Mutation of Alanine 623 in the Third Cytoplasmic Loop of the Rat Thyrotropin (TSH) Receptor Results in a Loss in the Phosphoinositide but Not cAMP Signal Induced by TSH and Receptor Autoantibodies*  

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Thyrotropin (TSH) and IgG preparations from patients with Graves’ disease increase inositol phosphate as well as cAMP formation in Cos-7 cells transfected with rat TSH receptor cDNA. Mutation of alanine 623 in the carboxyl end of the third cytoplasmic loop of the TSH receptor, to lysine or glutamic acid, results in the loss of TSH- and Graves’ IgG-stimulated inositol phosphate formation but not in stimulated cAMP formation. There is no effect of the mutations on basal or P2-purinergic receptor-mediated inositol phosphate formation. The mutations do not affect transfection efficiency or the synthesis, processing, or membrane integration of the receptor, as evidenced by the unchanged amount and composition of the TSH receptor forms on Western blots of membranes from transfected cells. The mutations increase the affinity of the TSH receptor for [125I]TSH and decrease B max; however, cells with an equivalently decreased B max as a result of transfection with lower levels of wild type receptor do not lose either TSH-induced inositol phosphate formation or cAMP signaling activity. Thus, in addition to discriminating between ligand-induced phosphati-
dylinositol bisphosphate and cAMP signals, the mutation appears to cause an altered receptor conformation which affects ligand binding to its large extracellular domain.

The thyrotropin (TSH)1 receptor is coupled to the PIP2 as well as the cAMP signal system in FRTL-5 rat thyroid cells; the α1-adrenergic receptor (α1-AR) couples only to the former (1–5). The rat TSHR, like the α1-AR, has seven transmembrane domains (6, 7); it is, however, an example of a single receptor coupled to more than one G protein (8). Thus, there is a single TSHR gene (6, 9, 10) and transfected TSHR cDNA

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† The abbreviations used are: TSH, thyrotropin; TSHR, thyrotropin receptor; AR, adrenergic receptor; PIP2, phosphatidylinositol bisphosphate.

**Fig. 1. Putative membrane topology of the rat TSHR showing the amino acid sequence and position of alanine 623 in the carboxyl end of the third intracellular loop.

The present report shows that alanine 623 in the third cytoplasmic loop of the TSHR seven-transmembrane domain (Fig. 1) is critical for the ability of TSH and Graves’ IgG to initiate the PIP2 but not the cAMP signal of Cos-7 cells transfected with rat TSHR cDNA. The selectivity and novelty of the mutation is underscored by studies of the α2A-AR, which showed that the same mutation of the comparably located alanine constitutively activated the PIP2 signal while minimally impacting the maximal hormone response (7), and by studies of the TSHR, which suggested all three intracytoplasmic loops were important for TSH-induced cAMP signal transduction (14).

EXPERIMENTAL PROCEDURES

Materials—Purified TSH, [125I]TSH, Econo-Pac column-purified normal or Graves’ IgG, and full-length, wild type rat TSH receptor (nucleotides –54 to 2780 in a pSG5 plasmid) were preparations previously described (6, 15).

Mutagenesis—Oligonucleotide-mediated, site-directed mutagenesis (15) was used to convert alanine 623 to glutamic acid (623E) or lysine (623K) and the derived clones inserted in the EcoRI site of pSG5. Amplified DNA was purified by CacCl gradient centrifugation; each mutant had the sequence predicted and produced RNA appropriate in size and amount, relative to both wild-type TSH receptor and β-actin RNA.

Transfection and Assays—Transfection of Cos-7 cells with mutant DNA was performed by electroporation (6, 15); 25 μg of purified
plasmid DNA was used unless otherwise noted. Transfection efficiency was measured by cotransfecting pSVGH and measuring growth hormone concentration in the media (10). The same batch of transfected cells was plated in Dulbecco's modified Eagle's medium with 10% fetal calf serum using six-well plates (5 × 10^5 cells/well) for TSH binding assays or 24-well plates (1 × 10^5 cells/well) for cAMP and inositol phosphate assays; medium was inositol-free in the latter assays and supplemented with 2.5 μCi/ml myo-[2-3H]inositol (Du Pont-New England Nuclear; specific activity approximately 20 Ci/mmol). All three assays were simultaneously initiated 48 h after transfection and after washing with assay buffer: NaCl-free Hanks' balanced salt solution containing 0.5% bovine serum albumin, 222 mM sucrose, and 20 mM HEPES at pH 7.4 (15). [125I]TSH binding was measured after incubation for 2 h at 22 °C in 1 ml of assay buffer containing 1.5 × 10^6 cpm [125I]TSH (60 μCi/μg) and 0 to 10^−7 M unlabeled TSH (6, 15). Specific TSH binding was obtained by subtracting values obtained in the presence of 10^−6 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 of assay buffer containing 10 mM LiCl, 0.5 mM 3-isobutyl-1-methylxanthine, and, as noted, 10−11 to 10−6 M TSH, 0.5 or 5.0 mg/ml Graves' or normal IgG, 10 μM ATP, 10 ng/ml choleratoxin, or 2 μM forskolin. After the addition of 1.0 ml of 5% perchloric acid, samples were centrifuged to remove insoluble salts. Total cAMP in aliquots of the supernatant was measured by radioimmunoassay (6, 15); inositol phosphate formation was determined using Dowex AG1-X8 columns (mesh size 100–200) eluted with 1 M ammonium formate, 0.1 M formic acid after removal of free inositol with H2O and glycerophosphoinositol with 5 mM sodium tetraborate, 60 mM sodium formate (5).

All assays were performed in duplicate, on at least three separate occasions with different batches of cells, and with simultaneously run positive and negative controls: cells transfected with wild type receptor or pSG5 vector alone. Values in each well were corrected for cell number by measuring cell surface activity, and the 180-kDa form appears to be a transmembrane but not Does Alter 125I]TSH Binding—Mutation of alanine 623 in the TSH Receptor

**RESULTS**

**Mutation of Alanine 623 Does Not Appear to Alter Synthesis, Processing, or Integration of the TSHR into the Plasma Membrane but Does Alter 125I]TSH Binding**—Mutation of alanine 623 to glutamic acid (623E) or lysine (623K) had no effect on transfection efficiency as measured by cotransfecting 0.1 μg of pSVGH; thus, growth hormone levels were 413 ± 32 ng (mean ± S.E.) in all transfections. In addition, the mutations did not alter the composition or amount of TSHR forms detected on Western blots of membranes from cells. Thus, similar amounts of the same three major TSHR forms, 230, 180, and 95 kDa, were identified (Fig. 2). The 230-kDa form is the unprocessed precursor of the receptor, the 95-kDa protein is the fully glycosylated receptor form associated with cell surface activity, and the 180-kDa form appears to be a biosynthetic intermediate, not a dimer of the 95-kDa form (17).

The two mutants exhibit specific and saturable [125I]TSH binding. Displacement data (Fig. 3A) or saturation binding assays using concentrations of [125I]TSH between 400 and 700,000 cpm/well (data not shown) indicated that Kd values for the 623K and 623E mutants were 16- or 8-fold lower than wild type receptor, 14 ± 2, 28 ± 7, and 231 ± 53 pm, respectively (Fig. 3A, insert); Bmax was, however, decreased by 8- and 5-fold to 12 ± 1.5 and 21 ± 1%, respectively, of wild type receptor (Fig. 3A, insert). Decreases in Bmax comparable to the mutants, i.e. to 45.1 ± 1.8 or 13.2 ± 2.1%, could be obtained by decreasing the amount of transfected wild type plasmid DNA from 25 to 2.5 or 1 μg, respectively; in this circumstance there was, however, no comparable change in Kd.

**Mutation of Alanine 623 Results in a Loss in TSH-induced Inositol Phosphate Formation but Not TSH-induced cAMP Signal Generation—TSH-increased inositol phosphate formation in Cos-7 cells transfected with wild type and mutant (623E, 623K) TSHR.**

**FIG. 2. Western blot of TSHR forms in membranes from Cos-7 cells transfected with 25 μg of wild type or mutant receptor plasmids or the pSG5 vector.** Enriched plasma membrane preparations from the same number of cells, transfected with equal efficiencies, were solubilized in Laemmli sample buffer containing 5% β-mercaptoethanol, and 10 μg of membrane protein was subjected to SDS-PAGE using a 4–12% gel (17). After blotting, nitrocellulose membranes were sequentially incubated with a rabbit antibody against the synthetic TSHR peptide (residues 352–366) and 125I-labeled donkey anti-rabbit Ig F(ab’2). The 230-, 180-, and 95-kDa proteins are not identified with preimmune serum nor with immune serum preincubated with peptide 352–366 (17).

**FIG. 3. Displacement of [125I]TSH binding by unlabeled TSH (A) and ability of TSH (B), a Graves' IgG (C), and ATP (D) to increase inositol phosphate formation in Cos-7 cells transfected with wild type and mutant (623E, 623K) TSHR.** In A, displacement regression curves are determined by LIGAND (16); data are from a single experiment performed in duplicate. Essentially identical results were obtained in five separate experiments; Kd and Bmax values (insert, panel A) are the mean of all of these. In B–D, each point is the mean ± S.E. of all experiments, the number of which is noted in Table I. WT(1), WT2(5), and WT(2) represent wild type transfectants made with 1, 2.5, and 25 μg of plasmid DNA, respectively. All other transfections included 25 μg of DNA including those with pSG5 vector alone.
The TSHR is one of a number of receptors for physiologically important hormones, i.e. parathormone, calcitonin, and
gonadotropins, which couple to different G proteins associated with stimulation of the cAMP and IP$_2$ cascade (8). This is the first report of a point mutation which discriminates between the two ligand-induced signals of this group of seven transmembrane receptors. Thus, we show that mutation of alanine 623 of the TSHR to glutamic acid (623E) or lysine (623K) results in a loss of TSH-increased inositol phosphate but not cAMP formation. These results may be compared to mutations that selectively perturb coupling to only one of the effector pathways used by $\alpha_{2\text{-AR}}$ and $\beta$-AR receptors. Thus, mutation of aspartic acid 79 in $\alpha_{2\text{-AR}}$ (18) results in a loss in agonist increased potassium currents but no change in agonist-inhibited adenylylcyclase or calcium currents; deletions of residues 222–229 and 258–270 in $\beta$-AR (19) lose $G_{s\text{-}}$dependent stimulation of adenylylcyclase but not non-$G$-coupled Na-H exchange.

Although the cAMP signal is important for growth and function of the thyroid, it is not the only signal implicated (1–6, 11, 12). The hyperfunction of Graves' patients does not correlate with goiter; neither correlates quantitatively with the TSHR 3C 441 residue. Three cytoplasmic loops of the TSHR have two-thirds fewer residues than the $\alpha_{1\text{-AR}}$ and no homologous insensitive G protein involved in activation of the PIP$_2$ cascade (20). The third cytoplasmic loop of the TSHR has two-thirds fewer residues than the $\alpha_{1\text{-AR}}$ and no homologous insensitivity to glutamic acid or lysine causes, in contrast to data herein, a significant enhancement of basal inositol phosphate formation, from approximately 2 to 200% above control, but no change in maximal ligand-increased activity despite a lower EC$_{50}$ (7). Nevertheless, it is clear that the comparable alanines in TSHR and $\alpha_{2\text{-AR}}$ are specifically linked to initiation of the IP$_2$ cascade, probably by influencing receptor affinity for a pertussis toxin-insensitive G protein involved in phospholipase C activation. These data are particularly relevant to gonadotropin receptors, whose sequences are identical in this area (6), but may be generally important to other seven-transmembrane domain receptors in the group.

Transfections with mutant receptors may result in decreased synthesis, processing, and insertion into the membrane. This is not the case here since there is no change in receptor forms present in the membranes on Western blots. Nevertheless, both alanine 623 mutations lower the $K_d$ and decrease the B$_{max}$ of ligand binding, as was the case when alanine 293 of the $\alpha_{2\text{-AR}}$ was mutated (7, 20). There is no comparable change in EC$_{50}$ and even an increase (623E) in the maximal TSH-increased cAMP response relative to basal.

Loss of coupling cannot be accounted for by alterations in absolute receptor number, since comparable changes are not evidenced when the $B_{max}$ of TSH binding is decreased nearly 90% by using less wild type receptor plasmid in the transfection. In the case of the $\alpha_{2\text{-AR}}$ (7), altered ligand binding is believed to result from an altered conformation associated with abnormal coupling to the G protein. In the case of the TSHR, altered ligand binding resulting from the conformation change may reflect the selective loss of coupling between mutant receptors and the G protein involved in phospholipase C activation but not $G_s$. The TSHR binds ligand via its large extracellular domain (15, 17). The present data appear, therefore, to establish that there is a conformational relationship between the signaling domain on the cytoplasmic surface of the cell and the large extracellular binding domain of the receptor. This was a possibility suggested for the lutropin receptor when aspartic acid 383 in the 2nd transmembrane domain was mutated (21).

The integrity of all three cytoplasmic loops of the TSHR is important for the TSH-induced increase in cAMP levels (14). In the case of the $\beta$-AR, receptor coupling to $G_s$ can thus be presumed to involve the C-terminal domain (19, 22). Moreover, it has been shown (14) that simultaneous substitution of all residues between 617–625, other than isoleucine 622 and alanine 623, results in a loss of the TSH-increased cAMP signal, whereas substitution of each residue individually has no effect (23). Caution must be used in interpreting the former TSHR result (14), since there is an associated 10-fold decrease in receptor affinity and since no measurements of $B_{max}$ or inositol phosphate formation were made nor was there evidence of normal receptor synthesis or degradation.

REFERENCES

1. Bone, E. A., Ailling, D. W., and Grollman, E. F. (1986) Endocrinology 119, 2123–2130.
2. Fieschi, J. B., Edery, P. A., Marshall, N. J., and Cockcroft, (1987) Biochem. J. 247, 519–524.
3. Conte, D., Marocco, C., Kohn, L. D., Axelrod, J., and Luini, A. (1985) J. Biol. Chem. 260, 9230–9236.
4. Tahara, K., Grollman, E. F., Saji, M., and Kohn, L. D. (1991) J. Biol. Chem. 266, 1430–1435.
5. Sho, K., Okajima, K., Majid, M. A., and Kondo, Y. (1991) J. Biol. Chem. 266, 12180–12184.
6. Akasaka, T., Iyama, S., Saji, M., Kosugi, S., Kozak, C. M., McBride, W. O., and Kohn, L. D. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 5679–5681.
7. Kielbasa, M. A., Cote, P., Okajima, K., Caron, M. G., and Lefkowitz, R. J. (1992) J. Biol. Chem. 267, 1430–1435.
8. Thompson, E. B. (1992) Mol. Endocrinol. 6, 501.
9. Rouesse-Mercier, M. F., Masashi, M., Leodahl, H., Ager, M., and Berger, R. (1990) Genomics 6, 223–236.
10. Iyama, S., Niller, H. H., Shimura, H., Akasaka, T., and Kohn, L. D. (1992) Mol. Endocrinol. 6, 785–804.
11. Van Sande, J., Raep, E., Ferreti, J., Lejeune, C., Maaenhout, C., Vassart, G., and Dumont, J. E. (1990) Mol. Cell. Endocrinol. 74, R1–R6.
12. Di Cerbo, A. Di, Giralamo, M., Guardabasso, V., De Filippis, V., and Corda, D. (1990) J. Clin. Endocrinol. Metab. 74, 585–591.
13. Laurent, E., Van Sande, J., Ludgate, M., Corvelin, B., Rocman, P., Dumont, J. E., and Mocek, J. (1991) J. Clin. Invest. 87, 1634–1642.
14. Chazenbalk, G. D., Nagayama, Y., Russo, D., Wadsworth, H. L., and Rapoport, B. (1990) J. Biol. Chem. 265, 20970–20975.
15. Kosugi, S., Ban, T., Akazuma, T., and Kohn, L. D. (1992) Mol. Endocrinol. 6, 106–110.
16. Munson, P. J., and Rodbard, D. (1980) Anal. Biochem. 107, 220–239.
17. Ban, T., Kosugi, S., and Kohn, L. D. (1992) Endocrinology 131, 815–829.
18. Stavnezer, A., Herzenz, D. A., Aabakken, H., and Limbird, L. E. (1992) Science 257, 977–980.
19. Barber, D. L., Ganz, M. B., Bongiorno, P. B., and Strader, C. D. (1992) Proc. Natl. Acad. Sci. U.S.A. 89, 2896–2900.
20. Cote, S., Ekum, S., Caron, M. G., and Lefkowitz, R. J. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 2896–2900.
21. J. E., and J. T. (1991) J. Biol. Chem. 266, 1495–14957.
22. O'Toole, B. F., Hrtonowitch, M., Regan, J. W., Leader, W. M., Caron, M. G., and Lefkowitz, R. J. (1992) J. Biol. Chem. 267, 1495–14957.
23. Chazenbalk, G. D., Nagayama, Y., Wadsworth, H. L., Russo, D., and Rapoport, B. (1991) Mol. Endocrinol. 5, 1523–1526.