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Thyrotropin internalization is directed by a highly conserved motif in the seventh transmembrane region of its receptor

Yufei Shi, Minjing Zou, Philip Ahring, Sultan T. Al-Sedairy & Nadir R. Farid

Molecular Endocrinology Laboratory, Department of Biological and Medical Research, King Faisal Specialist Hospital and Research Center, Riyadh 11211, Saudi Arabia

The thyrotropin (TSH) receptor is a member of G protein-coupled seven-transmembrane-segment receptors. It is characterized by a large extracellular domain linked to the seven transmembrane segments and ending with a cytoplasmic tail. Sequence alignment shows that a highly conserved motif, NPXXY where X is any amino acid, exists at the boundary between the seventh transmembrane domain and proximal part of the cytoplasmic tail of virtually all G protein-coupled receptors. This motif has been implicated as an internalization signal for several cell surface receptors, such as the low density lipoprotein (LDL), insulin and insulin-like growth factor-1 (IGF-1) receptors. The potential effects of this motif on the TSH receptor signal transduction and receptor-mediated TSH internalization was analysed by replacement of the tyrosine^{678} residue with an alanine residue. This mutation does not impair high affinity TSH binding, but completely abolishes the ability of cAMP response upon TSH stimulation. It also significantly reduces TSH internalization. The role of the cytoplasmic tail of the TSH receptor in receptor-mediated internalization was also assessed. Deletion of up to 56 amino acids from the C-terminus of the cytoplasmic tail enhances TSH internalization as compared to the wild-type receptor. We conclude that tyrosine^{678} in the NPXXY motif is required for efficient receptor-mediated TSH internalization and G protein coupling. The cytoplasmic tail of the TSH receptor may contain sequence domains which could modulate the effects of the NPXXY internalization signal.

Keywords: thyrotropin; TSH receptor; receptor internalization; endocytosis; NPXXY motif

Introduction

Thyrotropin (TSH) regulates differentiated thyroid cell function, such as iodide uptake, organification, thyroid hormone synthesis and secretion, as well as thyroid cell growth (Vassart & Dumont, 1992). These effects are mediated through interaction with its receptor on the surface of thyroid cells. The TSH receptor is a member of the family of G protein coupled seven-transmembrane-segments receptors (Libert *et al.*, 1989; Nagayama *et al.*, 1989; Misrahi *et al.*, 1990). The receptor shares an overall structural organization and substantial sequence homology with lutropin-choriogonadotropin (LH/CG) (Loosfelt *et al.*, 1989) and follicle-stimulating hormone (FSH) receptors (Sprengel *et al.*, 1990). All three glycoprotein hormone receptors have a large extracellular domain with multiple N-linked glycosulation sites, seven transmembrane segments and a cytoplasmic tail. Together, they represent a unique subclass of G protein-coupled receptor family.

TSH binding to its receptor causes a stimulation of adenylyl cyclase via activation of Gs protein and phospholipase C through Gq protein (Vassart & Dumont, 1992). Like other polypeptide hormones, the binding of TSH to its receptor also leads to subsequent receptor-mediated endocytosis, i.e. migration, aggregation, and concentration of ligand-receptor complexes in clathrin-coated pits (Avivi *et al.*, 1981, 1982; Fahraeus-Van Ree & Farid, 1990). The regions which control receptor-mediated TSH internalization, however, remain ill-defined.

The NPXY motif (where X is any amino acid), a tetrapeptide sequence located in the submembraneous region, was identified in the low density lipoprotein (LDL) receptor as an internalization signal (Chen *et al.*, 1990), and subsequent studies have indicated that the NPXY sequence is also an important recognition element for the internalization of insulin (Rajagopalan *et al.*, 1991), and insulin-like growth factor-1 (IGF-1) receptors (Hsu *et al.*, 1994). A similar motif (NPXXY) is present at the carboxyl terminus of the seventh transmembrane helix of the TSH receptor corresponding to residues 674–678 and is highly conserved in almost all G protein-coupled seven-transmembrane-segments receptors (Dohlman *et al.*, 1991; Probst *et al.*, 1992; Figure 1). The potential role of this motif in receptor-mediated TSH internalization has not been demonstrated.

Deletion of the cytoplasmic tail of the luteinizing hormone/chorionic gonadotropin (LH/CG) receptor on the receptor desensitization and internalization has been reported recently (Rodriguez *et al.*, 1992; Sanchez-Yague *et al.*, 1992; Zhu *et al.*, 1993). Rodriguez *et al.* (1992) reported that deletion of up to 43 amino acids from the carboxyl terminus of the cytoplasmic tail of the rat LH/CG receptor enhanced receptor-mediated hCG internalization. Once internalized, hCG was also degraded at a faster rate in cells expressing the truncated LH/CG receptors. Studies with other cell surface receptors have demonstrated that most sequence domains controlling internalization are located in the cytoplasmic tail (Davis *et al.*, 1987; Chen *et al.*, 1990; Collawn *et al.*, 1990; Canfield *et al.*, 1991; Girones *et al.*, 1991; Rajagopalan *et al.*,

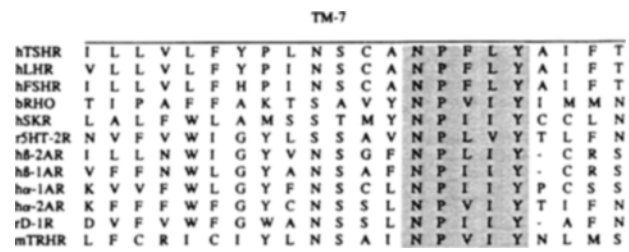

Figure 1 Alignment of a highly conserved NPXXY sequence in the seventh transmembrane segment. Shown are residues 661–682 in the seventh transmembrane segment of the human TSH receptor and the comparable regions of several other G-protein coupled seven-transmembrane-segment receptors. hLHR: human luteinizing hormone receptor, hFSHR: human folicle-stimulating hormone receptor, bRHO: bovine rhodopsin, hSKR: human substance K receptor, r5HT-2R: rat serotonin receptor, hβ-2AR: human β-2 adrenergic receptor, hβ-1AR: human β-1 adrenergic receptor, hα-1AR: human α-1 adrenergic receptor, hα-2AR: human α-2 adrenergic receptor, rD1R: rat D-1 dopamine receptor, mTRHR: mouse thyrotropin-releasing hormone receptor

Correspondence: Y. Shi
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1991; Jadot *et al.*, 1992; Lehmann *et al.*, 1992; Nussenzveig *et al.*, 1993; Hsu *et al.*, 1994). We have shown in our previous studies that deletion of up to 56 amino acids from the C-terminus of the human TSH receptor cytoplasmic tail did not affect TSH-induced homologous desensitization (Shi *et al.*, 1993). It is not clear whether this deletion would affect TSH internalization.

In the present studies, we investigated the potential role of NPXXY motif in receptor-mediated TSH internalization and signal transduction by replacing the tyrosine^{678} residue in the NPXXY sequence with an alanine residue by site-directed mutagenesis. The effects of truncation of the cytoplasmic tail of the TSH receptor on TSH internalization was also studied.

Results

Characterization of CHO cell lines expressing human wild-type and Y^{678} mutant receptors

CHO cells were stably transfected with the cDNAs encoding the wild-type or the tyrosine residue-mutated (A^{678}) TSH receptors. The surviving clones after G418 selection were pooled and the expression of both wild-type and mutant receptors were analyzed by Northern blot. The wild-type and mutant TSH receptors were expressed at similar level in CHO cells with the predicted size (Figure 2). They were further characterized by [^{125}I]-TSH binding and cAMP response upon bTSH stimulation. As determined by Scatchard analysis from the binding competition data shown in Figure 3, replacement of the tyrosine residue with an alanine residue in the NPXXY motif did not affect the high affinity TSH binding to the receptor, as compared to the wild-type receptor ($Kd = 3.5 \pm 0.9 \times 10^{-10}$ M *vs* $3.7 \pm 1.2 \times 10^{-10}$ M). The total binding was actually increased by about 2-fold ($Bmax = 35 \pm 12$ fmoles/10^5 cells *vs* 18 ± 5.6 fmoles/10^5 cells of the wild-type receptor). However, the Y^{678} mutation virtually abrogated the ability of the receptor to produce cAMP in response to bTSH stimulation (Figure 4).

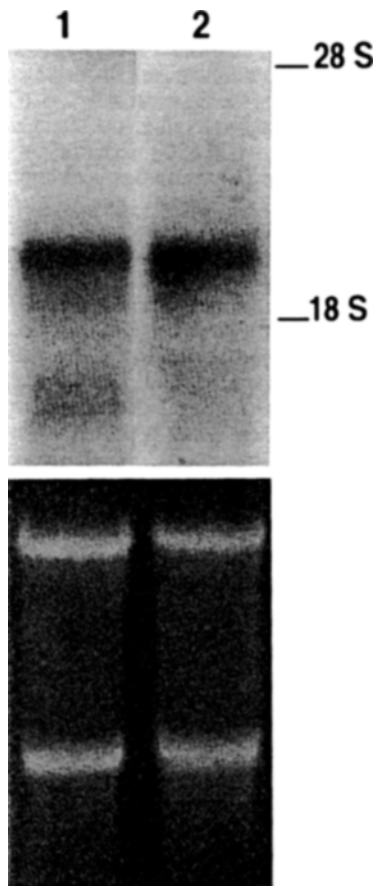

Figure 2 Northern blot analysis of wild-type (CHO-TSHR) and A^{678} mutant (CHO-TSHR-A^{678}) human TSH receptor expressed in CHO cells. Shown in upper panel is Northern blot hybridization of a full-length human TSHR cDNA probe to 20 μg of total RNA extracted from CHO-TSHR cells (lane 1) and CHO-TSHR-A^{678} cells (lane 2). Lower panel: ethidium bromide staining of the RNA loaded for Northern blot to monitor the actual RNA loading. The positions of 28S and 18S ribosomal RNA are indicated

TSH internalization

At 4°C, regardless of the incubation time, virtually all of the radiolabeled TSH bound to the TSH receptor was released by washing the cells with the acidic glycine buffer (Figure 5A). This demonstrates that the acid washing procedure used in these studies was effective at displacing the surface-bound TSH. Furthermore, it also demonstrates that TSH internalization does not occur at 4°C and that binding studies performed at this temperature reflect cell-surface binding alone. As shown in Figure 5B, when binding studies were performed at 37°C, less [^{125}I]-TSH was recovered by acidic glycine wash, and increasing quantities of [^{125}I]-TSH were becoming acid-resistant. Up to 40% of [^{125}I]-TSH became acidresistant after 6 h incubation. These findings indicate that TSH internalization had occurred and that the internalization process is both time and temperature dependent.

Effects of Y^{678} mutation on TSH internalization

The next study investigated whether replacement of the tyrosine^{678} residue in the NPXXY motif with an alanine would affect TSH internalization. Figure 6 shows that the

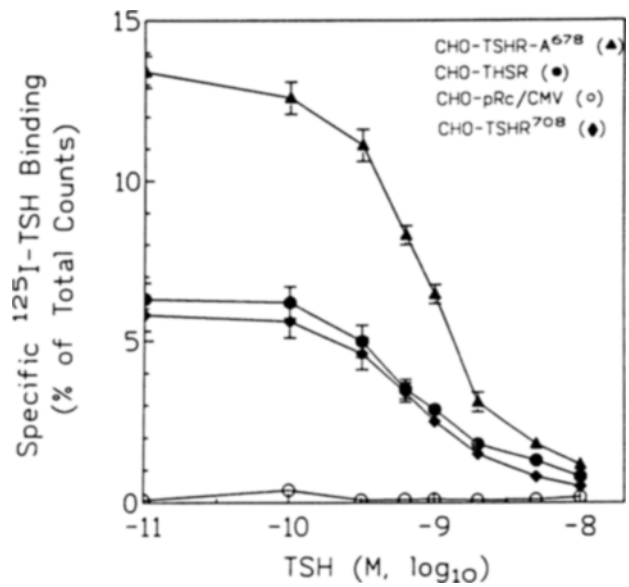

Figure 3 Displacement by TSH of [^{125}I]-TSH binding to the CHO cells transfected with wild-type hTSHR (CHO-TSHR), TSHR^{708} cDNA, and TSHR-A^{678} cDNA (CHO-TSHR-A^{678}). The CHO cells transfected with vector alone (CHO-pRc/CMV) were used as control. The cells in 24 well plates were grown to confluence. [^{125}I]-TSH binding was performed for 2 h at 37°C in 0.25 ml of modified Hank's buffer containing different concentrations of bTSH. Data are expressed as the percentage of total [^{125}I]-TSH added to each well. Each point represents the mean ± S.D. of four experiments and corrected for non-specific binding. Nonspecific binding was determined in the presence of 10^{-7} M TSH, and was subtracted from total counts bound to yield specific binding values

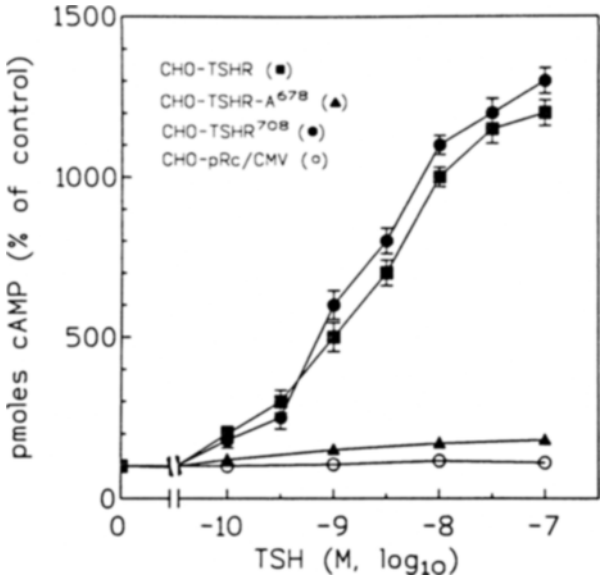

Figure 4 TSH-induced cAMP accumulation in CHO cells transfected with wild-type hTSHR cDNA, $TSHR^{708}$ cDNA, and TSHR-A^{678} cDNA. The CHO cells transfected with vector alone (CHO-pRc/CMV) were used as control. The cells were grown to confluence in 24 well plates and then washed twice with 1 ml Hank's buffer. Different concentrations of bTSH were added to 0.5 ml F12 medium with 1 mM IBMX and incubated for 2 h at 37°C. Cellular cAMP level was measured by radioimmunoassay. Data points represent the mean ± S.D. of four experiments

ability of the A^{678} mutant receptor to mediate TSH internalization is severely compromised. After 6 h incubation at 37°C, 42 ± 2.8% of [^{125}I]-TSH bound to the cells transfected with the wild-type receptor became acid-resistant, whereas only 24 ± 2.0% of the hormone became acid-resistant in the TSHR-A^{678} mutant receptor transfected cells ($P < 0.01$). This indicates that the tyrosine residue in the NPXXY motif is critical for efficient receptor-mediated TSH internalization.

Effects of truncation of the TSH receptor cytoplasmic tail on TSH internalization

Most internalization signals identified so far are present in the cytoplasmic tail of cell surface receptors (Davis *et al.*, 1987; Chen *et al.*, 1990; Collawn *et al.*, 1990; Canfield *et al.*, 1991; Girones *et al.*, 1991; Rajagopalan *et al.*, 1991; Jadot *et al.*, 1992; Lehmann *et al.*, 1992; Nussenzveig *et al.*, 1993; Hsu *et al.*, 1994). The TSH receptor has a relatively large cytoplasmic tail which contains 83 amino acids. To determine whether the cytoplasmic tail contains sequence domains important for TSH internalization, we analysed TSH internalization in CHO cells stably transfected with pTSHR^{708}/CMV cDNA in which 56 amino acids were deleted from the C-terminus of the cytoplasmic tail. Previous studies have indicated that such a deletion would not affect either cAMP production in response to TSH stimulation (Figure 4), or TSH binding affinity (Figure 3; Chazenbalk *et al.*, 1990; Shi *et al.*, 1993). Further deletions from the C-terminus, however, resulted in a receptor which may not be properly expressed on the cell surface (Chazenbalk *et al.*, 1990). As shown in Figure 6, after 6 h incubation at 37°C, 55 ± 3.5% of [^{125}I]-TSH bound to the cells transfected with $TSHR^{708}$ mutant receptor became acid-resistant, as compared to 42 ± 2.8% of the hormone resistant to acid wash (internalized) in the wild-type receptor transfected cells ($P < 0.01$). Thus, deletion of 56 amino acids from the C-terminus of the TSH receptor cytoplasmic tail indeed increases the efficiency of TSH internalization.

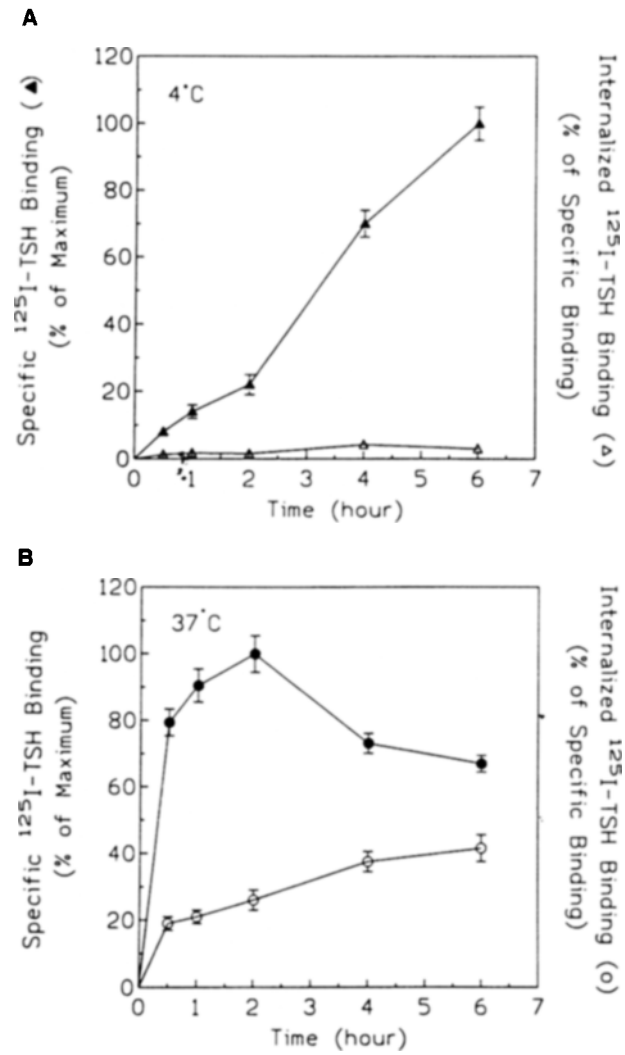

Figure 5 TSH internalization is time and temperature dependent in CHO cells transfected with wild-type human TSHR cDNA. The CHO cells were grown to confluence in 24 well plates and then washed twice with 1 ml modified Hank's buffer. The cells were incubated at 4°C (**A**) or 37°C (**B**) for up to 6 h with [^{125}I]-TSH. At the times indicated, the specific and acid-resistant binding were measured as described in Materials and methods. Binding data are expressed as the percentage of maximum [^{125}I]-TSH binding to the transfected CHO cells. Internalization data are expressed as the percentage of specific [^{125}I]-TSH binding which includes surface-bound and internalized TSH. Data points represent the mean ± S.D. of three experiments

Discussion

We have demonstrated in this study that a tyrosine residue, Y^{678}, in the NPXXY sequence is required for efficient receptor-mediated TSH internalization. As compared to the wild-type receptor, replacement of the tyrosine residue with an alanine residue in this sequence significantly reduced TSH internalization. This mutation also abrogates TSH stimulated cAMP production without affecting high affinity ligand binding, indicating that this motif is likely involved in the receptor coupling to Gs protein as well.

In contrast to the constitutively internalized receptors such as the transferrin (Class I) and the low density lipoprotein receptors (Class II), whereas their internalization process is independent of ligand binding and the receptors constantly recycle to plasma membrane (Goldstein *et al.*, 1985), internalization of receptors involved in signal transduction (Class

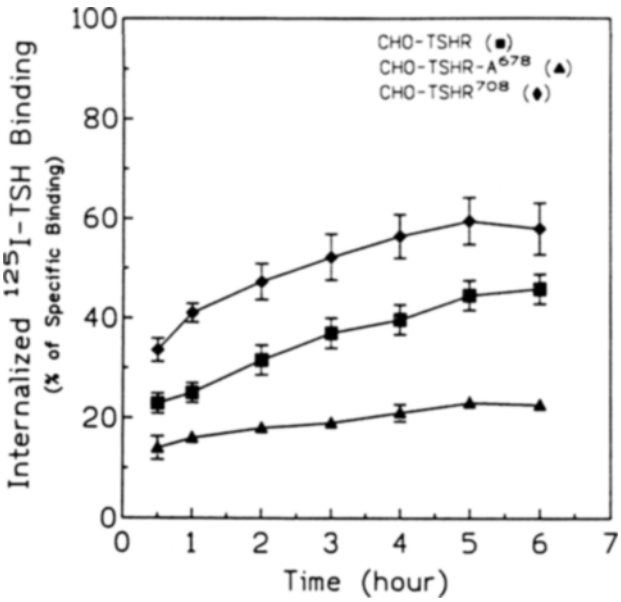

Figure 6 TSH internalization in CHO cells transfected with TSHR-A^{678} cDNA, $TSHR^{708}$ cDNA as compared to the cells transfected with wild-type TSHR cDNA. The CHO cells were grown to confluence in 24 well plates and then washed twice with 1 ml modified Hank's buffer. The cells were incubated at 37°C for the times indicated with [^{125}I]-TSH in the presence or absence of 10^{-7} M TSH. The specific and acid-resistant binding were measured as described in Materials and methods. Data are expressed as the percentage of specific [^{125}I]-TSH binding (including surface-bound and internalized TSH). Data points represent the mean ± S.D. of four separate experiments

III receptor) such as the epidermal growth factor (EGF), the platelet-derived growth factor (PDGF), and the insulin receptors, is ligand-dependant (Brown & Greene, 1991). The internalized receptor-ligand complex is ultimately delivered to and degraded in lysosomes (Brown & Greene, 1991; Mori *et al.*, 1994). The glycoprotein hormone receptors appears to be Class III receptor: the hormone-receptor complexes are degraded in lysosomes once internalized (Avivi *et al.*, 1982; Rodriguez *et al.*, 1992; Mori *et al.*, 1994). The increased total [^{125}I]-TSH binding in the TSHR-A^{678} transfected cells is probably due to reduced TSH receptor degradation resulting from impaired TSH internalization or increased TSH receptor insertion into the membrane. Northern blot analysis of mRNA from cells transfected with wild-type and TSHR-A^{678} cDNA did not show any significant difference of mRNA abundance (Figure 2), therefore excluding the possibility that the increased TSH binding is due to increased transcription or mRNA stability resulting from the mutation. The same results were obtained from two independent transfection experiments (data not shown), indicating that the difference in the efficiency of internalization and cAMP production or TSH binding between the wild-type and TSHR-A^{678} mutant receptors cannot be ascribed to transfection variation.

Receptor-mediated endocytosis allows cells to internalize surface-bound molecules rapidly. The initial step in the internalization process is thought to be aggregation of the receptors in specialized domains of the plasma membrane called clathrin-coated pits (Goldstein *et al.*, 1985). It has been suggested that the preferential association of receptors with clathrin-coated pits involves peptide motifs in the receptor interacting selectively with proteins associated with these invaginations, presumably the adaptins (Pearse *et al.*, 1990). These motifs are generally located in the cytoplasmic tail, within approximately 30 amino acids from the plasma membrane. Although heterogeneous, their primary sequence usually includes an aromatic amino acid. NPXY sequence has been implicated as one of these peptide motifs for internalization of insulin, insulin-like growth factor-I, and low density lipoprotein receptors (Chen *et al.*, 1990; Rajagopalan *et al.*, 1991; Hsu *et al.*, 1994). Several other kinds of tyrosine-based internalization motifs have been identified by extensive mutational analyses, e.g. YXRF for the transferrin receptor (Collawn *et al.*, 1990; Girones *et al.*, 1991), GPLY for the insulin receptor (Rajagopalan *et al.*, 1991), YXLV for the mannose-6-phosphate receptor (Jadot *et al.*, 1992) and PGYRHV for lysosomal acid phosphatase (Lehmann *et al.*, 1992). Studies have shown that the rate of internalization is affected partially by the replacement of the tyrosine residue with an aromatic amino acid residue i.e. phenylalanine, whereas that is completely blocked by a nonaromatic residue i.e. alanine substitution (Davis *et al.*, 1987; Canfield *et al.*, 1991; Girones *et al.*, 1991). It has been proposed that one essential feature of these motifs is to form a tight β-turn conformation (Collawn *et al.*, 1990; Bansal *et al.*, 1991; Eberle *et al.*, 1991; Backer *et al.*, 1992). This conformation is, however, destabilized by the substitution of the tyrosine residue by a phenylalanine residue, and the tendency to form the turn is further reduced when the tyrosine residue is substituted by an alanine residue (Eberle *et al.*, 1991; Lehmann *et al.*, 1992), indicating the critical roles of the tyrosine residue in these motifs for receptor-mediated internalization.

A very similar motif, NPXXY, is present in virtually all G protein-coupled receptors (Dohlman *et al.*, 1991; Probst *et al.*, 1992). In contrast to the NPXY sequence located in the cytoplasmic tail, this motif exists at the proposed junction of the seventh transmembrane domain and proximal part of the cytoplasmic tail of G protein-coupled receptors. The potential role of this sequence motif has recently been characterized in β_2-adrenergic receptor (β_2AR) (Barak *et al.*, 1994). Replacing the tyrosine residue in the NPXXY sequence with an alanine residue (β_2AR-Y^{326}A) completely abolishes agonist-mediated receptor sequestration (a rapid internalization of the surface receptor) without affecting the ability of the receptor to undergo rapid desensitization and down-regulation in response to agonist. The analysis of the internalization efficiency of NPXY related motifs indicates that the most efficient internalization signal is FXNPXY, requiring the presence of aromatic residues (at least one of which is a tyrosine) at both ends of the -XNPX- motif (Paccaud *et al.*, 1993). Sequence alignment (Figure 1) of selected G protein-coupled receptors shows that an aromatic amino acid residue phenylalanine is flanking the NPXXY motif in the β adrenergic receptor, whereas a nonaromatic residue alanine is in front of the NPXXY motif for glycoprotein hormone receptors (including the TSH receptor). This may explain the complete abolishment of the β_2-AR versus partial impairment of TSHR internalization by Y to A mutation in the NPXXY motif. It, however, remains to be determined whether the variations to the FXNPX(X)Y motif would reduce efficiency of agonist-induced internalization in G protein-coupled receptors. Although the mutant β_2AR-Y^{326} was effective as the wild-type β_2AR in mediating maximal agonist stimulation of adenylyl cyclase, the agonist isoproterenol was less potent in stimulating adenylyl cyclase via the mutant versus the wild-type receptors, indicating that the mutant receptor appears to be less well coupled to Gs protein than the wild-type receptor.

Based on the structural information and projection map of rhodopsin that has been obtained by electron crystallography of two-dimensional crystals, Baldwin proposed a tentative three-dimensional structure of the transmembrane helices in all G protein-coupled receptors (Baldwin, 1993, 1994). She predicted that the regions implicated in the G protein binding were close together; these include the highly conserved D/ERY sequence at the end of helix III, the N- and C-terminal portions of the loop between helices V and VI, and the portion of the C-terminus near helix VII. Y^{678} in the NPXXY motif maps to position 21 of the helix VII on the Baldwin's projection, which faces ERW (residue 518–520) motif at the end of helix III intracellularly (Baldwin, 1993, 1994). These

two motifs presumably present important contact residues for G protein coupling. ERW motif is identical among glycoprotein hormone receptors, and is equivalent to DRY motif in adrenergic receptors. Replacement of E with Q or D residue in ERW motif does not impair signal transduction of rLH/CG receptor (Wang *et al.*, 1993). It, however, reduced cAMP response upon TSH stimulation in TSH receptor (Haraguchi *et al.*, 1994), whereas Y^{678} to A mutation completely crippled this response. On the other hand, replacement of the corresponding D residue with N residue in the DRY motif significantly reduced the ability of adrenergic (Fraser *et al.*, 1988; Wang *et al.*, 1991) and muscarinic M1 (Fraser *et al.*, 1989) receptors to couple to their respective G proteins. The difference in the agonist-induced cAMP response between the TSH and β_2-adrenergic receptors mutated at the equivalent Y positions may be related to the fact that in β_2-adrenergic receptor the DRY motif contributes more in G protein coupling than NPXXY motif, thus partially compensate for the Y^{326} to A mutation, whereas in TSH receptor NPXXY plays more important role in G protein coupling than ERW, resulting in the complete loss of cAMP response with Y^{678} to A mutation. This model suggests that both DRY or ERW and NPXXY motifs are required for G protein association. Further studies are, however, needed to confirm the hypothesis. Our studies have shown that deletion of up to 56 amino acids from the C-terminus of the human TSH receptor enhanced TSH internalization as compared to the wild-type receptor. Although most sequence domains controlling receptor internalization are located in the cytoplasmic tail (Davis *et al.*, 1987; Chen *et al.*, 1990; Collawn *et al.*, 1990; Canfield *et al.*, 1991; Girones *et al.*, 1991; Rajagopalan *et al.*, 1991; Jadot *et al.*, 1992; Lehmann *et al.*, 1992; Nussenzveig *et al.*, 1993; Hsu *et al.*, 1994), and point mutations and/or deletions of these domains usually impair receptor-mediated internalization, enhancement of internalization resulting from deletions of cytoplasmic tail is also observed as in the cases of rLH/CG and avian β-adrenergic receptors (Hertel *et al.*, 1990; Rodriguez *et al.*, 1992). Deletion of up to 43 amino acids from the C-terminus of the rLH/CG receptor enhanced hCG internalization as compared to the wild-type receptor. The turkey β-adrenergic receptor has an extended cytoplasmic tail and is not internalized, whereas the mammalian forms of this receptor are internalized. However, when 18 amino acids are removed from the C-terminus of the avian receptor, it becomes internalized in response to agonist. Altogether, these results suggests that a single or multiple sequence motifs may exist in the cytoplasmic tail of these receptors, and inhibit or modulate receptor-mediated internalization. It has been shown that apart from internalization signals which mediate endocytosis, other factors are also involved in the control of internalization. These include receptor autophosphorylation (Goldstein *et al.*, 1985; Carpentier *et al.*, 1992), associated protein tyrosine kinases (Pelchen-Matthews *et al.*, 1992), inhibitory signal sequences intrinsic to the receptor (Miettinen *et al.*, 1992), and serine or threonine phosphorylation (Moro *et al.*, 1993; Carpentier *et al.*, 1991).

In summary, we have identified a tyrosine^{678} residue in the NPXXY motif, which is required for efficient TSH internalization. This residue is also important for G protein coupling. Replacement of the tyrosine residue with an alanine in the sequence significantly reduces TSH internalization as well as TSH-induced cAMP response. The cytoplasmic tail of the TSH receptor contains sequence domain or domains which may modulate the effects of the NPXXY internalization signal.

Materials and methods

Recombinant DNA

The full-length human TSH receptor cDNA (pBS-hTSHR) was kindly donated by Dr E. Milgrom, Faculty of Medicine, University of Paris, France (Misrahi *et al.*, 1990). The cDNA is inserted in the SmaI site of the Bluescript SK^+ vector. The 5' end faces the T3 promoter of the vector. The pRc/CMV eukaryotic expression vector, designed for high level stable expression, was obtained from Invitrogen Co., (San Diego, CA). The genes inserted into the vector are expressed using the CMV promoter and enhancer. The vector also contains Neomycin resistance gene for the selection of stable transformants.

Construction of the TSH receptor cDNA mutants

The pTSHR/CMV, the expression vector containing the full length human TSH receptor cDNA, and pTSHR^{708}/CMV, the TSHR cDNA truncated at residue 708 of cytoplasmic tail, have been previously described (Shi *et al.*, 1993). The TSHR-A^{678}, the tyrosine^{678} residue in the NPXXY motif of the TSH receptor was mutated to alanine, was created by site-directed mutagenesis as described previously (Shi *et al.*, 1993). The sequence of the mutant cDNA was verified by dideoxy sequencing.

Transfections

The CHO cell line was obtained from ATCC (Rockville, MD) and maintained in Ham's F12 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO^2. Transfections were initiated when the cells were 70% confluent, following the procedure of Chen and Okayama (1987). 72 h after transfection, the cells were cultured in the F12 growth medium containing G418 (400 μg/ml) for 3 weeks. Surviving clones were pooled and used for the experiments described.

RNA extraction and Northern hybridization

Total RNA was extracted from 10^7 stably transfected cells and used for Northern blotting experiment as described previously (Shi *et al.*, 1993). Briefly, 20 μg of total RNA was fractionated on 1% agarose gel containing 2.2 M formaldehyde and blotted onto a nylon membrane (Hybond-N, Amersham) by capillary transfer. The 2.4 kb full-length cDNA probe was labelled with [α-^{32}P]-dCTP to a specific activity of 10^9 cpm/μg using Pharmacia's random primer labelling kit. Hybridization was performed at 42°C for 18 h in 6 × SSPE, 10 mM EDTA, 5 × Denhardt's solution, 0.5% SDS, 100 μg/ml denatured salmon testis DNA and 50% formamide. The membrane was then washed twice in 2 × SSPE at 65°C and exposed to X-ray film at −70°C with intensifying screens.

[^{125}I]-TSH binding to transfected cells

[^{125}I]-TSH was obtained from RSR Limited, Cardiff, UK, with a specific activity of about 70 μCi/μg. 10^5 transfected cells were cultured in 24 well plates and washed twice with 1 ml modified Hank's buffer (NaCl replaced by 280 mM sucrose) containing 0.25% BSA. The cells were then incubated at 37°C for different periods of time in 0.25 ml of the same buffer containing 1.6×10^4 cpm of [^{125}I]-TSH and different concentrations of unlabelled bTSH (Sigma Co., St Louis, MO). At the end of the incubation, the cells were washed three times with 1 ml modified Hank's buffer, lysed in 0.5 ml 1 N NaOH, and radioactivity was measured in a γ-counter. Specific binding was calculated by subtracting counts obtained in the presence of 10^{-7} M unlabelled bTSH.

Cellular cAMP measurements

Cells were cultured in 24 well plates in the F12 growth medium. bTSH was added to 0.5 ml medium at different concentrations together with 1 mM isobutyl methylxanthine.

After 2 h incubation at 37°C, cells were washed twice with Hank's buffer. Cellular cAMP was extracted with 500 µl absolute ethanol and measured by radioimmunoassay using Du Pont-New England Nuclear's cAMP assay kit (Shi *et al.*, 1993).

TSH internalization

TSH internalization was measured as specifically bound [^{125}I]-TSH that was resistant to acid glycine wash (Rodriguez *et al.*, 1992). At the end of incubation, the cells were washed three times with 1 ml cold Hank's buffer. Surface-bound TSH was released by incubating the cells with 1 ml cold 50 mM glycine, 100 mM NaCl, pH 3, for 5 min on ice. The radioactivity that was removed by the acid wash treatment was taken as a measure of the surface-bound hormone. The cells were then solubilized with 0.5 ml 1 N NaOH. The radioactivity that remained associated with the cells after the acid wash was considered to be internalized hormone.

Statistical analysis

Data are represented as the mean ± S.D.. The significance was calculated by Student's *t* test.

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