Requirement of membrane-proximal amino acids in the carboxyl-terminal tail for expression of the rat AT$_{1a}$ angiotensin receptor

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Abstract A series of deletion mutants was created to analyze the function of the membrane-proximal region of the cytoplasmic tail of the rat type 1a (AT$_{1a}$) angiotensin receptor. In transiently transfected COS-7 cells, the truncated mutant receptors showed a progressive decrease in surface expression, with no major change in binding affinity for the peptide antagonist, [Sar^1,Ile^8]angiotensin II. In parallel with the decrease in receptor expression, a progressive decrease in angiotensin II-induced inositol phosphate responses was observed. Alanine substitutions in the region 307–311 identified the highly conserved phenylalanine^{309} and adjacent lysine residues as significant determinants of AT$_{1a}$ receptor expression.
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Key words: AT$_1$ angiotensin receptor;
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Receptor expression; Ca^{2+} mobilizing hormone

1. Introduction

The type 1 (AT$_1$) angiotensin II receptor is a G protein-coupled receptor (GPCR), that mediates the cardiovascular and other actions of the octapeptide hormone, angiotensin II (Ang II). Agonist binding to the AT$_1$ receptor stimulates phospholipase C activation via the G$_q$ family of G proteins, and promotes rapid internalization of the receptor [1,2]. Several conserved amino acids, mostly located in the membrane domains, are important determinants of GPCR function [3,4]. The membrane-proximal region of the cytoplasmic tail of many GPCRs contains a conserved aromatic residue and one or more positively charged adjacent amino acids. The function of the membrane-proximal region that includes these conserved structural elements was analyzed in the rat AT$_{1a}$ angiotensin receptor.

It has been demonstrated that the carboxyl-terminal tail of several GPCRs has a critical role in coupling of the receptor with its cognate G protein(s) [5–7]. In the β$_2$ adrenergic receptor and endothelin receptors, palmitoylation on cysteine residues of the cytoplasmic tail is required for G protein coupling [8–10]. In addition to palmitoylation, other structural elements in this region have been implicated in G protein coupling of adrenergic receptors [5], the substance P receptor [11], and endothelin receptors [8,10]. However, the participation of the carboxyl-terminal tail in G protein activation is not a general requirement for GPCR signaling. Truncation of the cytoplasmic tail did not affect second messenger generation by several GPCRs, including many Ca^{2+} mobilizing receptors, such as the C5a [12], parathyroid [13], neurotensin [14] and neurokinin-2 receptors [15]. Also, the GnRH receptor, a GPCR that lacks a cytoplasmic tail, shows normal G protein coupling and inositol phosphate signal generation [16].

The carboxyl-terminal tail of many GPCRs is also required for receptor internalization and desensitization [17]. However, the structural determinants for these processes appear to be different from those of G protein coupling. Serine-threonine rich regions, located relatively distant from the membrane domain in the cytoplasmic tail and in the third intracellular loop, are the major requirements for receptor internalization and desensitization in many GPCRs [5,17].

Reduced expression levels after deletions of the carboxyl-terminal tail have been reported for many GPCRs, including rhodopsin [18], β$_2$-adrenergic [19], muscarinic [20], histamine H$_2$ [21], neurokinin [22], C5a anaphylatoxin[12], parathyroid hormone [13] and AT$_1$ angiotensin [23,24] receptors. These findings indicate that the cytoplasmic tail may be a major determinant of the expression of GPCRs on the cell surface.

Previous studies have demonstrated the importance of the carboxyl-terminal tail in internalization of the AT$_1$ receptor [25–27]. However, the role of the cytoplasmic tail in AT$_1$ receptor signaling and expression is more controversial. It is generally agreed that C-terminal deletions of the cytoplasmic tail up to position 314 do not interfere with signal generation by the AT$_1$ receptor [23,26,28]. Larger deletions of the cytoplasmic tail have been found to impair the inositol phosphate responses of the rat AT$_{1a}$ receptor [29,30]. However, a study on the human AT$_1$ receptor reported that the cytoplasmic tail is selectively required for G$_i$ activation without affecting G$_q$-mediated inositol phosphate responses [24]. Although an earlier report described normal expression levels after deletion of the cytoplasmic tail of the rat AT$_{1a}$ receptor [29], other studies of rat AT$_{1a}$ [23] and human AT$_1$ [24] receptors found that truncation of the cytoplasmic tail decreased the expression level of the receptor.

The aim of the present study was to further investigate the role of the membrane-proximal portion of the carboxyl-terminal tail in AT$_{1a}$ receptor expression and activation, and to identify the functions of conserved amino acids in this region.

2. Materials and methods

2.1. Mutagenesis and expression of the rat smooth muscle AT$_{1a}$ receptor cDNA
The cDNA of the rat smooth muscle AT$_{1a}$ receptor (kindly pro-

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vided by Kenneth E. Bernstein [31]) was subcloned into the mammalian expression vector pcDNAI/Amp (Invitrogen, San Diego, CA) as described earlier [32]. Mutant AT_{1a} receptors were created using the Mutagene kit (Bio-Rad). The sequences of the mutants were identified by dideoxy sequencing using Sequenase II (Amersham). The COS-7 cells were transiently transfected with plasmids containing wild-type or mutant AT_{1a} receptor cDNAs using Lipofectamine (Life Technologies) as described previously [33].

2.2. Inositol phosphate measurements

The culture medium was replaced 24 h after transfection with 0.5 ml of inositol-free Dulbecco's modified Eagle's medium (DMEM) containing 1 g/l bovine serum albumin, 20 µCi/ml *myo*-[2-^{3}H]inositol (Amersham), 2.5% fetal calf serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. 24 h later the cells were washed twice, incubated in inositol-free DMEM in the presence of 10 mM LiCl for 30 min at 37°C, and stimulated with 1 µM Ang II for 20 min. Inositol phosphates were extracted as described earlier [32], and after neutralization the samples were applied to Bio-Rad AG 1-X8 columns. The columns were washed three times with 3 ml water and twice with 3 ml 0.2 M ammonium-formate in 0.1 M formic acid. After these washing steps, the combined $InsP_2$+$InsP_3$ fractions were eluted with 2×3 ml 1 M ammonium formate in 0.1 M formic acid, and radioactivities were detected by liquid scintillation counting.

2.3. [Sar^1,Ile^8]Ang II binding to intact cells

The number of Ang II binding sites was determined by incubating the transfected cells with ^{125}I-[Sar^1,Ile^8]Ang II (Hazleton Laboratories, Vienna, VA or DuPont-NEN) and increasing concentrations of unlabeled [Sar^1,Ile^8]Ang II in DMEM containing 25 mM HEPES (pH 7.4) for 6 h at 4°C. The cells were washed twice with ice-cold phosphate-buffered saline, and the radioactivity associated with the cells was measured by γ-spectrometry. The displacement curves were analyzed with the Ligand computer program using a one-site model [34].

2.4. Receptor internalization in transiently transfected COS-7 cells

To determine the internalization kinetics of the mutant and wild-type AT_{1a} receptors ^{125}I-Ang II was added to HEPES-buffered DMEM, and the cells were incubated at 37°C for the indicated times. Incubations were stopped by placing the cells on ice and rapidly washing them twice with ice-cold phosphate-buffered saline. Acid-released and acid-resistant radioactivities were separated and measured by γ-spectrometry as described earlier [32]. The percent of internalized ligand at each time point was calculated from the ratio of the acid-

resistant specific binding to the total (acid-resistant+acid-released) specific binding.

3. Results and discussion

3.1. [Sar^1,Ile^8]Ang II binding to cytoplasmic tail deletion mutant AT_{1a} receptors

Deletion mutant AT_{1a} receptors were created to study the membrane-proximal region (position 307–319) of the cytoplasmic tail (Fig. 1) by substituting the indicated amino acid with a stop codon (e.g. substitution of Tyr^{319} with a stop codon = $\Delta319$). To determine the surface expression level and structural integrity of wild-type and mutant AT_{1a} receptors, transiently expressed in COS-7 cells, the binding of the Ang II antagonist [Sar^1,Ile^8]Ang II was measured at 4°C. Scatchard analysis of the data showed that the K_d values of the mutant receptors were comparable to that of the wild-type AT_{1a} receptor (in nM: wild-type: 2.6 ± 0.6; $\Delta319$: 2.2 ± 0.5; $\Delta315$: 1.9 ± 0.2; $\Delta311$: 2.2 ± 0.1; $\Delta310$: 2.3 ± 0.3; $\Delta309$: 1.9 ± 0.3; $n=3$). These data suggest that the integrity of the receptor was not compromised by these mutations, since the complex structure of the binding site for the peptide ligand remained intact. Scatchard analysis of these data also showed that deletions of the cytoplasmic tail caused a progressive decrease of surface expression (Fig. 2). The reduced expression level of the $\Delta315$ mutant indicates that structures in the cytoplasmic tail distal to position 314 are required for the maximal level of receptor expression. Removal of 6 additional amino acids ($\Delta309$) further reduced expression to $4.3\pm0.7\%$ ($n=3$) of that of the wild-type receptor, and deletion of one additional amino acid, Lys^{308}, caused complete loss of cell-surface receptors.

These data suggest that the membrane-proximal region of the cytoplasmic tail is also required for normal AT_{1a} receptor folding and surface expression in COS-7 cells. The reduced expression levels of the truncated AT_{1a} receptors were consis-
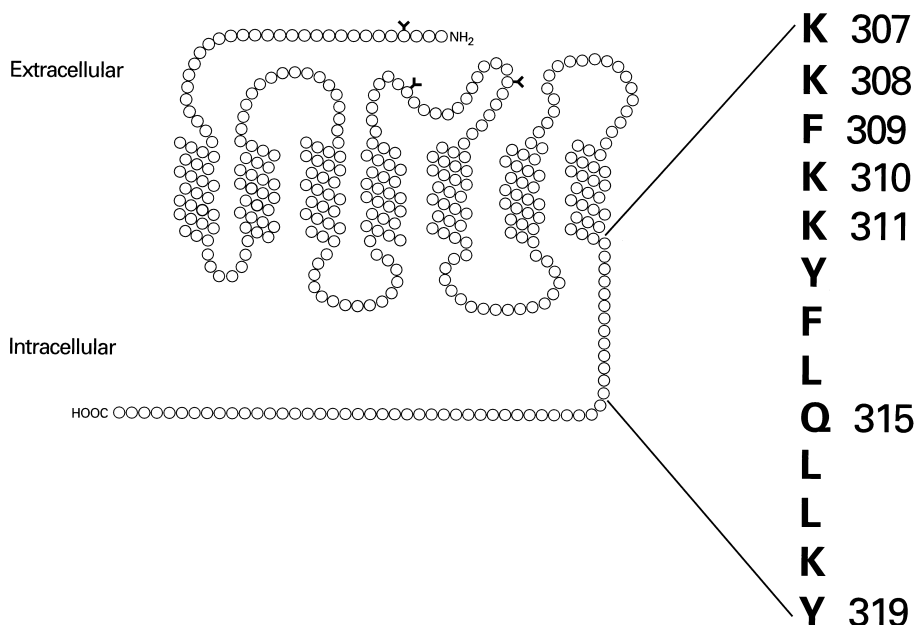

Fig. 1. The region of the carboxyl-terminal tail adjacent to the seventh transmembrane helix of the AT_{1a} receptor (positions 307–319) was analyzed in this study. The one letter codes of amino acids in this region and the positions of the mutated amino acids are shown. The putative glycosylation sites are also indicated.
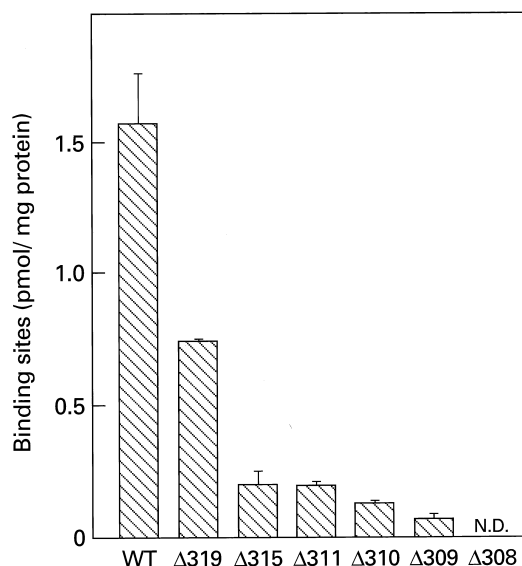
Fig. 2. Surface expression levels of wild-type and deletion mutant AT$_{1a}$ receptors were calculated by Scatchard analysis of [Sar^1,Ile^8]Ang II binding to transiently transfected COS-7 cells. The designation of each truncated mutant receptor represents the position of the amino acid that was replaced with a stop codon. Binding sites of wild-type and mutant AT$_{1a}$ receptors are shown as the means ± S.E.M. from three independent experiments, each performed in duplicate. N.D. indicates that no detectable surface expression was observed.

tent with findings of studies on other GPCRs, in which truncation of the membrane-proximal region reduced receptor expression levels [12,13,19–22]. Deletion of the cytoplasmic tail of the human AT$_1$ receptor at position Lys^{310} reduced the expression of the receptor in COS-7 cells [24]. The average expression level of the Δ315 mutant rat AT$_{1a}$ receptor was also reduced in permanently transfected CHO cells, but some of the individual colonies exhibited relatively high expression levels [23]. This may account for the relatively high expression levels of cytoplasmic tail deletion mutant AT$_{1a}$ receptors observed in a recent study on permanently transfected CHO cells [30].

Although our data do not provide evidence for the mechanism by which tail truncations affect receptor expression level, several explanations for this effect have been proposed. Studies on mutant rhodopsins have indicated that truncations of the carboxyl-terminal region of the molecule interfere with transport of the receptor from the endoplasmic reticulum, and with membrane insertion of the molecule [35]. A recent study on the H$_2$ histamine receptor reported that although there was no detectable ligand binding after truncation of the cytoplasmic tail adjacent to the membrane domain, synthesis of the mutant receptor mRNA was still observed, suggesting that the carboxyl-terminally truncated receptor is not efficiently synthesized, transported, or inserted into the membrane [21]. Similar mechanisms may operate in AT$_1$ receptors, since normal mRNA level and reduced expression of the Δ310 mutant human AT$_1$ receptor was observed in transiently transfected COS-7 cells [24]. However, increased degradation of the truncated receptor molecules is an alternative possibility that cannot be excluded at this stage.

3.2. Inositol phosphate responses of cytoplasmic tail truncated mutant AT$_{1a}$ receptors

Transiently transfected COS-7 cells were prelabeled with [^{3}H]inositol, and after LiCl pretreatment the cells were stimulated with Ang II for 20 min as described in Section 2. To evaluate the G protein coupling of wild-type and mutant AT$_{1a}$ receptors maximally effective concentration (1 μM) of Ang II was used in these experiments [33]. With the exception of the Δ319 mutant, all of the cytoplasmic tail deletion-mutant AT$_{1a}$ receptors showed reduced inositol phosphate responses after Ang II stimulation (Fig. 3A). The Δ319 mutant receptor was previously shown to mediate inositol phosphate responses comparable to those of the wild-type receptor [26]. The reduced inositol-phosphate responses of the Δ309, Δ310, Δ311 and Δ315 mutant receptors (Fig. 3A) are consistent with our earlier observation that inositol phosphate signaling in this system depends on the receptor expression level [33]. After normalization to the receptor expression level [33], the inositol phosphate responses for the truncated receptors were higher than those of the wild-type receptor (Fig. 3B). A similar finding has been reported in the truncated neurokinin-2 receptor, and was attributed to impaired phosphorylation and desensitization of the receptor [15]. This mechanism is probably responsible for the consistently higher inositol phosphate responses of the Δ309 to Δ319 mutant AT$_{1a}$ receptors. Such deletions eliminate the serine-threonine rich sequences of the cytoplasmic tail, which are the major targets for phosphoryl-
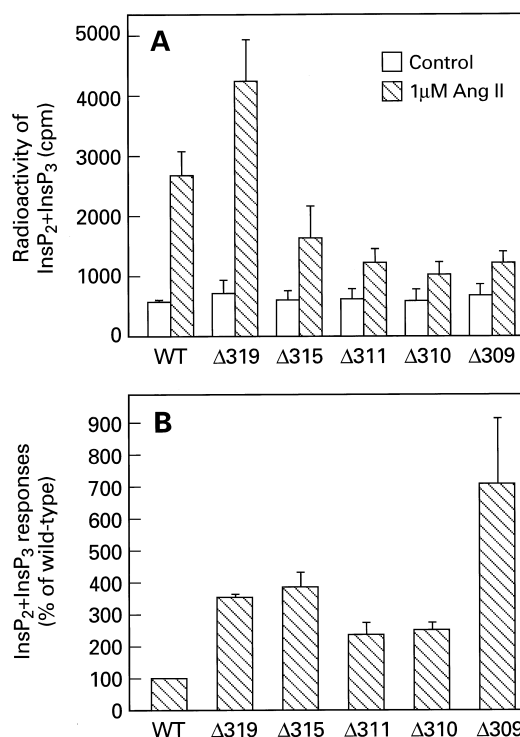

Fig. 3. Inositol phosphate responses of wild-type and truncated AT$_{1a}$ receptors (A) were measured as the combined accumulation of radioactivity in the InsP$_2$ + InsP$_3$ fraction in [^{3}H]inositol prelabeled and LiCl (10 mM, 30 min) pretreated COS-7 cells after stimulation with 1 μM Ang II for 20 min. B: Data were normalized to the number of expressed extracellular binding sites for each mutant, and are shown as percent of the wild-type response. Data are shown as means ± S.E.M. from three independent experiments, each performed in duplicate.

ation and desensitization of many GPCRs [17,36]. In accordance with this suggestion, it has been shown that protein kinase C-mediated desensitization of the AT_{1a} receptor depends on the presence of the cytoplasmic tail [28].

In a recent report, the inositol phosphate responses mediated by $\Delta 314$ and larger deletion mutant AT_{1a} receptors were found to be markedly impaired after 10 s stimulation with Ang II [30]. The present findings, in accordance with a study on the $\Delta 310$ mutant human AT_1 receptor [24], demonstrate that inositol phosphate accumulation is detectable after 20 min stimulation of the $\Delta 309$ mutant receptor in the presence of LiCl. A possible explanation for the impaired signaling detected after 10 s of Ang II stimulation is the slower kinetics of the inositol phosphate responses of the mutant receptors, similar to earlier reports on the function of Tyr^{302} in the AT_{1a} receptor [33,37,38].

3.3. [Sar^1,Ile^8]Ang II binding to KKFKK(307–311)AAAAA, KKFKK(307–311)AAFAA, KK(310–311)AA, F309A and $\Delta 309$ mutant AT_{1a} receptors

The membrane-proximal region of the cytoplasmic tail, adjacent to the seventh transmembrane helix (TM7), contains conserved structural elements. In a published collection of GPCRs [7], 84% of the mammalian receptors, excluding the odorant receptors, contained a conserved phenylalanine residue 3–5 amino acids away from the predicted boundary of TM7. Another conserved feature of this region is the accumulation of positively charged amino acids adjacent to this conserved phenylalanine. Of 59 mammalian GPCRs, all except the C5a anaphylatoxin receptor contained at least 1 lysine or arginine residue in the region corresponding to positions 307–311 of the AT_1 receptor, and 65% had two or more residues in this region.

Our data showed that the $\Delta 311$ and $\Delta 315$ mutant receptors were similar in all tested parameters, but deletion of additional residues progressively decreased the receptor expression, suggesting the importance of this conserved domain, located proximal from position 311. To study the role of this region $(Lys^{307}\text{-}Lys^{308}\text{-}Phe^{309}\text{-}Lys^{310}\text{-}Lys^{311})$ in AT_{1a} receptor function, alanine substitution mutant receptors were created. No [Sar^1,Ile^8]Ang II binding was detected after replacement of all five amino acids with alanine (KKFKK(307–311)AAAAA) or elimination of the positive charge of the region by substituting the four lysine residues with alanine (KKFKK(307–311)AAFAA) (Fig. 4A). These findings are consistent with the above-suggested function of this region in receptor expression.

To further analyze the function of the conserved amino acids, two more mutants were constructed. In mutant KK(310–311)AA, Lys^{310} and Lys^{311} were replaced with alanine to remove part of the positive charge in this region, and an F309A mutant AT_{1a} receptor was created to study the role of the conserved phenylalanine residue. The binding affinities of the KK(310–311)AA and F309A mutant AT_{1a} receptors for the peptide antagonist [Sar^1,Ile^8]Ang II were 1.6 ± 0.2 and 1.7 ± 0.1 nM ($n = 3$), similar to the affinity of the wild-type receptor (2.4 ± 0.7 nM, $n = 3$) as determined in the same experiments. However, the expression levels of the mutant receptors were markedly reduced (Fig. 4A). Cells expressing F309A or KK(310–311)AA mutant receptors also showed reduced inositol phosphate accumulation after agonist stimulation ($56.8 \pm 3.6\%$ and $42.9 \pm 2.4\%$ of that of the wild-type AT_{1a} receptor, respectively). Considering the lower expression
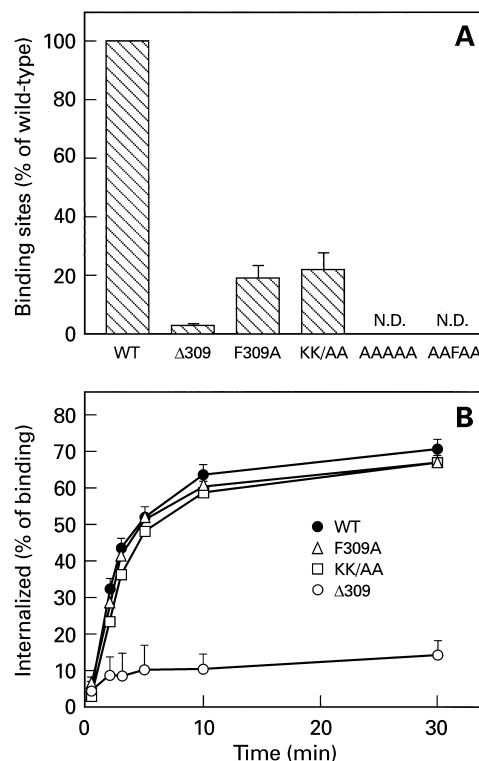

Fig. 4. Surface expression levels (A) of wild-type, $\Delta 309$, F309A, KK(310–311)AA (KK/AA), KKFKK(307–311)AAAAA (AAAAA) and KKFKK(307–311)AAFAA (AAFAA) mutant AT_{1a} receptors were calculated by Scatchard analysis of [Sar^1,Ile^8]Ang II binding to transiently transfected COS-7 cells. Data are shown as percent of the expressed wild-type AT_{1a} receptor binding sites ($n = 3$). N.D. indicates that no detectable surface expression was observed. Internalization kinetics (B) of the wild-type (●), F309A (△), KK/AA (□) and $\Delta 309$ (○) mutant AT_{1a} receptors were measured after addition of ^{125}I-Ang II at 0 min as described in Section 2. Internalized binding is shown as percent of total specific binding of each sample. Data represent the means ± S.E.M. from three independent experiments, each performed in duplicate.

levels of these receptors, we concluded that Phe^{309}, Lys^{310}, and Lys^{311} do not have a major role in the inositol phosphate signal generation of the AT_{1a} receptor.

Based on these data, the major loss of expression caused by truncating the AT_{1a} receptor at position 309 has at least two components. In addition to the role of Phe^{309} and the surrounding positively charged residues in the regulation of the expression level, the lower expression level of the $\Delta 315$ mutant receptor indicates that additional elements, located distal to position 314, are also required for maximal receptor expression.

The internalization kinetics of the alanine substitution KK(310–311)AA and F309A mutant receptors did not differ from those of the wild-type receptor (Fig. 4B). These findings confirm that the structure of the receptor, and its agonist-induced activation, are not affected by mutations in the membrane-proximal region of the cytoplasmic tail. The strongly impaired internalization kinetics of the $\Delta 309$ mutant are consistent with earlier findings on the role of the cytoplasmic tail in AT_1 receptor internalization [23,25–28].

In conclusion, these data provide further evidence that the cytoplasmic tail is required for normal expression and internalization of the AT_{1a} receptor. The present findings also

demonstrate that the conserved Phe[309] residue, and the adjacent positively charged amino acids in the membrane-proximal region of the cytoplasmic tail, are selectively involved in the regulation of the receptor expression level without affecting receptor internalization or inositol phosphate signal generation.

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