Ras homolog enriched in striatum inhibits the functional activity of wild type thyrotropin, follicle-stimulating hormone, luteinizing hormone receptors and activating thyrotropin receptor mutations by altering their expression in COS-7 cells

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ABSTRACT. Ras homolog enriched in striatum (Rhes) is a member of the Ras family of small GTPases detected in the thyroid. Rhes inhibits signal transduction from Gαs protein. In this study we investigated whether Rhes can interfere with stimulation of cAMP/protein kinase A (PKA) pathway of TSH, FSH and LH receptors (TSHr, FSHr, LHr) and of activated TSHr mutants. Receptors were transiently transfected in COS-7 cells with or without Rhes; cAMP was evaluated in basal conditions and after hormone stimulation. Constitutive and bovine TSH (bTSH)-stimulated activity of wild type (wt) and mutated TSHr was inhibited after Rhes co-transfection. Rhes decreased cAMP after FSH and hCG β-subunit (βhCG) stimulation in cells expressing the cognate receptors. In binding experiments Rhes, as another membrane protein, sodium/iodide symporter (NIS), reduced membrane expression of wt TSHr (wtTSHr). In conclusion, Rhes can interfere with the functional activity of wt and mutated TSHr and with the respective hormone-stimulated cAMP production of FSHr and LHr. This interference is not specific and due to the co-expression of two membrane proteins. (J. Endocrinol. Invest. 30: 279-284, 2007)

INTRODUCTION

Ras homolog enriched in striatum (Rhes) is a member of the Ras family small GTPases and it is predominantly expressed in the striatum. The expression of Rhes in the striatum is regulated by thyroid hormones (1, 2). Rhes protein is composed of 266 amino acids and belongs to the RASD subfamily of the Ras-related GTP-binding protein superfamily. Rat Rhes has 95% identity with human Rhes ortholog identified as a tumor endothelial marker (TEM2) (3) and 62% identity with dexamethasone-induced Dexras1, another member of the RASD subfamily. Ras family proteins are molecular switches that respond to extracellular signals and regulate intracellular signaling pathways controlling cell growth, gene transcription, mRNA stability and translation, cytoskeleton organization, peptide trafficking, and secretion. In the central nervous system, Ras protein controls pathways involved in synaptic plasticity, learning, and memory (4).

Rhes is prominently expressed in the striatum and other areas of the brain but, interestingly, it is also detected in the thyroid gland. Rhes is farnesylated and targeted to the plasma membrane via this post-translational modification (5).

The Rhes homolog Dexras1 is implicated in the activation of Gαi proteins (6). On the other hand, Rhes does not seem to influence signalling through Gαi proteins, but inhibits signal transduction from a Gαs protein coupled to cAMP production. This is supported by the finding that Rhes inhibits the activation of a cAMP responsive element (CRE) induced by a constitutively active β2-adrenergic receptor, but not when induced by forskolin or by a constitutively active Gαs protein suggesting that Rhes acts somewhere upstream of the activation of the heterotrimeric complex by the receptor. Moreover, it has been demonstrated that Rhes impairs the activation of the cAMP/protein kinase A (PKA) pathway mediated by the TSH (5).

Key-words: Rhes, G-protein coupled receptors, cAMP, PKA.

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The TSH receptor (TSHr), together with FSH receptor (FSHr) and LH receptor (LHr), is a member of a subfamily of seven transmembrane G protein-coupled receptors (GPCRs), characterized by a large N-terminal extracellular domain involved in hormone binding (7, 8); the receptor is mainly coupled to adenylyl cyclase via Gαs and, in some species including man, it also activates the inositol phosphate cascade (IPs) via a Gαq protein (9-11). Somatic and germline activating mutations of the TSHr gene have been identified as a major cause of toxic thyroid adenoma (12-14) and hereditary or sporadic non-autoimmune toxic thyroid hyperplasia (15, 16), respectively. Moreover, activating TSHr mutations have been identified in the majority of hyperfunctioning thyroid nodules of toxic multinodular goiter (17). All activating TSHr mutations have been shown to activate adenylyl cyclase when expressed in eukaryotic cells. Some of these mutations also represent constitutive activity for the inositol-phosphates pathway (12, 13).
In this paper we studied whether Rhes was able to interfere with stimulation of the cAMP/PKA pathway of glycoprotein hormone receptors (TSHr, FSHr and LHr). We also explored whether Rhes was able to inhibit the constitutive activity of activated TSHr mutants.

MATERIALS AND METHODS

Construction of mutant TSHr

In Figure 1, the localization of the TSHr mutations included in this study is shown. In particular, isoleucine/methionine substitution at position 486 of TSHr (I486M) mutation is localized in the 1st extracellular loop, serine/asparagine substitution at position 505 of TSHr (S505R) in the 3rd transmembrane segment, alanine/isoleucine substitution at position 623 of TSHr (A623I) in the 3rd intracellular loop, and proline/serine substitution at position 639 of TSHr (P639S) in the 6th transmembrane segment.
The constructs harboring the single mutated TSHr I486M, S505R, A623I, and P639S have been described (12, 13, 17, 18). In brief, a SpeI-CvnI segment (1322-1603) or a CvnI-BstEII segment (1604-2169) in the cDNA of the wild type (wt) TSHr (wtTSHr) in the expression vector pSVL, was replaced by a homologous segment harboring the mutation in position 486, 505, 623, and 639, respectively. These mutated sequences were directly cloned from DNA extracted from nodular tissues obtained from patients with toxic multinodular goiter (I486M, A623I, P639S) or from the blood of a patient affected by autosomal dominant toxic thyroid hyperplasia with a germline activating TSHr mutation (S505R). The SpeI restriction site in the wtTSHr was created by site-directed mutagenesis (the change in the coding region does not modify the encoded amino acid sequence).
The resulting constructs were directly sequenced by an ABI PRISM 310 Genetic Analyzer (PE Applied Biosystems, Foster City, CA) to verify the presence of the mutations.

Expression in eukaryotic cells of wt and mutated genes

For transient expression, COS-7 cells were seeded at the concentration of about 150.000 cells/3-cm dish and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 2.5 µg/ml fungizone, and 1 mM sodium pyruvate. Twenty-four h after seeding, the cells were transfected using the DEAE-Dextran method followed by exposure to 10% dimethylsulfoxide (DMSO) for 2 min (19); COS-7 cells were transfected with 250 ng of wtTSHr, wt FSH and LH receptor (wtFSHr, wtLHr) genes as well as mutated TSHr; when needed, the cells were co-transfected with increasing amounts (from 5 to 10000 ng) or with 5 µg of the Rhes gene in the pCEFL expression vector.
In order to investigate the effect of co-transfection of two different membrane proteins on cAMP production and on cell membrane expression through binding studies, COS-7 cells were also co-transfected with 250 ng of wtTSHr gene and 250 ng or 5 µg of the sodium/iodide symporter (NIS) gene cloned into the expression vector pcDNA3.
Forty-eight h after transfection, the cells were used for cAMP determinations and binding assay. Triplicate dishes were used for each condition and each experiment was repeated at least three times. Results were expressed as mean±SE from one representative experiment. When not shown, SE values were so small that they fall within the symbols.

Functional assays

cAMP and binding assays were performed as described in reference 17.

RESULTS

Rhes protein effect on the constitutive activity of TSHr

Both wild type and mutated TSHr displayed constitutive activity toward cAMP production when transfected in COS-7 cells. The activity of the mutated receptors

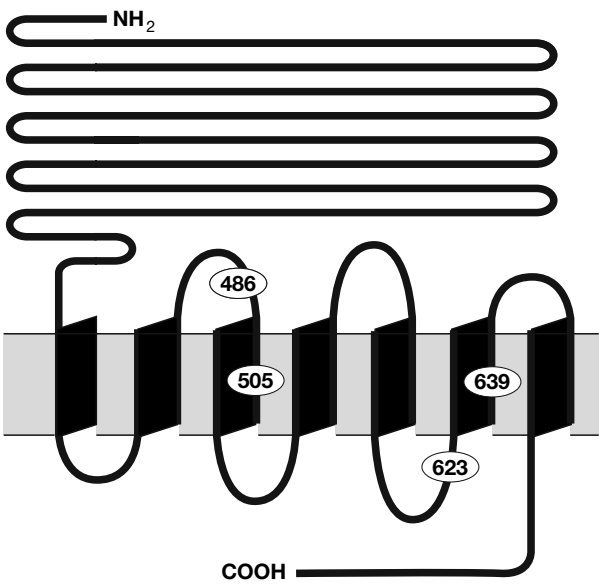


Fig. 1 - *Schematic localization of the TSH receptor activating point mutations included in the study.*

was 2- to 5-fold higher than that of wt receptor. The co-transfection of COS-7 cells with the wtTSHr gene and increasing amounts of the Rhes gene determined a significant decrease of basal constitutive cAMP levels starting from 500 ng of the Rhes gene (Table 1); the minimum level of cAMP production was observed after co-transfection with 5000 ng of the Rhes gene and this dose was used for further experiments.
After co-transfection with Rhes, the constitutive cAMP production by the wtTSHr and the mutated receptors decreased by 25-45% with respect to COS-7 cells transfected with the receptors alone (Table 2). As a positive control, COS-7 cells were transfected with the constitutively active β2-adrenergic receptor in the presence and in the absence of the Rhes protein; Rhes was able to reduce by 27% the cAMP production in co-transfected COS-7 cells (Table 2). Moreover, Rhes was not able to inhibit the direct activation of the PKA induced by forskolin (Table 2) or by a constitutively active Gαs protein (data not shown).

Rhes protein effect on hormone-stimulated FSHr and LHr

When expressed in COS-7 cells, wtFSHr and wtLHr, as expected, did not show constitutive activity in terms of cAMP production (Table 2).
The stimulation of the wtFSHr with sub-maximal concentrations of human reconbinant FSH (hrFSH) (100 mU/ml) increased cAMP production about 2-fold with respect to the basal values (Table 2). Co-transfection of the Rhes gene resulted in a significant decrease (about 36%) in cAMP accumulation after 100 mU/ml hrFSH stimulation (Table 2).
The stimulation of the wtLHr with sub-maximal concentrations of hCG β-subunit (βhCG) (2 mU/ml) increased cAMP production about 3-fold with respect to the basal values (Table 2). Co-transfection of the Rhes gene determined a significant decrease (about 39%) in cAMP level after 2 mU/ml βhCG stimulation (Table 2).

Rhes protein effect on bTSH-stimulated TSHr

Co-transfection of the Rhes gene was able to significantly reduce the cAMP production induced by bovine TSH

Table 1 - *Basal and bovine TSH (bTSH) (10 mU/ml) stimulated cAMP production in COS-7 cells co-transfected with wild type TSH receptor (wtTSHr) and increasing amounts of Ras homolog enriched instriatum (Rhes) gene. The minimum level of cAMP was produced after co-transfection with 5000 ng of Rhes gene. *p<0.05.*

	bTSH (mU/ml)	
	0	10
wtTSHr	49.3±3.2	278.9±11.1
wtTSHr + 5 ng Rhes	48.2±4.3	278.4±16.3
wtTSHr + 10 ng Rhes	46.1±4.2	271.4±14.8
wtTSHr + 50 ng Rhes	46.3±3.5	266.4±15.3
wtTSHr + 100 ng Rhes	44.2±2.5	261.5±16.7
wtTSHr + 250 ng Rhes	43.1±3.0	252.5±15.0
wtTSHr + 500 ng Rhes	39.4±3.4*	213.1±15.7*
wtTSHr + 1000 ng Rhes	36.8±3.6*	207.7±15.5*
wtTSHr + 5000 ng Rhes	33.2±3.1*	180.3±16.4*
wtTSHr + 10000 ng Rhes	33.3±2.8*	187.2±14.4*
Empty vector	15.5±1.8	

Table 2 - *cAMP production in COS-7 cells differently transfected [wt and mutated TSH receptors (wtTSHr), β2-adrenergic receptor, FSH receptor (wtFSHr), LH receptor (wtLHr)] and in COS-7 cells after forskolin stimulation in the absence and in the presence of the Ras homolog enriched in striatum (Rhes) protein. *p<0.05.*

	cAMP (pmoles/dish)	% inhibition
wtTSHr	31.8±4.2	25
wtTSHr + Rhes	23.8±2.5*	
I486M	153.2±13.2	43
I486M + Rhes	87.9±6.2*	
S505R	105.3±6.5	39
S505R + Rhes	64.4±4.4*	
A623I	144.8±17.6	38
A623I + Rhes	89.5±10.3*	
P639S	70.2±4.1	45
P639S + Rhes	38.5±1.5*	
β2-adrenergic receptor	97.6±8.1	27
β2-adrenergic receptor + Rhes	71.0±7.5*	
wtFSHr	13.1±1.3	4
wtFSHr + Rhes	12.6±1.7	
wtFSHr + 100 mU/ml FSH	29.4±3.2	36
wtFSHr + 100 mU/ml FSH+Rhes	18.8±2.0*	
wtLHr	16.5±1.5	5
wtLHr + Rhes	15.7±1.5	
wtLHr + 2 mU/ml βhCG	46.6±5.3	39
wtLHr + 2 mU/ml βhCG + Rhes	28.2±2.9*	
Forskolin	118±12.2	2
Forskolin+Rhes	115.2±15.1	
Empty vector	14.9±2.0	

I486M: isoleucine/methionine substitution at position 486 of TSH receptor; S505R: serine/asparagine substitution at position 505 of TSH receptor; A623I: alanine/isoleucine substitution at position 623 of TSH receptor; P639S: proline/serine substitution at position 639 of TSH receptor.

(bTSH) stimulation in COS-7 cells transfected with the wt and the mutated receptors (Table 3). We observed a 17 to 60% inhibition of cAMP levels induced by increasing concentrations of bTSH (Table 3). This inhibitory effect of Rhes required targeting to the cell membrane, since a mutated soluble form of Rhes (C263A) did not affect the TSHr effect (data not shown).

Rhes protein effect on binding of ^{125}I-bTSH

To measure the total number of receptors (or TSH binding capacity, Bmax) expressed at the surface of the cells transfected with the different constructs, and their relative dissociation constants (Kd), binding studies were performed with a bovine ^{125}I-TSH tracer as described in Materials and Methods.
Cells transfected with the wtTSHr alone exhibited a higher level of expression as compared to cells co-transfected with the wtTSHr and Rhes. As shown by Bmax values, Rhes reduced the expression of the wtTSHr at the cell surface of about 50% (Fig. 2).

Effects of other membrane proteins on constitutive activity and bTSH-stimulated cAMP production and on binding of ^{125}I-bTSH

The effect of co-transfection of two different membrane proteins in COS-7 cells on cAMP production and on cell membrane expression were studied using NIS and TSHr genes.
A reduction of about 40% of basal cAMP production was observed when COS-7 cells were co-transfected with the wtTSHr and 5 µg of the NIS gene, while a reduction of 50% of cAMP level was observed after stimulation with 10 mU/ml bTSH (Fig. 3); NIS protein determined a reduced expression of wtTSHr at the cell surface in COS-7 cells co-transfected with wtTSHr and 5 µg of the NIS gene with respect to cells transfected only with the wtTSHr. As shown by Bmax values, NIS co-transfection reduced the expression of wtTSHr of about 80% (Fig. 3). Similarly, NIS co-transfection reduced the expression of FSHr, LHr and β-adrenergic receptor at a similar level (data not shown).

DISCUSSION

Rhes is a novel member of the Ras family of small GTPases. Recently, it has been demonstrated that Rhes seems to be implicated in the inhibition of signal transduction mediated by Gαs protein. Moreover, it has been demonstrated that Rhes impairs the activation of the cAMP/PKA pathway mediated by TSH in PC12 cells (5). In this study we investi-

Table 3 - *cAMP production in COS-7 cells transfected with the wild type (wt) and the mutated receptors, in the absence and in the presence of Ras homolog enriched in striatum (Rhes), both in basal conditions and after stimulation with increasing doses of bovine TSH (bTSH) (0.1; 1; 10 mU/ml).*

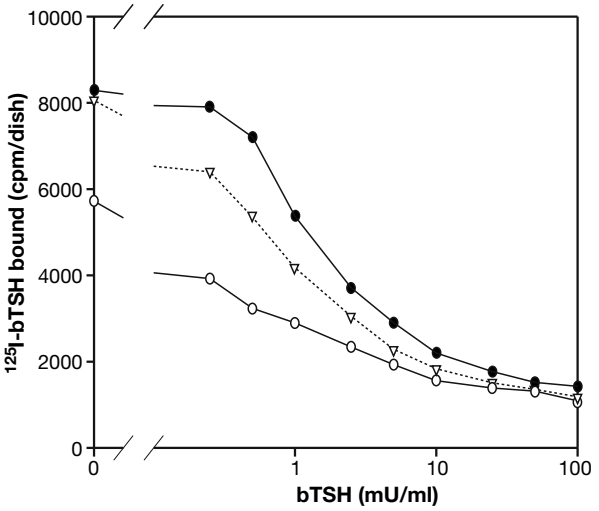
	bTSH (mU/ml)			
	0	0.1	1	10
wtTSHr	31.8±4.2	62.3±13.8	93.4±8.8	138.6±16.8
wtTSHr + Rhes	23.8±2.5	36.4±1.8	56.9±11.5	61.6±10.5
% inhibition	25	42	39	56
I486M	153.2±13.2	206.5±8.6	221.2±24.7	328.2±50.9
I486M + Rhes	87.9±6.2	98.3±3.3	118.0±18.4	130.1±7.7
% inhibition	43	52	47	60
S505R	105.3±6.5	107.9±6.2	145.8±5.8	289.9±34.3
S505R + Rhes	64.4±4.4	86.1±3.1	121.3±4.3	155.7±9.8
% inhibition	39	20	17	46
A623I	144.8±17.6	190.3±7.7	247.8±23.5	260.8±25.5
A623I + Rhes	89.5±10.3	86.5±3.4	147.0±15.7	176.1±1.9
% inhibition	38	53	41	32
P639S	70.2±4.1	91.2±13.4	103.9±8.9	150.0±11.9
P639S + Rhes	38.5±1.5	48.6±2.9	69.5±1.4	102.4±6.9
% inhibition	45	47	33	32

wtTSHr: wild type TSH receptor; I486M: isoleucine/methionine substitution at position 486 of TSH receptor; S505R: serine/asparagine substitution at position 505 of TSH receptor; A623I: alanine/isoleucine substitution at position 623 of TSH receptor; P639S: proline/serine substitution at position 639 of TSH receptor.

gated the action of the Rhes protein on the activity of wtTSHr, wtFSHr and wtLHr. We also investigated the activity of Rhes protein in previously described constitutively active TSHr mutants identified in patients with thyroid toxic adenomas.

Our results indicate that the Rhes gene, when co-transfected in COS-7 cells with the wtTSHr, is able to decrease the spontaneous constitutive activity of the receptor by about 20-30% in terms of cAMP production. At the same time Rhes was able to inhibit cAMP production in COS-7 cells transfected with wtTSHr challenged with increasing concentrations of bTSH. It is known that LHr and FSHr have no basal constitutive activity when transiently expressed in COS-7 cells (20); as expected, in this case Rhes protein was not able to modify the cAMP production of COS-7 cells transfected with wtFSHr and wtLHr, but was able to inhibit cAMP production after stimulation with hrFSH and βhCG, respectively. These data would suggest that Rhes protein is able to regulate signal transduction from G-protein coupled receptors. Previous data (5), however, were based on the final stimulation of a CRE reporter gene after transfection of adenylate cyclase linked receptors.

In this paper we show that Rhes inhibits cAMP production, a more direct measurement of the activity of Gαs-linked receptors. Rhes was not able to inhibit the function of a constitutively active Gαs or the forskolin-induced cAMP production due to the direct activation of adenylate cyclase. However, data obtained after co-transfection of NIS gene together with the wtTSHr (or FSHr, LHr) suggest a possible phenomenon that could explain this observation. As previously demonstrated (21), the co-expression of two membrane proteins alters their expression at the cell surface reducing it, probably by a saturation of the translational or post-translational machinery. It has been hypothesized that when cells are co-transfected with plasmids encoding two membrane proteins, the ribosome acceptor sites of the rough endoplasmic reticulum (ER) are saturated and a lower quantity of proteins can be synthesized (21). This effect seems to be restricted when co-expressing two membrane proteins because the expression does not decrease when the membrane protein is co-expressed with a cytosolic protein (21). In fact, the NIS gene, as the Rhes one, when co-expressed with the wtTSHr, reduced the Bmax value in binding experiments indicating a reduction of the receptor expression at the cell membrane. Consequently, the reduction of basal and bTSH-stimulated cAMP values may be due to the reduced expression of the receptor at the cell surface.



	Bmax (mU/ml)	Kd (mU/ml)
wtTSHr	0.12±0.05	1.24±0.13
wtTSHr + Rhes	0.058±0.01	0.64±0.08
wtTSHr + NIS	0.021±0.004	0.37±0.04

Fig. 2 - *Binding of ^{125}I-bTSH on COS-7 cells co-transfected with the wild type TSH receptor (wtTSHr) and 5 μg of the Ras homolg enriched in striatum (Rhes) and the sodium/iodide symporter (NIS) genes. Co-transfection of the Rhes gene reduced of about 50% the number of receptors (Bmax) expressed at the cell surface, while NIS gene reduced the Bmax value by about 80%. bTSH: bovine TSH; Kd: dissociation constant.*

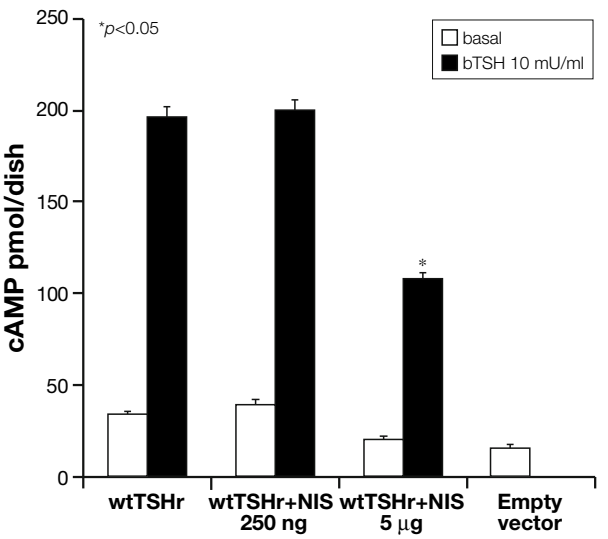


Fig. 3 - *cAMP production in COS-7 cells co-transfected with the wild type TSH receptor (wtTSHr) and 250 ng or 5 μg of the sodium/iodide symporter (NIS) gene. Co-transfection of the NIS gene at 5 μg reduced the basal cAMP production of about 40% and the cAMP production after bovine TSH (bTSH) stimulation of about 50%.*

In conclusion, Rhes protein is able to interfere with the functional activity of wild type and mutated TSHr and with the respective hormone-stimulated cAMP production of wtFSHr and wtLHr when expressed in COS-7 cells. This interference is not specific and due to the co-expression at the cell surface of two membrane proteins that alter their own expression.

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