

Correlation between Basal Signaling and Internalization of Thyrotropin-Releasing Hormone Receptors: Evidence for Involvement of Similar Receptor Conformations

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Previous studies have shown that rat thyrotropin-releasing hormone (TRH) receptor type 2 exhibits higher basal signaling activity and internalizes more rapidly upon agonist binding than rat TRH receptor type 1. The mouse TRH receptor type 2 (mR2) was recently cloned and, similar to its rat homolog, shows a higher basal signaling activity than mR1. Taking advantage of the high degree of sequence homology between mR1 and mR2, we used chimeras/mutants of these receptors to gain insight into the properties of the receptors that influence internalization and basal signaling. Chimeric receptors that have the mR1 extracellular and transmembrane domains with the carboxyl terminus and intracellular loops of mR2 (R1/R2-tail; R1/R2-I3,tail; R1/R2-I2,3,tail; R1/R2-I1,2,3,tail) exhibited internalization rates and basal activities that were similar to that of mR1. In contrast, a chimeric receptor with the extracellular and transmembrane domains of mR2 and the carboxyl terminus of mR1 exhibited the more

rapid internalization rate and higher basal signaling activity characteristic of mR2. We showed previously that mutation of a highly conserved tryptophan to alanine caused mR1 to exhibit a high basal signaling activity and rapid internalization rate. In contrast, mutation of this tryptophan to alanine in mR2 decreased the rate of internalization and inhibited basal signaling activity. The rates of receptor internalization did not correlate with the binding affinities, coupling efficiencies, or potencies of the receptors. Thus, we observed that receptors with more rapid internalization rates showed relatively higher basal signaling activities, whereas receptors with lower basal signaling activities showed slower internalization rates. These data suggest that similar receptor conformations are required for productive coupling to signaling G proteins and to proteins involved in internalization. (*Endocrinology* 143: 2886–2892, 2002)

RECEPTOR INTERNALIZATION IS a common response that follows agonist binding to G protein-coupled receptors (GPCRs) and appears to modulate several aspects of receptor function such as termination of signaling, activation of MAPKs, resensitization, and down-regulation (1–4). Although components of the internalization pathway of several GPCRs have been documented, the structural motifs within GPCRs that mediate internalization are not understood in most receptors. A number of studies have shown that the intracellular carboxyl terminus in many GPCRs is important for internalization because truncations or mutations of the amino acid residues in this region impaired receptor endocytosis (5–12). Other studies indicated that mutations in the intracellular loops of the human GnRH receptor (GnRH R) (13) and the human and rat lutropin/choriogonadotropin receptor (LH R) (14), the extracellular domain of the human LH R (15, 16), and the transmembrane region of the FSH receptor (FSH R) (17) are capable of affecting receptor internalization.

Abbreviations: CREB, cAMP response element binding protein; EM, Epstein-Barr macrophage scavenger receptor; E/TM, extracellular and transmembrane; FSH R, FSH receptor; GnRH R, GnRH receptor; GPCRs, G protein-coupled receptors; I1, I2, or I3, first, second, and third intracellular loops; HEK, human embryonic kidney; IP, inositol phosphate; LH R, lutropin/choriogonadotropin receptor; mR1 or mR2, mouse TRH receptor type 1 or 2; R1 or R2, receptor type 1 or 2; TM 2 or 6, transmembrane helix 2 or 6; TRH, thyrotropin-releasing hormone; TRH R, TRH receptor.

Studies on mouse LH (18), β_2 -adrenergic (19), and m3 muscarinic receptors (20) have shown that weak partial agonists induced slower rates of receptor internalization than full agonists. In addition, mutations of LH (21), β_2 -adrenergic (22), and thyrotropin-releasing hormone (TRH) receptors (TRH R) (23) that impaired signaling activities also impaired agonist-induced receptor internalization. Therefore, it has been proposed that receptor activation is required for agonist-induced internalization. However, different conclusions have been drawn about receptor activation and internalization based on evidence that some mutations of the β_2 -adrenergic receptor (24) and type 1 angiotensin II receptor (25, 26) caused impairment of signaling activity but did not affect internalization. Yeast α -factor (27), mammalian β_2 -adrenergic (28), and mouse TRH Rs (29) are capable of internalization in the absence of G proteins. In addition, antagonist-bound type 1 angiotensin receptors (26) and antibody-bound TRH R (30) have also been shown to undergo internalization. More recent experiments conducted on signaling-impaired and phosphorylation-deficient mutants of the human LH R suggested that phosphorylation and activation play a redundant role in agonist-induced internalization (31), and the association of nonvisual arrestins, which is necessary for internalization, with human LH R depends more on receptor activation than receptor phosphorylation (32). In addition, studies of rat FSH Rs have demonstrated that agonist-induced activation and phosphorylation are not required for internalization, whereas the

interaction of the rat FSH R with a nonvisual arrestin is essential for internalization (33).

TRH receptors (TRH Rs) are members of the rhodopsin/ β -adrenergic receptor subfamily of GPCRs (34). The binding of TRH activates signaling primarily via G-proteins of the $G_{\alpha_q/11}$ family (35–37). Like other GPCRs, binding of agonists to TRH Rs triggers internalization of the agonist-receptor complex, most likely via the β -arrestin mediated clathrin pathway (29, 30, 38, 39). The internalized TRH Rs are rapidly recycled to the plasma membrane, with only a fraction of the internalized TRH Rs targeted to a degradation pathway (38). Several mutations of TRH R1 have been shown to be defective in internalization, including receptors with mutations in the second transmembrane helix (TM 2), the third intracellular loop (I3), and a truncated receptor missing the carboxyl terminus (6, 23, 30, 38). Coupling to G protein is not sufficient to cause internalization (23). There are contradictory conclusions about whether receptor-G protein coupling is necessary for TRH R internalization (23, 29, 30, 40).

Two types of TRH Rs have been cloned. The cDNA of TRH R1 was initially cloned from mouse (41) and subsequently from rat (42–44), human (45, 46), chicken (47), and bovine tissues (48). Type 2 TRH R (TRH R2) was recently cloned from rat (49–51) and mouse (52). Amino acid sequence alignments of the two types of TRH Rs from the same species reveal a 50% overall identity. Despite their similarities in binding affinities for agonists and intracellular signaling pathways (51), TRH R1 and R2 exhibit several marked differences. First, unlike TRH R1, which is expressed in lactotrophs and thyrotrophs of the anterior pituitary, TRH R2 mRNA is extensively distributed in the brain, particularly in the cerebral cortex, with less abundance in the anterior pituitary (50, 51). Second, TRH R2 has a much higher basal signaling activity than TRH R1 (51–53). Third, rat TRH R2 (rR2) is more rapidly internalized and more markedly down-regulated after binding TRH than rR1 (51). Because TRH R2 is widely distributed in the brain, the high basal signaling activity and rapid agonist-induced internalization of TRH R2 may play an important role in mediating the function of TRH in the brain.

The focus of this study was to identify domains/residues of mouse receptors that influence their rates of internalization. We constructed mutant receptors in which we shifted the carboxyl terminus of mR1 and mR2, constructed chimeras containing the extracellular and transmembrane (E/TM) domains of mR1 and various intracellular domains of mR2, and made point mutations of mR1 and mR2. All receptors were tested for ligand binding, basal and stimulated signaling, and agonist-induced internalization in transfected HEK (human embryonic kidney) 293 EM (Epstein-Barr macrophage scavenger receptor) cells. The results showed that changes in the intracellular domains of mouse receptors that did not change their basal signaling activity had small effects on agonist-induced receptor internalization, whereas mutation of a highly conserved tryptophan residue in transmembrane helix 6 (TM6) that affected basal signaling greatly affected the rate of agonist-induced receptor internalization.

Materials and Methods

Materials

[³H][methyl-His]TRH ([³H]MeTRH) was purchased from NEN Life Science Products (Boston, MA). Myo-[³H]inositol was obtained from Amersham Pharmacia Biotech (Arlington Heights, IL). Restriction endonucleases, Vent DNA polymerase and deoxynucleotides were obtained from New England Biolabs, Inc. (Beverly, MA). DMEM and fetal bovine serum were purchased from Sigma (St. Louis, MO). The mammalian expression vector pcDNA3.1 was from Invitrogen (Carlsbad, CA). All reagents were analytical grade.

DNA

The full-length, mR1 in pMT4 (54) was subcloned into the pcDNA3.1 vector. The full-length mR2 in pcDNA3 was a gift from Dr. Thomas Bruhn (52). These plasmids were used as templates for mutagenesis. Plasmid pCDM8 containing an insert encoding mR1 with Trp at position 279 mutated to Ala (R1-W279A) was constructed as previously described (55). Overlapping PCR was used to generate fragments containing R2-W267A, R1/R2-tail, R2/R1-tail. The fragments were digested with *Eco*R I and *Not*I and then subcloned into pcDNA3.1. To construct R1/R2-I3 and R1/R2-I3,tail, three DNA fragments, which were obtained as follows, were ligated together. The mR1 or R1/R2-tail in pcDNA3.1 was digested with *Bst*EII, and the resulting 5.9-kb and 711-bp fragments were purified by QIAGEN gel extraction kit (QIAGEN, Valencia, CA). The 711-bp fragment was then digested with *Msc*I, and a 439-bp fragment was extracted and purified. The I3 of mR2 was generated by PCR using mR2 in pcDNA3 as template, and digested with *Bst*EII and *Mcs*I. The 5.9-kb, 439-bp, and I3 of mR2 were subsequently ligated to yield plasmids encoding the chimeric receptors (R1/R2-I3 and R1/R2-I3,tail). To generate R1/R2-I2,3,tail and R1/R2-I1,2,3,tail, overlapping PCR was employed using R1/R2-I3,tail as template. The R1/R2-I1,2,3,tail receptor was obtained the same way using R1/R2-I2,3,tail as template.

Cell culture and transfections

HEK 293 EM cells were grown in DMEM containing 10% fetal bovine serum (Life Technologies, Inc., Grand Island, NY). On the day before transfection, the cells were seeded in 24-well dishes (30,000 cells/well). After 16 h, the medium was aspirated and the cells were transfected using calcium phosphate. The concentration of receptor-encoding plasmid DNA in transfection cocktails varied from 0.1–2 μ g/ml. Where appropriate, 1 μ g/ml pFR-Luc and 1 μ g/ml pFA2-CREB (cAMP-response element binding protein) (PathDetect *In Vivo* Signal Transduction Pathway *trans*-Reporting System, Stratagene, La Jolla, CA) was added to the transfection cocktail. The pFA2-CREB encodes a chimeric protein of the yeast Gal4 binding domain and the activation domain of CREB, and the pFR-Luc contains yeast Gal4 binding elements upstream of the luciferase gene. Total DNA was kept constant by adding empty plasmid. Mock transfections were performed with empty plasmid. The cells were exposed to the transfection cocktail for 6 h and were then incubated in DMEM containing 1% fetal bovine serum for 16–24 h.

Measurement of TRH R expression

Expression of mR1 and mR2 was measured as maximal binding of [³H]MeTRH in intact cell monolayers as described (56). The concentration of [³H]MeTRH was 0.1–10 nM. The data were analyzed using PRISM software (GraphPad Software, Inc., San Diego, CA).

Measurement of phosphoinositide hydrolysis

Acute stimulation of phosphoinositide hydrolysis by TRH was measured as accumulation of ³H-labeled inositol phosphates (IPs) over 60 min in the presence of 10 mM LiCl in myo-[³H]inositol labeled cells as described (56). Basal phosphoinositide hydrolysis was measured for the times indicated in the presence of 10 mM LiCl.

Assay of luciferase activity

Cells in 24-well plates were washed with PBS and lysed with 0.5 ml of lysis buffer (25 mM GlyGly, pH 7.8; 15 mM MgSO₄·6H₂O; 4 mM EGTA;

1 mM dithiothreitol; 1% Triton X-100). Cell lysates (0.025 ml) were combined automatically with 0.125 ml reaction buffer (25 mM GlyGly, pH 7.8; 15 mM MgSO₄·6H₂O; 4 mM EGTA; 1 mM dithiothreitol; 15 mM KH₂PO₄; 2 mM ATP) and 0.025 ml luciferin (0.4 mM; Sigma) in reaction buffer and the luminescence was measured for 10 sec in a TR717 Microplate Luminometer (Tropix, Bedford, MA).

Assay of agonist-induced receptor internalization

Internalization of TRH Rs was measured as specifically bound [³H]MeTRH that was resistant to acid wash (6). At the end of the incubation with 2 nM [³H]MeTRH for the time shown, free ligand was removed by aspirating the binding buffer and washing the cells with 2 ml ice-cold buffer. Cells were then exposed to 1 ml 50 mM glycine (pH 3.5), 0.5 M NaCl for 3 min at 4 C, and then washed with 2 ml binding buffer twice. Acid-resistant [³H]MeTRH was counted and compared with total specific binding. Internalized receptors were presented as the fraction of acid-resistant [³H]MeTRH-bound receptors divided by total [³H]MeTRH-bound receptors.

Data analysis

Statistical significance was determined using Student's *t* test with a probability criterion of *P* < 0.05.

Results

Roles of the carboxyl termini and intracellular loops in agonist-induced receptor internalization

The rates of agonist-induced internalization of many GPCRs are greatly affected by alterations in their carboxyl termini. In a previous report (6), we characterized two domains within the C-tail of mR1 that affect agonist-induced internalization. Truncations of the C-tail, or mutations of two cysteine residues in this region impaired agonist-induced internalization of mR1. The intracellular loops of many GPCRs have also been shown to play a role in agonist-induced internalization (13, 14, 23) and may interact with proteins involved in internalization (2). We began to examine whether changes in the carboxyl termini or intracellular loops of mR1 and mR2 could alter the rate of internalization. We exchanged the carboxyl terminus between mR1 and mR2. We also shifted the third, second, and first intracellular loop of mR2 sequentially (I3, I2, I1, respectively) to the chimeric receptor R1/R2-tail. The structures of these chimeric recep-

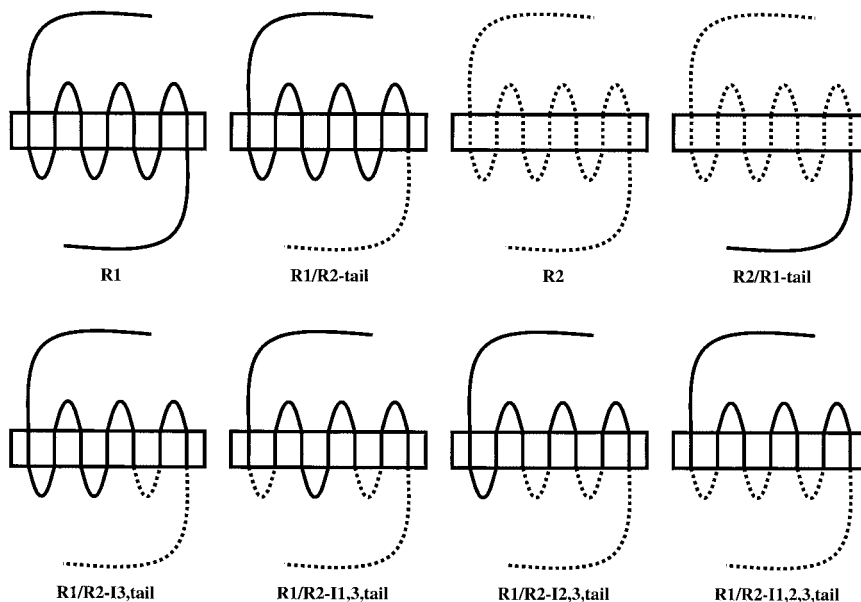


FIG. 1. Two-dimensional topologies of mR1 and mR2 chimeric receptors. The domains from mR1 and mR2 are shown in *solid* and *dash*, respectively.

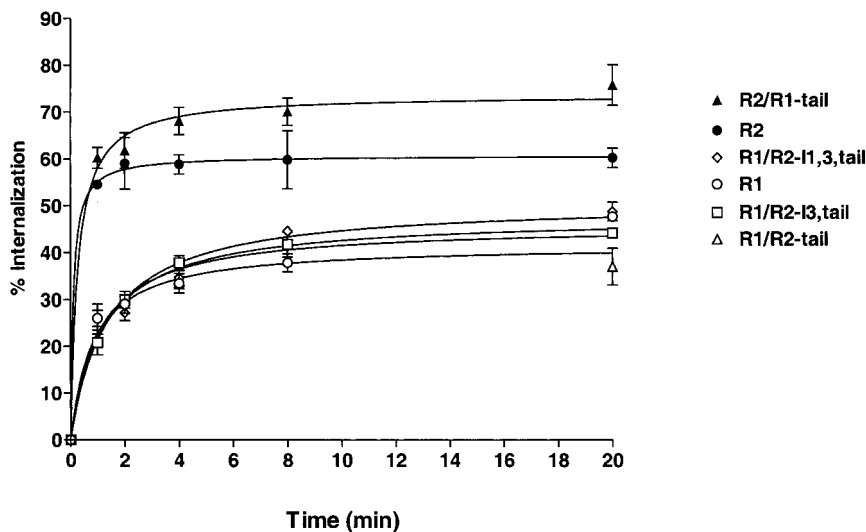


FIG. 2. Receptor internalization in HEK 293 EM cells transiently expressing wild-type (R1 and R2) and chimeric receptors. Transfected cells were incubated with 2 nM [³H]MeTRH at 37 C for the times indicated and specific and acid-resistant binding were measured as described in *Materials and Methods*. Results are expressed as mean \pm SD of assays performed in triplicate in a representative experiment.

tors are illustrated in Fig. 1. As shown in Fig. 2, the chimeric receptors exhibited agonist-induced internalization in a fashion similar to the receptor from which the E/TM domains were derived: mR2 ($t_{1/2} = 0.1 \pm 0.07$ min) and R2/R1-tail ($t_{1/2} = 0.3 \pm 0.08$ min) internalized more rapidly than the wild-type mR1 ($t_{1/2} = 1.0 \pm 0.2$ min) and the chimeric receptors with E/TM domains of R1 and various portions of R2's intracellular domains ($t_{1/2} = 1.0 \pm 0.2$ min for R1/R2-tail; $t_{1/2} = 1.0 \pm 0.1$ min for R1/R2-I3,tail; $t_{1/2} = 1.0 \pm 0.2$ min for R1/R2-I1,3,tail). The internalization rates of R1/R2-I2,3,tail and R1/R2-I1,2,3,tail were similar or slower than mR1 (not shown).

Internalization rates are correlated with basal activities of the receptors

We compared the basal activities of the more rapidly internalized receptors (mR2 and R2/R1-tail) with those of receptors that showed similar internalization rates to mR1 (R1/R2-tail, R1/R2-I3 and R1/R2-I3,tail). Basal signaling was estimated as the effects of increases in the number of expressed receptors (produced by varying the amount of plasmid in the transfection cocktail) on basal IP accumulation (Fig. 3A). The slopes of the lines describing the relationships between receptor expression and basal activities fell into two groups: for receptors displaying a rapid internalization (mR2 and R2/R1-tail), the slopes were significantly greater than zero, indicating that these receptors were constitutively active. For receptors displaying similar internalization rates as mR1, the slopes were near zero, indicating that these receptors were not measurably basally active in this assay. These observations were confirmed by using a luciferase assay in which basal activity is assessed as induction of transcription of the luciferase reporter gene (Fig. 3B), a system more sensitive than IP formation (53, 56). The receptors fell into the same two groups using this assay as with measurement of IPs. Thus, of the receptors that were tested in the present study, the rates of agonist-induced internalization were closely correlated with their basal activities; receptors with higher agonist-induced internalization rates showed greater basal activities. The internalization rate of a receptor was not correlated with its affinity, potency, or coupling efficiency assessed as the ratio of potency to affinity. As shown in Table 1, receptors that displayed different dissociation constants (K_d s), half-maximally effective concentrations (EC_{50} s), or maximal binding capacities, but the same basal activities showed similar internalization rates. Lastly, rapidly internalized receptors with higher basal activities responded to TRH stimulation to a lesser extent than the more slowly internalized receptors with lower basal signaling activities that showed greater responses to TRH stimulation (Table 1).

Roles of the conserved tryptophan residues in transmembrane helix 6 in agonist-induced receptor internalization

We next examined whether mutations in the transmembrane domains of the receptors could influence the rate of receptor internalization. Our previous study showed that a highly conserved tryptophan residue (W279) in TM 6 of mR1

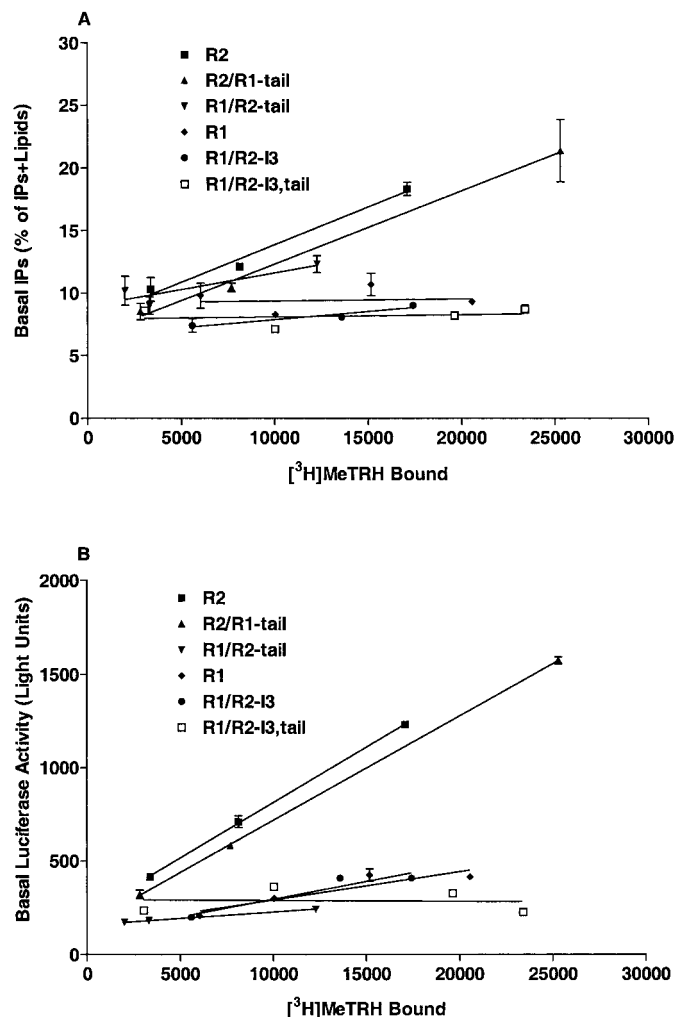


FIG. 3. Basal IP second messenger formation and reporter luciferase activity as a function of wild-type or chimeric receptor expression levels. HEK 293 EM cells were cotransfected with plasmid containing pFA2-CREB, pFR-Luc, and various amounts of plasmid encoding wild-type or chimeric/mutant mRs. Cells were assayed for IP formation (A) and luciferase activity (B) as described in *Materials and Methods*. Results are expressed as mean \pm SD of assays performed in triplicate in a representative experiment.

is critical for maintaining the conformation of mR1, and mutation of W279 to an alanine residue (R1-W279A) disturbed the hydrophobic interaction between TM5 and TM6, changing the receptor conformation and causing it to become constitutively active (57). We mutated the corresponding tryptophan in mR2 to an alanine (R2-W267A) and analyzed the internalization rate of this receptor mutant. In contrast to R1-W279A, which showed a more rapid internalization compared with mR1 (data not shown), R2-W267A displayed a slower internalization rate than mR2 (Fig. 4A, $t_{1/2} = 8 \pm 2$ min for R2-W267A; $t_{1/2} = 0.06 \pm 0.05$ min for R2, $t_{1/2} = 1.0 \pm 0.2$ min for R1). We showed in a previous report that R1-W279A was basally active (57). The basal activities of R2-W267A were then compared with mR1 and mR2. Consistent with our previous observation, mR2 was constitutively active, whereas R2-W267A and mR1 were not basally active (Fig. 4B).

TABLE 1. Internalization rates, affinities, maximal binding capacities, potencies, and fold increases in TRH-stimulated signaling activities of wild-type (R1 and R2) and chimeric/mutant receptors

	Internalization		Binding		Signaling	
	$t_{1/2}$ (min)	K_d (nM)	B_{max} (dpm)	EC_{50} (nM)	TRH response (fold stimulation)	
mR1	1.0	3.5	38,000	1.8	18.0	
R1/R2-tail	1.0	2.9	26,000	2.5	8.0	
R1/R2-I3,tail	1.0	2.0	13,000	1.0	11.0	
R1/R2-I2,3,tail	n.d.	2.6	1,200	10.0	7.0	
R1/R2-I1,2,3,tail	n.d.	3.7	2,200	6.0	17.0	
R1/R2-I1,3,tail	1.0	1.4	7,600	1.3	10.0	
R1/R2-I3	0.8	0.4	3,700	1.0	10.0	
mR2	0.1	2.9	27,000	1.0	2.0	
R2/R1-tail	0.3	1.2	19,000	0.8	2.0	

$t_{1/2}$, Time (min) to attain half-maximal internalization in the presence of 2 nM [3 H]MeTRH. EC_{50} , Half-maximally effective concentration of TRH in nM for stimulation of IPs. K_d , Dissociation constant for [3 H]MeTRH measured in equilibrium binding experiments. B_{max} , Maximal binding of [3 H]MeTRH (dpm). TRH response, fold stimulation by 1 μ M TRH. n.d., Not determined; these values were greater than 1.5 min but could not be accurately estimated because of low B_{max} .

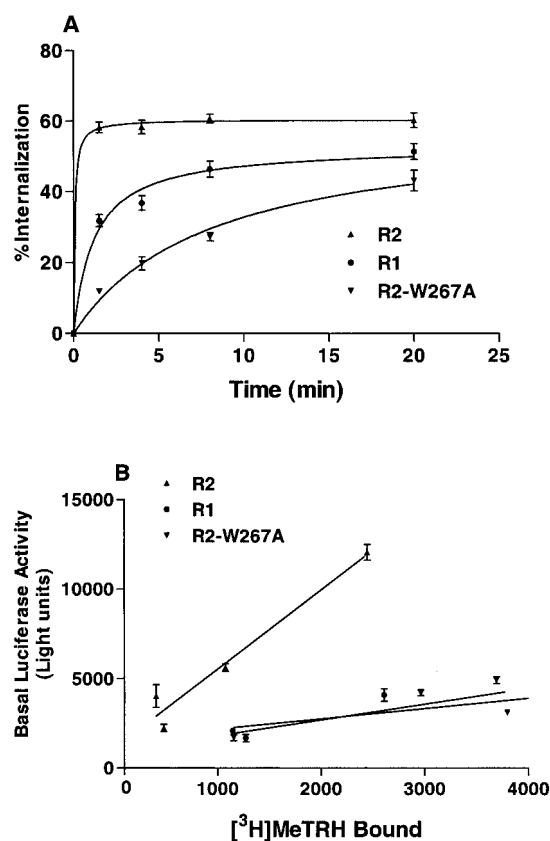


FIG. 4. Effect of mutation of the conserved tryptophan in TM 6 on internalization and basal signaling activity of mR2. **A**, Transfected cells were incubated with 2 nM [3 H]MeTRH at 37 C for the times indicated and specific and acid-resistant binding were measured as described in *Materials and Methods*. **B**, Cells were cotransfected with plasmid containing pFA2-CREB, pFR-Luc, and various amounts of plasmid encoding wild-type or R2-W267A and were assayed for luciferase activity as described in *Materials and Methods*. Results are expressed as mean \pm SD of assays performed in triplicate in a representative experiment.

Discussion

Chimeras of mR1 and mR2 were constructed and used in experiments to determine the structural basis of the different rates of agonist-induced receptor internalization. We dem-

onstrated that the rate of agonist-induced internalization of a given chimera is more dependent on the origin of the extracellular domains and/or the transmembrane helices than on the origin of the intracellular domains of the receptors. These findings appear to contradict those of previous studies that have shown that the structural features of mR1 that determine the rate of agonist-induced internalization are located in the carboxyl terminus (6). However, these conclusions were based on studies using deletions of the carboxyl terminus of mR1. In the present study, we shifted the entire carboxyl termini of mR1 and mR2 to create chimeric receptors R1/R2-tail and R2/R1-tail that were shown to undergo agonist-induced internalization at a rate closely resembling mR1 and mR2, respectively. These data suggest that, although an intact carboxyl terminus is required for agonist-induced receptor internalization, it is not the structural feature(s) responsible for determining the internalization rate of mR1 and mR2; that is, other structural features may be more important in determining the rate of agonist-induced internalization in the presence of an intact carboxyl terminus. The conserved Trp of helix 6 in mR1 (R1-W279) had been shown in a previous study from our laboratory to form a hydrophobic cluster with amino acids of helix 5, and the interaction between helix 5 and 6 constrains the mR1 in an inactive conformation. Substitution of Trp 279 by Ala rendered mR1 constitutively active (57). The agonist-induced internalization rate of R1-W279A is 10-fold higher than that of wild-type mR1 (data not shown). In contrast, substitution of this conserved Trp with Ala in mR2 (R2-W267A) decreased both the basal signaling activity and the agonist-induced internalization rate. These findings indicated that the conserved Trp in helix 6 not only plays a role in determining the basal signaling activity of mR1 and mR2 but also greatly affects the rate of agonist-induced internalization of these receptors. In supporting our findings that the amino acids in the helical domains are important for determining basal activities and internalization rates of TRH Rs, we mutated G200 (TM5) and A252 (TM6) in mR2 to their corresponding amino acid in mR1, leucine, and valine, respectively, and the mutated receptors showed decreased basal activities and internalization rates (our unpublished observation). It is important to note that other structural features could affect agonist-

induced internalization rates of TRH Rs. In fact, there is accumulating evidence indicating that the domains/residues involved in receptor internalization are multisite in nature. It is tempting to speculate that there is a specific conformation of GPCRs destined for internalization, just as there is an active conformation of GPCRs responsible for G protein activation and that these conformations are similar. Therefore, any mutation that facilitates or impairs the conformational change leading to this specific state would affect receptor internalization and basal activity. Different domains may function independently or cooperatively in determining the rate of receptor internalization. It will be of interest to examine whether the mutation of the carboxyl terminus of mR1 could impair the high receptor internalization rate of mR2 in the chimeric receptor R2/R1-tail, or whether R1-W279A could rescue the slow internalization of the carboxyl-terminal deletion form of mR1.

TRH Rs that were only minimally basally active responded to TRH stimulation to a greater extent than constitutively active receptors. Relative refractoriness to further stimulation by agonist has also been reported for constitutively active forms of human LH Rs (31, 58). It is known that internalization of TRH is involved in the termination of its actions; therefore, the lack of responsiveness detected in cells expressing constitutively active receptors may be due in part to the rapid internalization of the TRH/TRH R complex. This would support the hypothesis that TRH R signaling occurs only when the active receptor is on the cell surface. In addition, the constitutively active receptors with rapid internalization rates might be down-regulated to a greater extent (59).

Although we have not found the explanation for the correlation between basal signaling activity and the rate of agonist-induced internalization, we suggest that a likely explanation is that the conformation of the active states of these receptors allows for more efficient coupling to G proteins and binding to the proteins involved in internalization. This is in agreement with the finding of several studies that have demonstrated that mutations that impair signaling of GPCRs reduce agonist-induced receptor internalization, whereas mutations that yield constitutively active receptors generate receptors that are internalized faster than wild-type receptors (21, 22, 31, 60). Another possibility is that active receptors, which are known to be posttranslationally modified, are more efficiently bound to the internalization machinery because they are modified. For example, phosphorylation of a number of GPCRs has been shown to be necessary for agonist-induced receptor internalization (12, 61). It is interesting to note that some constitutively active mutants of GPCRs showed a high level of basal phosphorylation (62, 63). It is possible, therefore, that the constitutively active TRH Rs are basally phosphorylated and interact with the internalization components in the ligand-free state and upon ligand binding more readily undergo agonist-induced internalization.

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