UD
B_{max} (%WT)	100	37 ± 5	40 ± 7	UD	UD	UD

Data are expressed as means ± SEM of three independent experiments. UD, undetectable.

WT(2) and D474E mutant, indicated that the impaired TSH- and TSAb-stimulated cAMP responses in cells with D474E mutant receptors was not attributable to poor surface expression but to the mutation itself.

4. Discussion

Ji et al. reported [6] that (i) substitution of Asp-397 of the LHR to Lys resulted in complete loss of hCG-induced cAMP response without affecting hCG binding in transfected cells, and (ii) that cAMP responses in cells expressing WT LHR by mutant hCG with substitution of Lys-91 of the α-subunit were markedly decreased and insensitive, but the mutant hCG bound to WT LHR with similar affinity to WT hCG. (iii) When the hCG mutant was used with the Asp-394-to-Lys LHR mutant, the hCG-induced cAMP response was restored. However, substitution with other amino acids which eliminated the counterionic nature failed to induce cAMP responses. Therefore, they concluded that Asp-397 of the LHR interacted with Lys-91 of αhCG [6].

The first aim of the present study was to examine whether the role of the corresponding Asp-474 in the TSHR is the same as that in the LHR. Cells expressing D474E mutant bound TSH with a similar K_d to that of WT but with a smaller B_{max}. However, the most conservative substitution mutant D474E showed a poor TSH-cAMP response with an EC_{50} value more than 100-times higher than that of WT cells with a similar receptor density. This is in contrast to the corresponding Asp-to-Glu mutant of the LHR with only a 3-fold increase in EC_{50} of hCG-induced cAMP activation [12]. The Asn, Arg and Lys LHR mutants showed almost completely abolished hCG-induced cAMP response without affecting hCG binding [12]. TSHR mutants D474K, D474N and D474L lost all TSHR functions including TSH binding, providing no informative evidence. Both Asp in WT and Glu in the mutant are negatively charged, and thus it is difficult to explain the electrostatic interaction with positively charged Lys residue of TSH. The present findings suggest a different mechanism of activation mediated by this hormone. Hydrogen bonding formed by the β-carbonyl group of Asp-474 rather than electrostatic bonding might be important. However, D474N does not provide any information of TSHR structure/function as described above. The role of Asp-474 in the TSHR may be similar to that of FSHR (follitropin receptor) rather than LHR; the corresponding Asp-405-to-Glu mutant showed marked impairment of FSH-induced cAMP generation [13]. Investigation of substitution mutants of adjacent amino acids needs to be elucidated for detailed mechanism of receptor activation involving Asp-474.

The other aim of the present study was to examine the hypothesis that TSAb but not TSH may activate Asp-474 mutants because this Asp was postulated to interact with the α-subunit of glycoprotein hormone. However, this was not the case because D474E showed only a slight response to TSAb at higher concentrations, and decreases in TSH- and TSAb-induced cAMP occurred in parallel.

It is clear that Asp-474 is important for signal transduction but unclear and interesting how Asp-474 works. There are, however, at least two possible mechanisms: (a) Asp-474 may interact with agonist-extracellular domain complex to provoke conformational changes of transmembrane helices or (b) Asp-474 may not be involved in direct interaction with agonist or the extracellular domain. However, the interaction of Asp-474 with other portion(s) of the transmembrane domain may be necessary for the conformational change of the transmembrane domain during activation. Assuming an interaction between the agonist-extracellular domain complex and the transmembrane domain, direct interaction of the transmembrane domain may occur with (i) TSH after initial binding to the extracellular domain, (ii) the extracellular domain conformationally changed by TSH binding, or (iii) both. Multiple interactions are suspected from the present findings and the data obtained from substitution mutation in the third exoplasmic loop [14].

In sum, D474E does not affect TSH binding affinity but does impair TSH- and TSAb-simulated cAMP responses. Asp-474 appears to be important for activation of the TSH receptor. The role of Asp-474 in receptor activation by TSH does not seem to be different from that by TSAb.

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