Vol. 200, No. 1, 1994 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 15, 1994 Pages 401-407

THE AMINO-TERMINAL HALF OF THE CYTOPLASMIC TAIL OF THE THYROTROPIN RECEPTOR IS ESSENTIAL FOR FULL ACTIVITIES OF RECEPTOR FUNCTION

Shinji Kosugi and Toru Mori

Department of Laboratory Medicine, Kyoto University School of Medicine, Kyoto 606-01, Japan

Received February 27, 1994

SUMMARY: We investigated the role of the cytoplasmic tail of the thyrotropin receptor (TSHR) in signal transduction using mutants with truncation at the cytoplasmic tail. Mutant without residues 700-764 completely lost inositol phosphate response to agonists and showed decreased basal cAMP level. Mutant without residues 710-764 showed full cAMP responses but blunted inositol phosphate responses. However, mutant without residues 722-764 retained all signal transduction activities. These findings suggest that the amino-terminal half of the cytoplasmic tail (up to residue 721) is essential for full expression of functional activities.

The thyrotropin receptor (TSHR) activates phosphatidylinositol bisphosphate (PIP_2) as well as cAMP signals when stimulated by TSH or TSHR autoantibodies from patients with Graves' disease (1-4). Both signals are involved in growth and various cellular functions of 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 cytoplasmic loops of the TSHR, by substituting 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 three cytoplasmic loops are important for PIP_2 signaling, and (iii) the three loops are important for regulating basal cAMP levels (2,4,6).

We have extended mutagenesis study to the intracellular region to characterize the roles of this region in signal transduction activity.

MATERIALS AND METHODS

Mutagenesis Oligonucleotide-mediated, site-directed mutagenesis was used to create truncation mutants by inserting a stop codon as previously described (2,4,6).

0006-291X/94 $5.00

Transfection Transfection of Cos-7 cells with DNA was performed by electroporation (2,4,6); 25 μg purified plasmid DNA was used in each. To evaluate transfection efficiency, pSVGH was co-transfected with mutant or wild type (WT) TSHR or pSG5 vector cDNA. Aliquotes of the same batch of transfected cells were plated for Northern blot, Western blot, TSH binding assay, 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).

Assays All the assays were initiated simultaneously 48 hours 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 hours 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, 0.5 or 5.0 mg/ml Graves' or normal IgG, 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 batches of cells, and with simultaneously run of positive and negative controls: cells transfected with WT TSHR DNA or pSG5 vector alone. Values in each well were corrected for cell protein. The program LIGAND (7) 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 in 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 (2,4,6) using total RNA and WT, full length rat TSHR cDNA probe.

Western blot Western blot of membrane proteins derived from an aliquot of Cos-7 cells transfected with WT or mutant receptor cDNA was performed as described (8).

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

RESULTS

We created three truncation mutants involving the cytoplasmic tail by inserting a stop codon (Fig. 1). The mutants are denoted as residue number (counted from methionine start site) where a stop codon is inserted and "ST" indicating a stop codon. For example, 700ST has a stop codon at residue 700 and has a correct amino acid sequence at residues 1-699. The same designation is used for description of the mutant cDNA itself and the Cos-7 cells transfected with the mutant cDNA. The shortest truncation mutant 700ST was designed to preserve Cys-699 whose equivalent cysteine residue is palmytolated to form "the fourth cytoplasmic loop" which is essential for signal transduction in adrenergic receptors (10).

To accurately compare functional activities among transfectants, cells were transfected by electroporation in batches, and aliquotes from one transfectant were used for GH measurement, Northern blot, Western blot, TSH

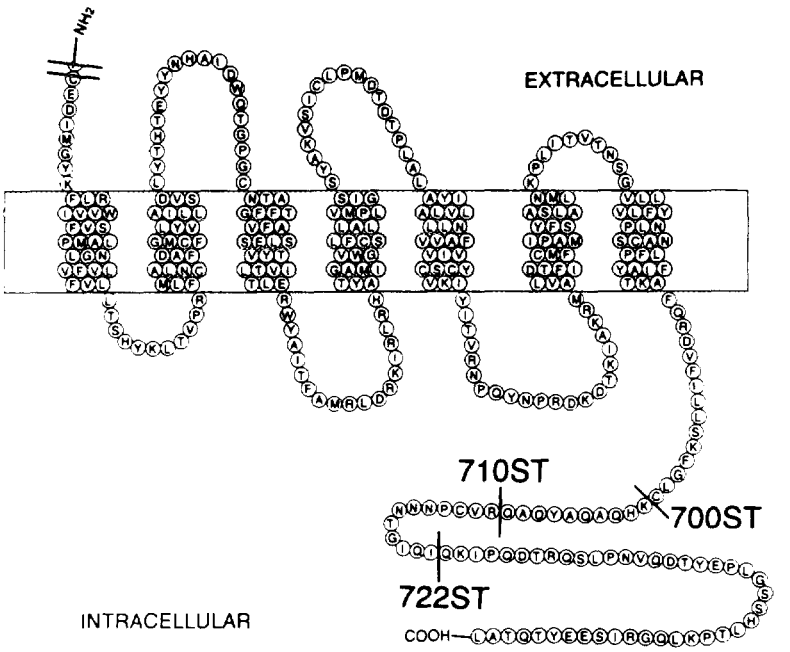

FIGURE 1. Putative membrane topology of the transmembrane and intracellular regions of the rat TSH receptor (TSHR) and the sites of truncation.

binding and cAMP/inositol phosphate assay simultaneously. Transfection efficiency, determined by co-transfecting 0.1 μg pSVGH and measuring the GH concentrations in the culture media, was always within ± 13% of the mean. The mRNA from Cos-7 cells transfected with the mutants, estimated by Northern blot analysis of 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.

As shown in Fig. 2, WT exhibited three major TSHR forms, 230, 180, and 95 kDa forms as described previously (8). These three bands migrated differently in the mutants from WT. The 95 kDa form, which directly relates the mature receptor (8), is apparently smaller than that of WT. Approximate sizes of the band are 87, 89 and 91 kDa in 700ST, 710ST and 722ST, respectively. Differences in size correspond well to those in predicted molecular weight. However, the major 3 forms appeared essentially the same in amounts among three different batches of transfected cells. In contrast, the 54kDa form which is a proteolytic fragment of the extracellular domain of the TSHR (8) is in identical position among WT and the mutants.

Ten μM ATP increased inositol phosphate levels 3.7 ± 0.4 fold (mean ± S.E.) in Cos-7 cells transfected with WT or pSG5 vector. Every mutant transfectant exhibited essentially the same response to ATP (data not

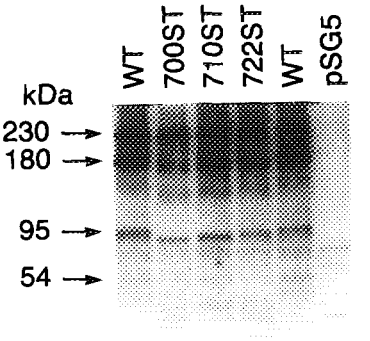

FIGURE 2. Western blot of TSHR forms in membranes from Cos-7 cells transfected with 25 μg wild type and mutant plasmid or the pSG5 vector.

shown), indicating inositol phosphate response to TSH or Graves' IgG is TSHR specific.

Functional Activities of Mutant Receptors

As shown in Fig. 3A and Table 1, 700ST and 710ST exhibited significantly decreased TSH binding Bmax compared with WT. There were however, significant increases in affinity. 722ST did not show any difference in TSH binding from that in WT. The EC_{50} for TSH in cAMP assays in the three mutants was not statistically different from WT. However, the maximal response relative to the level in pSG5 control transfectant was significantly decreased only in 700ST because the basal cAMP level was decreased in 700ST (Fig. 3B). In contrast, WT, 710ST and 722ST increased the basal cAMP level compared with pSG5 control transfectant. The maximal

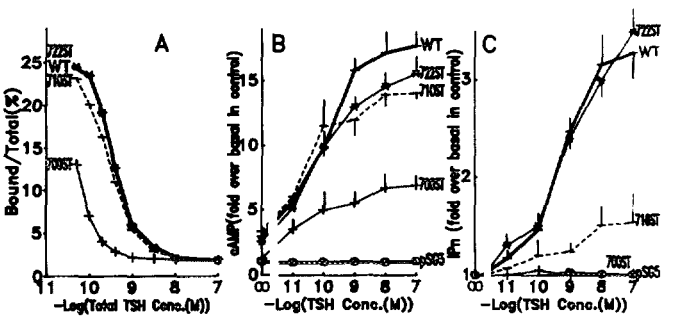

FIGURE 3. Displacement of [^{125}I]TSH binding by unlabeled TSH (A) and the ability of TSH to increase cAMP (B) and inositol phosphate formation (C) in Cos-7 cells transfected with wild type (WT) and the truncation mutants in the cytoplasmic tail (700ST, 710ST and 722ST) (Fig. 1). The data in A are from a single experiment performed in duplicate. Essentially identical results were obtained in at least 3 separate experiments. The Kd and Bmax values determined by the program LIGAND (7) are noted in Table 1 and are the means ($\pm$ S.E.) of all experiments. In B and C, each point is the mean $\pm$ S.E. 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.

TABLE 1. Summary of mutant activities

Mutant	pSG5	WT	700ST	710ST	722ST
TSH binding					
Kd (pM)	-	213±23	8±3 **	94±14 *	147±54
%Bmax (/WT)	-	100	6±3 **	41±4 **	69±18
cAMP increase					
EC50 (pM)	-	125±18	25±20	31±23	67±28
%Basal(/pSG5)	100	294±26	127±29 **	333±38	256±31
Max Resp(/pSG5)+	-	17.8±1.2	6.9±0.7 *	14.1±0.3	15.6±1.3
Max Resp(/basal)`	-	6.0±0.7	5.5±0.6	4.2±0.2	6.1±0.6
IPn increase					
EC50 (pM)	-	571±62	NR#	1190±304	663±103
%Basal(/pSG5)	100	96±2	99±1	98±4	90±8
Max Resp(/pSG5)+	-	3.3±0.2	-	1.5±0.3 **	3.5±0.3
Number of Experiments	9	9	3	3	3

Values are expressed as means ± S.E. of all experiments whose numbers are shown in the bottom line.
Nonresponsive.
** 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).

responses, when compared with the basal levels of the same transfectant in the absence of TSH, were not significantly different among WT and the three mutants (Table 1). On the other hand, increase in inositol phosphates induced by TSH was almost completely abolished in 700ST (Fig. 3A and Table 1). 710ST significantly decreased maximal response but did not change EC_{50} significantly in the TSH-inositol phosphate assay. 722ST shows inositol phosphate response similar to WT in terms of both EC_{50} and maximal response.

As shown in Table 2, Graves' IgG #1, which was used in our previous studies (2,4,6), stimulated cAMP response in all the transfectants. cAMP responses by Graves' IgG in 710ST and 722ST were similar to those in WT. In 700ST, the response when compared with the basal level of the pSG5 control transfectant was markedly lower again. However, when compared with the basal level of each transfectant in the absence of TSH, 700ST responded to the Graves' IgG similarly to WT. On the other hand, 700ST and 710ST showed no or very weak inositol response to Graves' IgG, respectively (Table 2). However, 722ST and WT responded well to Graves' IgG. Generally, cAMP and inositol phosphate responses induced by Graves' IgG approximated the responses by TSH.

DISCUSSION

The three truncation mutants involving the cytoplasmic tail reasonably retained agonist-induced cAMP response. But the basal cAMP level was

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

Mutant		pSG5	WT	700ST	710ST	722ST
cAMP(/pSG5)+						
IgG#	0.5 mg/ml	1.0	9.7	4.7	10.1	9.9
	5.0 mg/ml	1.0	14.0	5.8	12.2	12.9
cAMP (/normal IgG)`						
IgG#	0.5 mg/ml	1.0	3.3	3.7	3.1	3.8
	5.0 mg/ml	1.0	4.8	4.6	3.8	5.0
IPn (/pSG5)+						
IgG#	0.5 mg/ml	1.0	1.5	1.0	1.1	1.6
	5.0 mg/ml	1.0	2.5	1.0	1.3	2.4

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

decreased in 700ST and the inositol phosphate response was lost or decreased in 700ST and 710ST. They significantly decreased TSH binding Bmax despite of exhibiting similar amount of completely processed TSHR form on Western blot. It should be noted that the WT TSHR transfectant with 1/8 TSH binding Bmax of the usual transfectant by decreasing the amount of DNA for transfection responded well to TSH and Graves' IgG giving a similar EC_{50} in terms of both cAMP and inositol phosphate responses (2).

These results lead to the conclusion that residues 700-709 are essential for increasing basal cAMP level and inositol phosphate response and that residues 710-722 are also important for full inositol phosphate response. Together with our unpublished observation that residues 684-672 are also essential for inositol response, amino-terminal half of the cytoplasmic loop of the TSHR is necessary for full functional expression of the receptor. In contrast, residues 722-764 are not necessary for or do not influence cAMP and PIP_2 responses and TSH binding.

Chazenbalk et al. (9) reported that a mutant (Δ709-764) showed slightly higher TSH binding affinity than WT and TSH-cAMP response similar to that of WT. This is consistent with our observation of 710ST. They also showed that a mutant with substitution at residues 699-707 had TSH binding affinity and EC_{50} in the TSH-cAMP response similar to those of WT but markedly decreased maximal cAMP response. In adrenergic and muscarinic receptors, the amino-terminal portion of the cytoplasmic tail adjacent to the transmembrane helices, as well as the 3rd and 2nd cytoplasmic loops, are considered to be important for G protein coupling (10).

The evidences obtained in the present study seem to be applicable to gonadotropin receptors which are homologous to TSHR in the amino-terminal

of the cytoplasmic tail, like the three cytoplasmic loops and unlike the carboxyl-terminal of the cytoplasmic tail (11-15).

The data obtained from all substitution mutants involving one of the three cytoplasmic loops and the amino-terminal portion of the intracellular region 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, the amino- and carboxyl-terminals of the 3rd cytoplasmic loop, and the amino-terminal half of the intracellular region, 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.

Cytoplasmic tail contains a possible phosphorylation site for protein kinase C at Thr-748 (15). 722ST is a good tool for characterizing carboxyl-terminal phosphorylation because it is essentially the same as WT in terms of TSH binding and signal transduction activities and it lacks above mentioned C-kinase site and other possible phosphorylation sites such as Thr-728, Thr-738, Ser-756, Thr-760.

REFERENCES

(1) Van Sande, J., Raspe, E., Perret, J., Lejeune, C., Maenhaut, C., Vassart, G. and Dumont, J.E. (1990) Mol. Cell. Endocrinol. 74, R1-R6.
(2) Kosugi, S., Okajima, F., Ban, T., Hidaka, A., Shenker, A. and Kohn, L.D. (1992) J. Biol. Chem. 267,24153-24156.
(3) Van Sande, J., Lejeune, C., Ludgate, M., Munro, D.S., Vassart, G. and Dumont, J.E. (1992) Mol. Cell. Endocrinol. 88,R1-R5.
(4) Kosugi, S., Okajima, F., Ban, T., Hidaka, A., Shenker, A. and Kohn, L.D. (1993) Mol. Endocrinol. 7,1009-1020.
(5) Vassart, G. and Dumont, J.E. (1992) Endocr. Rev. 13,596-611.
(6) Kosugi, S., Kohn, L.D., Akamizu, T. and Mori, T. Mol. Endocrinol. in press.
(7) Munson, P.J. and Rodbard, D. (1980) Anal. Biochem. 107,220-239.
(8) Ban, T., Kosugi, S. and Kohn, L.D. (1992) Endocrinology 131,815-829.
(9) Chazenbalk, G.D., Nagayama, Y., Russo, D., Wadsworth, H.L. and Rapoport, B. (1990) J. Biol. Chem. 265,20970-20975.
(10) Strosberg, A.D. (1991) Eur. J. Biochem. 196,1-10.
(11) Yarnev, T.A., Sairam, M.R., Khan, H., Ravindranath, N., Payne, S. and Seidah, N.G. (1993) Mol. Cell. Endocrinol. 93,219-226.
(12) Gudermann, T., Birnbaumer, M. and Birnbaumer, M. (1992) J. Biol. Chem. 267,4479-4488.
(13) Libert, F., Lefort, A., Gerard, C., Parmentier, M., Perret, J., Ludgate, M., Dumont, J.E. and Vassart, G. (1989) Biochem. Biophys. Res. Commun. 165,1250-1255.
(14) Parmentier, M., Libert, F., Maenhaut, C., Lefort, A., Gerard, C., Perret, J., Van Sande, J., Dumont, J.E. and Vassart, G. (1989) Science 246,1620-1622.
(15) Akamizu, T., Ikuyama, S., Saji, M., Kosugi, S., Kozak, C., McBride, O.W. and Kohn, L.D. (1990) Proc. Natl. Acad. Sci. U.S.A. 87,5677-5681.