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IDENTIFICATION OF RESIDUES ESSENTIAL FOR PROGESTERONE BINDING TO UTEROGLOBIN BY SITE-DIRECTED MUTAGENESIS

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Summary—In order to identify amino acids directly involved in progesterone binding to rabbit uteroglobin we have mutated Phe 6, Tyr 21 and Thr 60 by site-directed mutagenesis of the uteroglobin cDNA. These residues have been postulated previously to participate in progesterone binding. High-level expression of the mutated uteroglobin cDNAs in *Escherichia coli* yields recombinant protein mutants that, like natural uteroglobin, form stable dimers, suggesting that the tertiary structure of the protein has not been altered. Substitution of Phe 6 by Ser or Ala does not change the progesterone binding characteristics. In contrast, replacement of Tyr 21 by Phe or Ala, drastically decreases progesterone binding. In addition, replacement of Thr 60 by Ala reduces the affinity for progesterone by a factor of three. These data suggest a direct interaction of progesterone with these two amino acids and support the idea of direct hydrogen bonding of the carbonyl (C3 and C20) of progesterone with the hydroxyl groups of Tyr 21 and Thr 60, respectively.

INTRODUCTION

Uteroglobin is a dimeric protein expressed in the rabbit endometrium during early pregnancy. It consists of two identical 70 amino acid polypeptide chains linked by two disulfide bridges. Uteroglobin has long been known to bind progesterone with high specificity [1]. It was recently shown that uteroglobin has a limited sequence similarity with the steroid binding domain of the progesterone receptor [2]. Therefore, uteroglobin may also serve as a general model for the analysis of specific interactions between progesterone and proteins.

Progesterone binding to uteroglobin is optimal in the presence of reducing agents. However, reduction of the disulfide bridges does not separate the polypeptide chains that are also held together by non-covalent bonds. To date, rabbit uteroglobin is the only steroid-binding protein for which the three-dimensional structure has been determined by X-ray diffraction analysis [3–5]. In these crystals uteroglobin exists as an antiparallel dimer, where Cys 3 of one chain is connected by Cys 69 of the other chain. Each monomer comprises four amphipatic α-helices separated by short β-turns. The α-helices are arranged such that almost all of the hydrophobic residues are positioned on one side of the monomer, producing a well-defined hydrophobic surface which generates a hydrophobic cavity in the dimer. Although the crystals analyzed so far did not contain progesterone, it was proposed that a single progesterone molecule binds to this cavity [3].

In order to enable a more detailed study of the interaction of progesterone with uteroglobin we have recently developed a system for the expression of the protein in *Escherichia coli* that permits large-scale purification. The recombinant uteroglobin has seven additional amino acids at the NH_2-terminal end but behaves like the naturally occurring protein purified from pseudopregnant rabbits, in terms of dimer formation and affinity for progesterone [6]. In the present study we have used this recombinant uteroglobin for mutational analysis and have identified amino acids that are directly involved in progesterone binding.

EXPERIMENTAL

Materials

DNA modifying enzymes were obtained from Pharmacia, Boehringer Mannheim and

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Amersham-Buchler and used according to the manufacturers specifications. Radiolabeled compounds were supplied by Amersham-Buchler. Superose 12 and Mono S ion exchange column were purchased from Pharmacia LKB.

Site-directed mutagenesis and sequence analysis

An EcoRI-XbaI fragment of pUG7 [6], containing the T5-promoter, the ribosomal binding site, the coding region for uteroglobin, the 3'-untranslated region and the transcriptional terminator, was ligated to EcoRI-XbaI cleaved M13 mp 19 double stranded DNA. Single stranded recombinant M13 DNA was prepared and mutated with synthetic oligonucleotides using the Amersham mutagenesis kit following the supplier's instructions. Recombinant clones were screened by sequencing with T7 DNA-polymerase [7] using a primer complementary to 17 base pairs of the T5 promoter. Double stranded DNA from positive clones was prepared, the mutated EcoRI-XbaI fragment isolated and recloned into the pUG7 expression plasmid (see Fig. 1A). *E. coli* strain W3110 $lacI^Q$ was transformed and DNA prepared from single clones. The sequences of the new constructs were verified by sequence analysis [8].

Purification of uteroglobin variants

Uteroglobin variants were purified from 500 ml *iso*propyl-β-thiogalacto-pyranoside (IPTG)-induced cultures. Pelleted bacteria were resuspended in 20 ml H_2O and subjected to lysis in a French press at 1000 psi (flow rate: 1.5 ml/min). After centrifugation (4°C, 30 min, 40,000 *g*) the supernatant was lyophilized. After solubilization in 4.5 ml TBS/DTE (100 mM Tris/Cl pH 7.5, 150 mM NaCl, 10 mM DTE) for 30 min at 37°C and additional 20 min on ice, undissolved proteins were removed by centrifugation (15 min, 10,000 *g*, 4°C) and the supernatant passed through a Superose 12 FPLC HR 16/50 column (flow rate: 0.8 ml/min). Fractions containing uteroglobin were pooled and dialyzed against 25 mM ammonium acetate, pH 4.2.

After filtration through a 0.2 μm nitrocellulose filter, the solution was subjected to Mono S ion exchange chromatography. Elution was performed with a linear pH and salt gradient ranging from 25 mM ammonium acetate, pH 4.2 to 1 M ammonium acetate pH 8. Uteroglobin containing fractions were dialyzed against water, lyophilized, weighted and stored at −20°C.

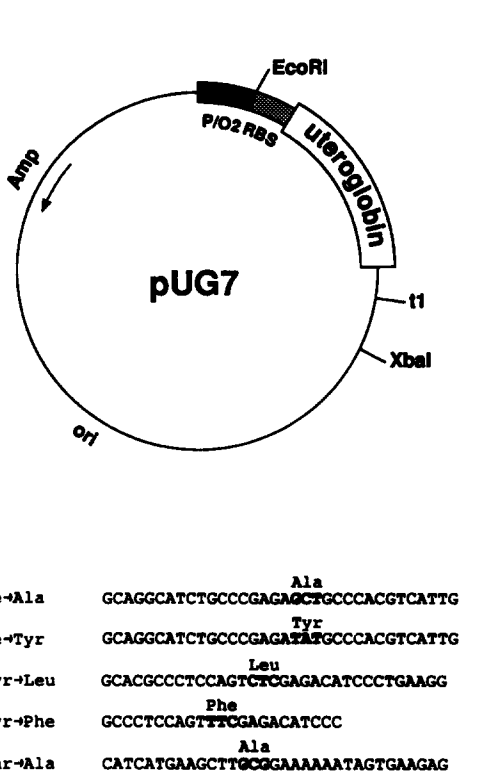

Fig. 1. Plasmid map of the *E. coli* expression vector and oligonucleotides for site-directed mutagenesis: (A) the *E. coli* expression vector (pUG7) contains the T5 phage derived promoter (P/O2), the ribosomal binding site (RBS), the mature uteroglobin coding sequence (uteroglobin) and a transcriptional terminator (t1). The pBR322 derived β-lactamase gene (Amp) and the origin of replication (ori) are indicated; and (B) oligonucleotides used for site-directed mutagenesis. Codons that change the amino acid sequence and the corresponding amino acid are indicated by bold letters. The sequence changes performed were: ^{6}Ala (TTT→GCT), ^{6}Tyr (TTT→TAT), ^{21}Leu (TAC→CTC), ^{21}Phe (TAC→TTC) and ^{60}Ala (ACG→GCG).

The concentration of solubilized purified protein was determined by scanning Coomassie blue stained polyacrylamide gels with bovine serum albumin as reference standard.

Immunoblotting

Immunoblots (Western blots) were performed as previously described [6]. After electroblotting, filters were incubated with a sheep antiserum to rabbit uteroglobin as first and a donkey anti-sheep IgG–alkaline phosphatase conjugate (Sigma) as second antibody. Visualization was performed with nitroblue tetrazolium (NTB) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) according to Blake *et al.* [9].

Progesterone binding assay

Equilibrium dialysis was used for determination of association constants [1]. Purified

uteroglobin variants (20–80 μg in 100 μl 20 mM Tris-Cl, pH 7.5, 10 mM NaCl and 10 mM DTE) were dialyzed in closed Plexiglass microchambers against 100 μl of increasing concentrations of [^{3}H]progesterone (0.2–10 $\times$ 10^{-6} M, sp. act. 1 Ci/mmol). The chambers were shaken for 24 h at 4°C. From each side of the dialysis membrane (Spectropor 3500, Roth) 80 μl aliquots were used for radioactivity determination in 4 ml of Rotiszint 2200 (Roth).

RESULTS

Site directed mutagenesis and expression of mutant proteins in E. coli

In a first attempt to identify residues of uteroglobin which are involved in progesterone binding, we have produced uteroglobin variants mutated at Phe 6, Tyr 21 and Thr 60. These amino acids are buried inside the uteroglobin dimer and are part of the central cavity surface that has been proposed to bind progesterone [4]. Recombinant uteroglobin variants were constructed by replacing Phe 6 by Ala and Tyr, Tyr 21 by Phe and Leu, and Thr 60 by Ala. Computer-assisted analysis of the probable changes in secondary structures of these uteroglobin variants according to Garnier *et al.* [10] revealed that these mutations should not disturb the α-helical structure of the protein.

All substitutions were generated using the oligonucleotide primer synthesis method on single-stranded M13 phage DNA templates [11]. The expression vector and the sequence of the oligonucleotides together with the mutated amino acid are shown in Fig. 1. DNA sequence analysis with double stranded DNA confirmed the expected mutations. *E. coli* strain W3110 lacI^Q was transformed with the mutated pUG7 plasmids and the expression of recombinant uteroglobin variants in the presence and absence of IPTG examined. All uteroglobin variants are expressed properly in a soluble form and with a similar yield.

Purification and biochemical characterization of uteroglobin mutants

The recombinant uteroglobin variants were purified through FPLC Superose 12 and Mono S columns as described above. The chromatographic properties of the uteroglobin variants were indistinguishable from the recombinant wild-type protein. The final yield of the purification was about 2–3 mg protein per 500 ml of induced bacteria. Purity was assessed by SDS–PAGE and estimated as about 90% for all preparations.

Natural uteroglobin from rabbit endometrium and recombinant uteroglobin UG7 form stable dimers under non-reducing conditions [6]. In these dimers Cys 3 (in the recombinant uteroglobin at position 10) is connected to Cys 69′ and vice versa. The ability of uteroglobin variants to form dimers after purification was tested. Figure 2 shows the electrophoretic analysis of the mutant proteins together with natural mature uteroglobin from rabbit endometrium and the recombinant wild-type. The ability to dimerize is not affected in the mutants. All uteroglobin variants migrate under reducing conditions as monomers and under non-reducing conditions as dimers like mature rabbit uteroglobin. This behavior strongly indicates that all uteroglobin variants have similar, if not identical structural characteristics as the mature natural uteroglobin. Note that the somehow slower migration of recombinant wild-type and the variants ^{6}Ala, ^{6}Tyr,

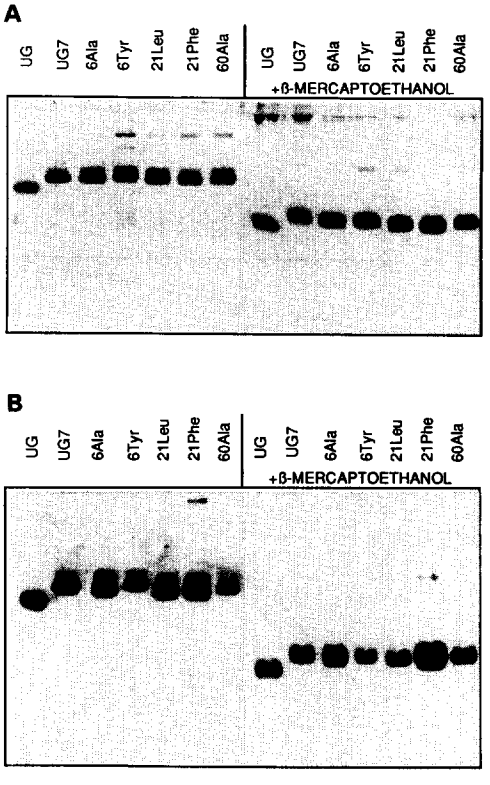

Fig. 2. Analysis of recombinant uteroglobin variants: (A) Coomassie-blue stained 18% SDS polyacrylamide gel with 2.5 μg purified mature uteroglobin (UG), recombinant wild-type uteroglobin (UG7) and various recombinant uteroglobin variants (^{6}Ala, ^{6}Tyr, ^{21}Leu, ^{21}Phe and ^{60}Ala) prior (left) and after reduction with β-mercaptoethanol (right); and (B) immunostained Western blot of (A).

^{21}Leu, ^{21}Phe and ^{60}Ala is due to NH_2-terminal extension of seven residues of these proteins [6].

Progesterone binding characteristics

Recombinant purified uteroglobin (UG7) and natural uteroglobin bind progesterone with the same association constant as determined by equilibrium dialysis [6]. The binding data and a Scatchard analysis [12] of the uteroglobin variants ^{6}Ala, ^{6}Tyr, ^{21}Leu, ^{21}Phe and ^{60}Ala together with the rabbit-derived protein and recombinant UG7 are presented in Fig. 3. The ^{6}Ala and ^{6}Tyr mutations have association constants for progesterone ($4.7 \times 10^6\ M^{-1}$ and $5.8 \times 10^6\ M^{-1}$,

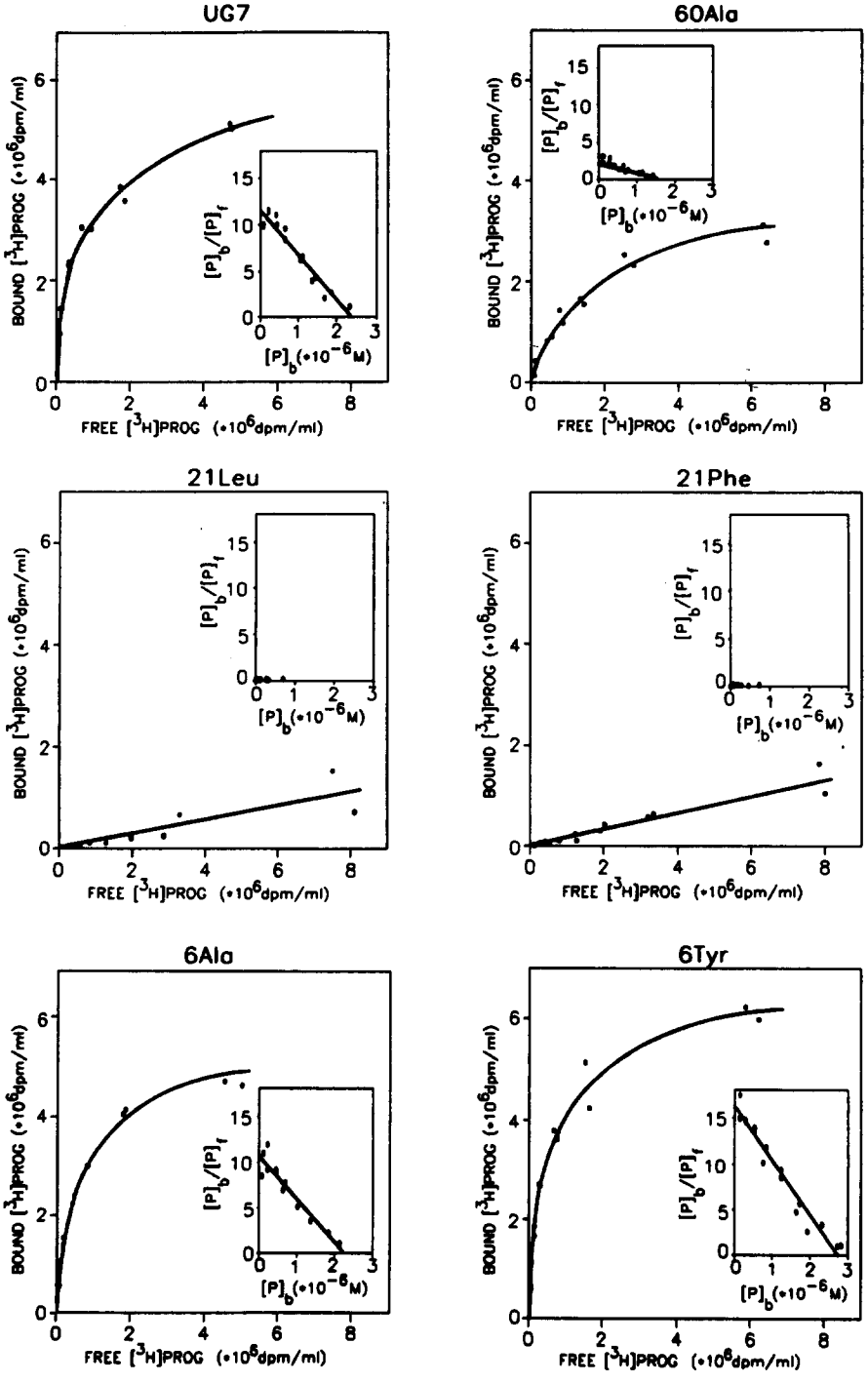

Fig. 3. Binding of [^{3}H]progesterone. Purified recombinant uteroglobin variants (UG7, ^{60}Ala, ^{21}Leu, ^{21}Phe, ^{6}Ala and ^{6}Tyr) were subjected to equilibrium dialysis with different concentrations of [^{3}H]progesterone (sp. act. 1 Ci/mmol). The inserts show Scatchard presentations of the binding data.

respectively), within the limit of the technique, equivalent to that of the natural and the unmodified recombinant proteins ($3.5 \times 10^6\ M^{-1}$ and $4.8 \times 10^6\ M^{-1}$, respectively).

In contrast, substitution of Tyr 21 and Ala and Phe (^{21}Ala, ^{21}Phe) causes drastic changes in the progesterone binding ability of recombinant uteroglobin. Increasing amounts of progesterone (from 2×10^{-7} to 1×10^{-5} M) cause a weak linear increase in binding, however, saturation of uteroglobin with progesterone is not observed. Much higher concentrations of progesterone could not be used due to the low progesterone solubility. Therefore we could not determine the affinity constant for the Tyr 21 mutants. Replacement of Thr 60 by Ala (^{60}Ala) influences binding of progesterone to uteroglobin less obviously, but nevertheless significantly. With increasing amounts of progesterone a saturation curve can be obtained and the affinity constant was determined by Scatchard plot analysis. In an average of two independent experiments a K_a of $1.5 \times 10^6\ M^{-1}$ was determined. This value is about 2–3-fold lower compared to the wild-type.

Our binding data obtained with the different uteroglobin variants suggest an important direct interaction of progesterone with Tyr 21 and Thr 60, respectively. Our experiments further support a model that has been postulated on the basis of crystallographic coordinates [4, 13]. In Fig. 4 progesterone was modeled in the central cavity of an uteroglobin dimer as described [4]. For this model a small displacement of Met 41 and Met 41′ is necessary. Four hydrogen bonds are observed: O3 is hydrogen bonded with Tyr 21 and Thr 60′ and O20 with Thr 60 and Tyr 21′.

DISCUSSION

We have used genetically engineered rabbit uteroglobin as starting material for site-directed mutagenesis. Amino acid residues which are part of the uteroglobin hydrophobic cavity surface were replaced by amino acids that did not alter the overall structure of the protein. All purified recombinant uteroglobin variants form dimers indistinguishable from the native or wild-type recombinant proteins.

Substitution of Phe 6 by Ala and Tyr had no effect on the progesterone binding properties of uteroglobin. Thus, the interaction of Phe 6 with progesterone that we observe when progesterone is modeled in the central hydrophobic pocket of the uteroglobin dimer, is not important.

In contrast, replacement of Tyr 21 and Thr 60 reduced progesterone binding. Previously it was shown that a change in the u.v. absorbance spectrum of the Tyr residue takes place upon progesterone binding and that Tyr 21 might therefore be a crucial residue for the interaction between uteroglobin and progesterone [14]. In

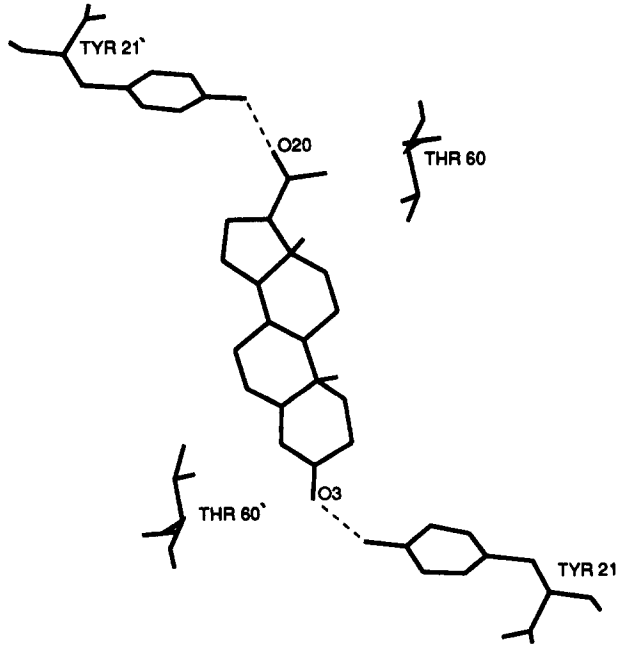

Fig. 4. Model for the interaction of uteroglobin with progesterone.

addition, a model was proposed from crystallographic data in which progesterone may be hydrogen bonded to OH of Tyr 21 and Tyr 21′ through O3 and O20, respectively [13]. Our mutational analysis strongly supports this idea as Tyr 21 substitution drastically reduces progesterone binding.

A weaker interaction seem to take place between Thr 60 and progesterone as the replacement of Thr 60 by Ala reduces the affinity for progesterone by a factor of two to three. This observation is also in agreement with progesterone binding models obtainted on the basis of the crystal structure of uteroglobin [3–5]. Like for Tyr 21 one can postulate hydrogen bonding between the OH-group of Thr 60 and 60′ and progesterone O20 and O3, respectively. The fact that the Thr 60 replacement does not alter the binding affinity as drastically as the Tyr 21 replacement may be explained by the larger distance between these atoms and the less optimal angles for the hydrogen bonds.

Recently we have cloned a rat protein homologous to rabbit uteroglobin, the 10 kDa Clara cell secretory protein, that shares similar tissue distribution [15], physico-chemical characteristics and steroid binding properties [13]. Although the cDNA-derived amino acid sequence identity to rabbit uteroglobin is only 54%, both the Tyr 21 and Thr 60 residues are conserved [15]. In contrast, in the human homologue to rabbit uteroglobin Thr 60 is replaced by a methionine [16] and the affinity for progesterone seems to be reduced (Katyal, personal communication). This observation fits well with our results.

Uteroglobin does not only bind progesterone but also certain methylsulfonyl metabolites of polychlorinated biphenyls (PCBs) with affinities at least one order of magnitude higher than progesterone [17]. Although methylsulfonyl-PCBs appear to be structurally dissimilar from steroids, it was suggested that the progesterone-binding domain of uteroglobin or even the same residues that bind steroids interact with this compound. Binding studies with ^{3}H-labeled methylsulfonyl-PCBs and the described uteroglobin variants are in progress.

Independently of the ability of uteroglobin to bind steroids and methylsulfonyl-PCBs, uteroglobin has been described as an inhibitor of phospholipase A_2 [18–20]. The specificity of this effect remains controversial [21] and an interaction of uteroglobin with phospholipase A_2 has not been shown. One possible explanation for the inhibitory activity on phospholipase A_2 could be that uteroglobin interacts with phospholipids, the substrates for PLA_2 and that the observed inhibitory effect would be unspecific. This has been suggested for lipocortins [22] that share some similarity to uteroglobin and that have been described originally also as PLA_2 inhibitors. Recombinant uteroglobin expressed in *E. coli* can exert phospholipase A_2 inhibition [23] and it is of certain interest to test the Tyr 21 and Thr 60 variants for inhibitory activity. Reduction of inhibition would point to an unspecific effect.

Tyr 21 is part of the second α-helix of uteroglobin which shows some similarity to the progesterone receptor steroid binding domain [2]. A tyrosine residue (Tyr 849) lies in that region of the human progesterone receptor and the question arises whether this residue is also directly involved in ligand binding to the progesterone receptor.

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