Vol. 211, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
June 6, 1995 Pages 205-210

CONSTITUTIVE ACTIVATION OF THE THYROTROPIN RECEPTOR BY DELETION OF A PORTION OF THE EXTRACELLULAR DOMAIN

Mei-Luo Zhang, Hideo Sugawa, Shinji Kosugi* and Toru Mori

Department of Laboratory Medicine, Kyoto University School of Medicine,
54 Shogoin Kawaharacho, Sakyo-ku, Kyoto 606-01, Japan

Received April 18, 1995

Summary: Mutations involving the transmembrane domain of the thyrotropin receptor (TSHR) confer constitutive activation of the receptor and can cause human diseases. Naturally occurring activating mutations identified to date are located only in the transmembrane domain of the receptor. We now report a mutant involving the extracellular domain of the TSHR which also shows constitutive activation. This mutation is missing residues 339-367 located in the C-terminal portion of the extracellular domain. When expressed in COS-7 cells, the mutated TSHR (M3B) retained similar TSH binding ability to that of the wild-type receptor. However, the basal cAMP production without TSH stimulation in COS-7 cells transfected with M3B cDNA was significantly higher than that of COS-7 cells with wild-type receptor, indicating that the mutant receptor is constitutively activated. Our results provide new insight into the mechanism of receptor activation. © 1995 Academic Press, Inc.

The thyrotropin receptor (TSHR) as well as lutropin receptor (LHR) and follitropin receptor (FSHR) belong to a large family of G protein-coupled receptors (GPCRs) with a characteristic structure of seven transmembrane helices (1,2). They are distinct from other GPCRs in terms of a long extracellular domain (~400 amino acids) which seems to confer specificity for binding of ligands about MW 30 kDa. We have reported previously that mutations involving the transmembrane domain of TSHR induce constitutive activation of the receptor (3). Subsequently, similar activating mutations were found in human diseases such as hyperfunctioning thyroid adenomas (4-8), non-autoimmune autosomal dominant hyperthyroidism (9) and congenital hyperthyroidism (10). To date, activating mutations in the TSHR gene were observed only in the transmembrane region, predominantly in the sixth transmembrane helix, and in the third cytoplasmic loop (3-8, 10). In LHR which is the closest to TSHR in structure among the GPCRs, similar activating mutations were found in familial male precocious puberty (FMPP) (11-15) in its transmembrane domain.

*To whom all correspondence should be addressed. Fax: 81-75-751-3233.

0006-291X/95 $5.00
Copyright © 1995 by Academic Press, Inc.
All rights of reproduction in any form reserved.

We now report that a mutation involving the extracellular domain of the TSHR also shows constitutive activation of the receptor.

Materials and Methods

Mutated Receptor cDNA and Plasmid

Full-length wild-type rat TSHR cDNA (WT), its antisense gene (AS), and mutated receptor cDNA (M3B) with deletion of residues 339-367 (residue numbers are counted from the methionine start site) in the C-terminal portion of the extracellular domain, which are the same as those described previously (16),were inserted into the EcoRI site of the SV-40 driven pSG5 vector.

Cell Culture and DNA Transfection

COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. At the late log growth phase, cells were collected and transfected with WT, AS, M3B or pSG5 vector DNA by electroporation (17, 18). The amount of each DNA used was 20 μg for maximal receptor expression. Transfected cells were aliquoted into 6-well plates for TSH binding assay and 96-well plates for cAMP measurement. The assays were carried out 48 hours after transfection.

cAMP Measurement

Forty-eight hours after transfection, the cells were rinsed once with assay buffer (Hanks' balanced salt solution (HBSS) containing 0.5% crystalline BSA and 20 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) pH 7.4) and incubated with 0-1000 μU/ml bovine TSH (bTSH, Sigma Chem Co. St. Louis) in the assay buffer with 0.5 mM 3-isobutyl-methyl-xanthine (IBMX) at 37°C for 2 hours. cAMP concentration in the medium was measured using a commercial kit (EIKEN Chem., Osaka, Japan). The assays were performed in triplicate and the transfection experiments were repeated 10 times.

TSH Binding Assay

Forty-eight hours after transfection, the cells were rinsed once with NaCl-free HBSS containing 0.5% BSA, 222 mM sucrose, and 20 mM HEPES at pH 7.4, then incubated in 1 ml of the same buffer containing 1.5 X 10^5 cpm [^{125}I] TSH (~60 μCi/μg) and 0-10^{-7} M unlabeled bTSH for 2 hours at room temperature. At the end of this incubation period, the cells were rapidly rinsed twice with ice-cold buffer and solubilized with 1N NaOH. The radioactivity was measured using a gamma counter. Specific TSH binding was calculated by subtracting values obtained in the presence of 10^{-7} M unlabeled TSH. The binding affinity and maximal binding capacity were calculated using program Ligand (19). The total protein contents were measured by the dye-developing method (Bio-Rad Richmond, CA).

Statistical Analysis

Paired t-test was used for statistical analysis of the basal cAMP production between COS-7 cells transfected with WT and M3B receptor cDNA.

Results

cAMP production with and without TSH stimulation was measured in COS-7 cells transfected with WT, AS, M3B or pSG5 vector. As shown in Fig. 1, significant TSH-stimulated cAMP production was induced in a dose-dependent manner in COS-7 cells transfected with WT or M3B. TSH-stimulated cAMP production at 100 μU/ml, when compared to the basal level of each transfectant, was about 2-fold in both WT- and M3B-containing cells. COS-7 cells transfected with vector only or antisense TSH receptor gene showed no significant increase in cAMP production. Basal cAMP production without TSH-stimulation in COS-7 cells with WT was higher than that in control cells as reported previously (3, 4, 20). Further, basal cAMP level in COS-7 cells transfected with M3B seemed slightly higher than that in WT transfectants.

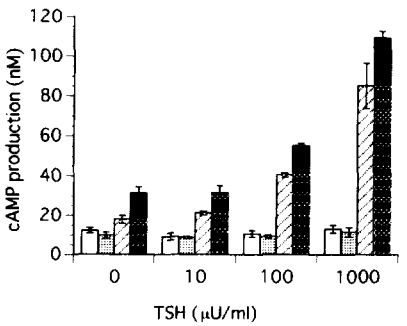

Fig. 1. TSH-stimulated cAMP production in COS-7 cells transfected with WT, AS, M3B and vector. Vector (□), WT (▨), AS (▤) and M3B (■) genes were transfected into COS-7 cells by electroporation as described in Materials and Methods. Forty-eight hours later, cAMP concentration was measured by RIA. Values are expressed as means ± SD of three determinations.

To exclude the possibility that this increase in basal cAMP level in COS-7 cells expressing M3B might have been due to increased transfection efficiency, overexpression or a simple difference in cell number, we repeated the experiments 10 times and measured TSH binding and cell protein amount in addition to basal cAMP levels. The basal cAMP level was calibrated by TSH binding Bmax and total cellular protein amount to show cAMP/Bmax and cAMP/ protein (Fig. 2). Dissociation constants (Kd) in WT and M3B were $1.00 \pm 0.42 \times 10^{-9}$ and $1.05 \pm 0.51 \times 10^{-9}$, respectively, which were not different significantly ($P = 0.24$). Basal cAMP levels in COS-7 cells with M3B in the absence of TSH were consistently higher than those in COS-7 cells with WT, when they were expressed as raw values, cAMP/Bmax or cAMP/protein. Paired t-test showed that these differences were highly significant ($P < 0.01$) in each group. These observations confirm that M3B is slightly but significantly activated relative to WT.

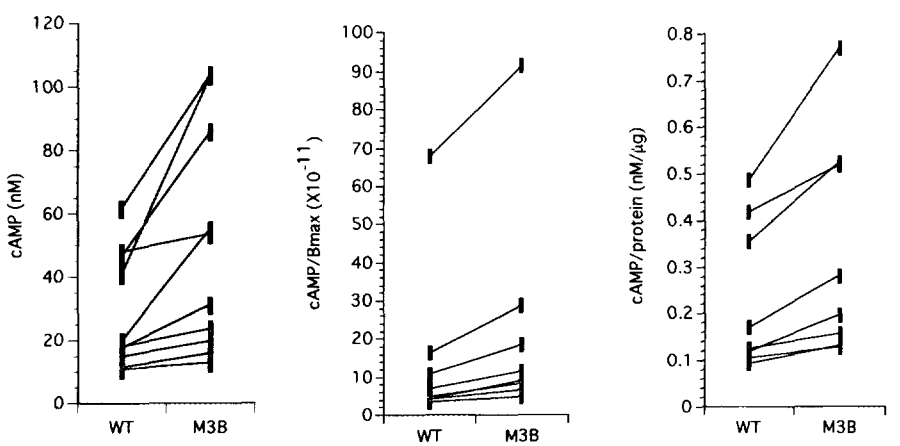

Fig. 2. Basal cAMP production and the ratio of cAMP/Bmax, cAMP/protein of COS-7 cells with WT and M3B. Paired t-test was used to analyze the 10 experiments. P values were 0.0057 (cAMP), 0.0094 (cAMP/Bmax) and 0.0061 (cAMP/protein).

Discussion

There is increasing evidence that some specific mutations involving the transmembrane domain of GPCRs are constitutively activating and can cause human diseases. Activating mutations of Asp-619 and Ala-623 in the third cytoplasmic loop and Phe-631, Thr-632 and Asp-633 in the sixth transmembrane helix of TSHR were identified in hyperfunctioning thyroid adenomas (4-8) and congenital hyperthyroidism (10). Mutations of Val-509 in the third transmembrane helix and Cys-672 in the seventh transmembrane helix were found in non-autoimmune autosomal dominant hyperthyroidism (9). These residues are perfectly conserved among all glycoprotein receptors. Activating mutations in the sixth transmembrane helix and the third cytoplasmic loop of LHR were also observed in FMPP and spontaneous testoxicosis without familial history (12-15). All activating mutations of glycoprotein hormone receptors reported to date are located in the transmembrane domain which interacts directly with G proteins and regulates signal transduction. The mechanism of the induction of constitutive activation by mutation involving the transmembrane region is still unknown but may be explained by increases in affinity with G proteins and/or conformational changes which mimic normal activation by agonists. Specific interaction between transmembrane helices may be critical for maintaining the inactive receptor conformation.

We observed constitutive activation caused by a deletion mutation of a portion in the extracellular domain of the TSH receptor. The increase in agonist-independent cAMP production in M3B was not documented but was happened to be shown previously (Fig. 5 in Ref. 16).

In initial attempts to characterize the extracellular domain of the TSHR by site-directed mutagenesis shortly after cloning of the receptor, we and others (16, 21) found that a portion in the C-terminal region of the extracellular domain of the receptor could be deleted without any loss of receptor function. In our previous report (16), in COS-7 cells transfected with the mutant M3B missing residues 339-367, specific TSH binding was similar to that in WT transfectants. The ability of TSH and Graves' IgG to increase cAMP levels in the M3B transfectants was reasonably preserved relative to that in WT. The deletable region extended from residues 303 to 382 (22), and the flanking the region cysteine-301 and cysteine-390 are postulated to form a disulfide bond making the region a loop (22, 23). It is clear that deletion of this region does not affect the 3-dimensional structure of the TSHR molecule (22, 23, 24). However, it is of interest that synthetic peptides with sequence of the C-terminal deletable region, surrounding or overlapping residues 339-367, specifically bound to IgGs from patients with Graves' disease (16, 25, 26). The C-terminal deletable region is not necessary for TSHR stimulation by Graves' IgG. However, there may be an alternative mechanism of activation of TSHR by TSAb; e.g. stabilizing activity of this region may be changed by binding of Graves' IgG.

This is the first report that a portion in the extracellular domain of the TSHR also plays a role in preventing receptor activation without agonist stimulation. We speculate that this

portion interacts with the transmembrane domain and contributes to maintain the inactive conformation. It is interesting that a portion in the TSHR with no corresponding sequence in the LH/CG receptor plays this role because wild-type TSHR but not wild-type LHR undergoes spontaneous isomerization.

Acknowledgments

We thank Ms. Eriko Ito for secretarial and technical assistance. This work was in part supported by grants-in-aid from the Japan Research Foundation for Clinical Pharmacology and the Japanese Ministry of Education (No. 06044128 and No. 06671024).

References

1. Vassart, G., Dumont, J.E. (1992) Endocr. Rev. 13, 596-611.
2. Birnbaumer, L., Abramowitz, J., Brown, A.M. (1990) Biochim. Biophys. Acta 1031: 163-224.
3. Kosugi, S., Okajima, F., Ban, T., Hidaka, A., Shenker, A., Kohn, L.D. (1993) Mol. Endocrinol. 7, 1009-1020.
4. Parma, J., Duprez, L., Van Sande, J., Cochaux, P., Gervy, C., Mockel, J., Dumont, J., Vassart, G. (1993) Nature 365, 649-651.
5. Porcellini, A., Ciullo, I., Laviola, L., Amabile, G., Fenzi, G., Avvedimento, V.E. (1994) J. Clin. Endocrinol. Metab. 79, 657-661.
6. Kosugi, S., Shenker, A., Mori, T. (1994) FEBS letters 356, 291-294.
7. Paschke, R., Tonacchera, M., Van Sande, J., Parma, J., Vassart, G. (1994) J. Clin. Endocrinol. Metab. 79, 1785-1789.
8. Paschke, R., Tonacchera, M., Dumont, J., Vassart, G. (1994) Thyroid 4, S27.
9. Duprez, L., Parma, J., Van Sande, J., Allgeier, A., Leclère, J., Schvartz, C., Delisle, M.J., Decoulx, M., Orgiazzi, J., Dumont, J., Vassart, G. (1994) Nature Genetics 7, 396-401.
10. Kopp, P., Van Sande, J., Parma, J., Duprez, L., Gerber, H., Joss, E., Jameson, J.L., Dumont, J.E., Vassart, G. (1995) New Eng. J. Med. 332, 150-154.
11. Laue, L., Hsueh, A., Cutler Jr, G.B., Chan, W.Y. (1994) FASEB J. 8, A1320.
12. Shenker A, Laue, L., Kosugi, S., Merendino Jr, J.J., Minegishi, T., Cutler Jr, G.B. (1993) Nature 365, 652-654.
13. Kremer, H., Mariman, E., Otten, B.J., Moll Jr, G.W., Stoellnga, G.B.A., Wit, J.M., Jansen, M., Drop,S.L., Faas, B., Ropers, H.H., Brunner, H.G. (1993) Human Mol. Genetics 2, 1779-1783.
14. Kosugi, S., Van Dop, C., Geffner, M.E., Rabl, W., Carel, J.C., Chaussain, J.L., Mori, T., Merendino Jr, J.J., Shenker, A. Human Mol. Genetics (1995) 4, 183-188.
15. Yano, K., Hidaka, A., Saji, M., Polymeropoulos, M.H., Okuno, A., Kohn, L.D., Cutler Jr, G.B. (1994) J. Clin. Endocrinol .Metab. 79, 1818-1823.
16. Kosugi, S., Akamizu, T., Takai, O., Prabhakar, B.S., Kohn, L.D. (1991) Thyroid 1, 321-401.
17. Akamizu, T., Ikuyama, S., Saji, M., Kosugi, S., Kozak, C., McBride, O.W., Kohn, L.D. (1990) Proc. Natl. Acad. Sci. USA 87, 5677-5681.
18. Kosugi, S., Ban, T., Kohn, L.D. (1993) Mol. Endocrinol. 7, 114-130.
19. Munson, P.J., Rodbard, D. (1980) Analytical Biochem. 107, 220-239.
20. Kosugi, S., Okajima, F., Ban, T., Hidaka, A., Shenker, A., Kohn, L.D. (1992) J. Biol. Chem .267, 24153-24156.
21. Wadsworth, H.L., Chazenbalk, G.D., Nagayama, Y., Russo, D., Rapoport, B. (1990) Science 249, 1423-1425.
22. Kosugi, S., Ban, T., Akamizu, T., Kohn, L.D. (1991) J .Biol. Chem. 266, 19413-19418.

23. Kosugi, S., Ban, T., Akamizu, T., Kohn, L.D. (1992) Biochem. Biophys. Res. Commun. 189, 1754-1762.
24. Kosugi, S., Ban, T., Akamizu, T., Kohn, L.D. (1991) Biochem. Biophys. Res. Commun. 180, 1118-1124.
25. Ueda,Y., Sugawa, H., Akamizu, T., Okuda, J., Kiho, Y., Mori, T. (1993) Thyroid 3, 111-117.
26. Nagy, E.V., Burch, H.B., Lukes, Y.G., Car, F.E., Kosugi, S., Kohn, L.D., Burman, K.D. (1993) J. Endocrinol. Invest. 16, 485-493.