Use of a Sandwich Enzyme-linked Immunosorbent Assay Strategy to Study Mechanisms of G Protein-coupled Receptor Assembly*

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All G protein-coupled receptors are predicted to consist of a bundle of seven transmembrane helices (I–VII) that are connected by various extracellular and intracellular loops. At present, little is known about the molecular interactions that are critical for the proper assembly of the transmembrane receptor core. To address this issue, we took advantage of the ability of coexpressed N- and C-terminal m3 muscarinic receptor fragments to form functional receptor complexes (Schöneberg, T., Liu, J., and Wess, J. (1995) J. Biol. Chem. 270, 18000–18006). As a model system, we used two polypeptides, referred to as m3-trunk and m3-tail, that were generated by “splitting” the m3 muscarinic receptor within the third intracellular loop. We initially demonstrated, by employing a sandwich enzyme-linked immunosorbent assay strategy, that the two receptor fragments directly associate with each other when coexpressed in COS-7 cells. Additional studies with N- and C-terminal fragments derived from other G protein-coupled receptors showed that fragment association was highly receptor-specific. In subsequent experiments, the sandwich enzyme-linked immunosorbent assay system was used to identify amino acids that are required for proper fragment (receptor) assembly. Point mutations were introduced into m3-trunk or m3-tail, and the ability of these mutations to interfere with efficient fragment assembly was examined. These studies showed that three highly conserved proline residues (located in transmembrane helices V, VI, and VII) are essential for proper fragment association (receptor assembly). Interestingly, incubation with classical muscarinic agonists and antagonists or allosteric ligands led to significant increases in the efficiency of fragment association (particularly upon substitution of the conserved proline residues), indicating that all of these ligands can act as “anchors” between the m3-trunk and m3-tail fragments. The approach described here should be generally applicable to gain deeper insight into the molecular mechanisms governing G protein-coupled receptor structure and assembly.

All G protein-coupled receptors (GPCRs) are predicted to share a common three-dimensional fold consisting of seven transmembrane (TM) helices (TM I–VII) linked by alternating intracellular (i1–i3) and extracellular (o2–o4) loops (Fig. 1) (1–3). TM I–VII are thought to be sequentially arranged in a ringlike fashion, thus forming a tightly packed TM receptor core (4, 5). Residues located on the inner surfaces of different TM helices are known to be involved in the binding of a great number of ligands that act on GPCRs (2–6). Moreover, ligand-induced conformational changes in the TM receptor core are thought to be intimately involved in receptor activation (7–9). However, detailed structural information about the molecular interactions that allow TM I–VII to assemble in the proper orientation and geometry is not available at present, primarily due to the lack of high resolution structural data for any GPCR.

Several studies have shown that GPCRs, like other polytopic transmembrane proteins (10), can be assembled from two or more independently stable receptor fragments (11–19). For these studies, GPCRs were “split” in various intracellular and extracellular loops by using recombinant DNA techniques. When the resulting fragment pairs were coexpressed in cultured cells, high affinity ligand binding and ligand-dependent G protein activation were observed in several cases (11–19). The most straightforward explanation for these findings is that GPCRs consist of multiple autonomous folding domains, probably due to the ability of individual TM helices to properly fold independent of the structural context within the full-length receptor protein. Taken together, these findings strongly support the notion that the folding of GPCRs (as has been proposed for other polytopic transmembrane proteins; Ref. 10) occurs in two consecutive steps. In step I, individual TM helices are established across the lipid bilayer, which, in step II, are then assembled, by specific helix-helix interactions, to form a functional receptor protein.

Over the past few years, we have carried out studies with split m3 muscarinic (12, 14) and V2 vasopressin receptors (16) to gain deeper insight into the molecular mechanisms governing GPCR structure and assembly. In one study (14), we split the rat m3 muscarinic receptor in all three intracellular (i1–i3) and all three extracellular loops (o2–o4). Coexpression in COS-7 cells of three of the six resulting polypeptide pairs (sites of split: i2, i3, or o3) led to the appearance of a significant number of specific high affinity radioligand binding sites (14). In addition, fragment pairs generated by splits within the i3 or o3 regions still retained the ability to activate G proteins in an agonist-dependent fashion. Moreover, immunofluorescence microscopic studies using intact and permeabilized cells showed that the different receptor fragments were individually stable.
and were properly inserted (in the correct orientation) into lipid bilayers (14).

In this study, we have used two m3 muscarinic receptor fragments, referred to as m3-trunk (containing TM I–V) and m3-tail (containing TM VI and VII), as model systems to learn more about the molecular mechanisms underlying GPCR assembly. The m3-trunk and m3-tail polypeptides were generated by splitting the rat m3 muscarinic receptor within the i3 loop (Fig. 1; Refs. 12 and 14).

Initially, we established a sandwich ELISA that allowed us to monitor the association between the two m3 receptor fragments with high sensitivity. We demonstrated that the strength of the ELISA signals strictly correlated with the amount of expressed receptor fragments. In addition, by including fragments derived from other GPCRs in this analysis, we verified that fragment assembly was highly receptor-specific.

Subsequently, we used the sandwich ELISA to identify m3 receptor residues located within the TM receptor core that are required for proper receptor assembly. Toward this goal, we coexpressed m3-trunk and m3-tail polypeptides that contained point mutations within individual TM helices and studied fragment association via ELISA. These studies showed that three proline residues that are highly conserved among GPCRs of the rhodopsin family are critical for efficient fragment assembly.

Another question that we addressed was whether muscarinic ligands (agonists, antagonist, or allosteric ligands) were able to affect the interaction between the m3-trunk and m3-tail fragments. We found that most ligands were able to stabilize the interaction between the m3-trunk and m3-tail polypeptides, as evidenced by increased ELISA signals in the presence of ligands. This stabilizing effect was most pronounced when fragment association was impaired due to Pro → Ala mutations.

The approach described here provides new insights into the molecular mechanisms governing GPCR assembly and should be applicable to other classes of GPCRs. The outlined experimental strategy offers the unique opportunity to study, in a quick and reliable fashion, the structural role of essentially every amino acid within a given GPCR.

**EXPERIMENTAL PROCEDURES**

**DNA Constructs—**All mutant muscarinic receptor constructs were derived from Rm3pcD-N-HA, a mammalian expression plasmid coding for the rat m3 muscarinic receptor containing a 9-amino acid hemagglutinin (HA) epitope tag (YPYDVPDYA) at its N terminus (14). Previous studies showed that the presence of the HA tag had no significant effect on the ligand binding and G protein coupling properties of the wild type m3 receptor (14). The construction of two pc-D-based expression vectors coding for m3-trunk (rat m3 receptor residues 1–272) and m3-tail (rat m3 receptor residues 388–589) (see also Fig. 1) has been described previously (12, 14). The m3-trunk polypeptide, like the full-length m3 receptor, carried an HA epitope tag at its N terminus. To allow proper translation of the m3-tail fragment, an in-frame translation start codon was inserted immediately upstream of codon 388 (12). Point mutations that had been generated previously in Rm3pcD (20–24) were introduced into m3-trunk-pcD or m3-tail-pcD by simple subcloning procedures.

The construction of pc-D-based expression vectors coding for V2-trunk, V2-tail, and Gnr-HRH-tail has been described previously (16, 17). V2-trunk and Gnr-HRH-tail are fragments derived from the human V2 vasopressin receptor and is structurally homologous to m3-tail (16). The different receptor fragments are composed as follows (amino acid numbers are given in parentheses): V2-trunk (1–241), V2-tail (242–371), and Gnr-HRH-tail (1–284).

Since it proved difficult to reliably detect the full-length m3 receptor (Rm3-N-HA) via Western blotting analysis, a construct was generated in which the central portion of the I3 loop (amino acids Ala274–Lys469) was deleted as described previously (25). The resulting plasmid codes for a modified version of the m3 receptor, which, for the sake of simplicity, is referred to as m3(′)wt receptor. Radioligand binding and second messenger assays showed that the m3(′)wt receptor displayed ligand binding and G protein coupling properties similar to the wild type m3 receptor (25).

The identity of all mutant constructs was verified by restriction endonuclease analysis and DNA sequencing.

**Cell Culture and Transfections—**COS-7 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM t-glutamine, 100 µg/ml penicillin, and 100 units/ml streptomycin, at 37 °C in a humidified 5% CO2 incubator. For transfections, 104 cells were seeded into 100-mm dishes. Cells were transfected about 20–24 h later by using a DEAE-dextran method (26). In the case of the m3(′)wt receptor, cells were transfected with 4 µg of plasmid DNA/dish. In cotransfection experiments involving receptor fragments, 4 µg of each plasmid were used per dish, unless indicated otherwise.

**Preparation of Membrane Lysates—**Approximately 70 h after transfections, cells were washed once with phosphate-buffered saline (PBS), scraped into 1.5 ml of sterile water, collected in Eppendorf tubes, and centrifuged for 30 min at 20,000 × g (4 °C). Pellets were resuspended in 1 ml of buffer A (PBS supplemented with 0.05% Tween 20, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml pepstatin A, and 0.2% digitonin), followed by a 30-min incubation on ice and a 30-min spin at 20,000 × g (4 °C) to remove soluble as well as peripheral membrane protein fragments. Cell lysates were resuspended in 0.2 ml of a 1% Triton X-100 solution as buffer A except that the digitonin concentration was increased to 4% and 1% sodium deoxycholate was added, followed by a 3-h incubation at 4 °C (under rotation) and a 30-min centrifugation at 20,000 × g (4 °C). Supernatants containing solubilized receptors/receptor fragments were used for further experiments.

**Western Blotting Analysis—**Receptor/receptor fragment expression was monitored via immunoblotting. 12 µl of membrane lysates (prepared as described in the previous paragraph) were mixed with 3 µl of 15% SDS (final SDS concentration: 3%) and incubated for 3 h at room temperature. Subsequently, samples were mixed with Laemmli buffer (27), heated for 5 min at 65 °C, and then subjected to 10% or 15% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes via electroblotting. Membranes were blocked overnight with PBS containing 5% bovine serum albumin (BSA) and then incubated with primary antibody (12CA5 anti-HA mouse monoclonal antibody (Boehringer Mannheim) or anti-C-m3 rabbit polyclonal antibody; see Fig. 1) at a concentration of 1 µg/ml in PBS-T-BSA (PBS containing 0.05% Tween 20 and 1% BSA) for 1 h. Bound antibody was then probed with a secondary antibody conjugated to horseradish peroxidase [goat anti-mouse IgG (Amersham Pharmacia Biotech); final dilution in PBS-T-BSA: 1:3,000]. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection kit (Amersham Pharmacia Biotech). The intensities of immunoreactive bands were quantified by scanning densitometry using the program Scion Image (Scion Corp.).

**Sandwich ELISA—**COS-7 cell microtiter plates (Nunc-Immuno Plate, MaxiSorp™) were coated with the anti-C-m3 (a rabbit polyclonal antibody directed against the C-terminal segment of the rat m3 muscarinic receptor) or the anti-C-V2 antibodies (a rabbit polyclonal antibody directed against the C-terminal 29 amino acids of the human V2 vasopressin receptor; Ref. 16) by incubating plates overnight at room temperature (under shaking) with 100 µl of antibody solution/well (anti-body concentration: 5 µg/ml). Plates were then washed twice with PBS containing 0.05% Tween 20 (PBS-T; 200 µl/well), incubated with 5% BSA in PBS (200 µl/well) for 1 h at room temperature, and washed again twice with PBS-T (200 µl/well). In parallel, 50 µl of membrane lysates prepared from transfected cells as described above (containing solubilized receptors/receptor fragments) were mixed with 50 µl of V2-trunk and incubated for 1 h at 37 °C with 50 µl of the 12CA5 monoclonal antibody (final concentration: 1 µg/ml). Subsequently, samples were incubated for another 1 h at 37 °C. Samples were then added to antibody-coated microtiter plates (see above), followed by a 2-h incubation at 37 °C. After this incubation step, plates were washed again twice with PBS-T (200 µl/well). Subsequent washes were carried out at room temperature by adding H2O2 and o-phenylenediamine (2.5 mM each in 0.1 M phosphate-citrate buffer, pH 5.0) (100 µl of each reagent/well). Reactions were stopped after 30 min by the addition of 50 µl of 1 M H2SO4 containing 0.05% NaNO2. Color

2 F.-Y. Zeng and J. Wess, unpublished observations.
development was measured bichromatically at 490 and 630 nm (background) using the BioKinetics reader (EL 312, Bio Tek Instruments, Inc.).

In a subset of experiments, sandwich ELISA measurements were carried out in the presence of different classes of muscarinic ligands (agonists, antagonists, or allosteric ligands). Ligands were included during the preparation of membrane lysates and all steps of the sandwich ELISA, except for the final color reaction.

Ligand Binding Assays—Radioligand binding studies were carried out using membranes prepared from transfected cells. For the preparation of cell membranes, pellets from the first centrifugation step described under "Preparation of Membrane Lysates" were resuspended in 5 ml of binding buffer, homogenized with a polytron homogenizer (two times for 30 s), and recentrifuged for 30 min at 23,500 \( \times g \) (4 °C). The resulting pellets were resuspended in binding buffer (composition: 136 mM NaCl, 5 mM KCl, 10 mM HEPES, 5 mM sodium phosphate buffer, 1 mM MgCl\(_2\), pH 7.4). Equilibrium dissociation constants (\( K_d \)) and number of binding sites (\( B_{max} \)) were determined in saturation binding experiments using \( ^3H \)-methylscopolamine (\( ^3H \)-NMS, 79 Ci/mmol; NEN Life Science Products) as a radioligand (concentration range used: 0.05–1.5 nM). Incubations were carried out at room temperature for 1 h (volume of the incubation mixture: 0.8 ml). Bound and free radioligand were separated by filtration through Whatman GF/C glass fiber filters. Nonspecific binding was determined in the presence of 1 μM atropine. Binding data were analyzed by nonlinear regression analysis using the program Lotus 1–2–3 (IBM Corp.).

**Results**

**Use of a Sandwich ELISA to Study the Association between N- and C-terminal m3 Muscarinic Receptor Fragments**—In this study, we have investigated the interaction between an N-terminal m3 muscarinic receptor fragment (referred to as m3-trunk) and the corresponding C-terminal m3 receptor polypeptide (referred to as m3-tail). The structures of these two polypeptides that were generated by splitting the rat m3 receptor within the i3 loop are given in Fig. 1. As a full-length
receptor (highlighted in 96-well-plates were coated with a rabbit polyclonal antibody (anti-C-m3) directed against the N-terminal HA epitope tag (see Figs. 1 and 2). 2) Membrane lysates prepared from COS-7 cells cotransfected with m3-trunk and m3-tail were added to coated plates. The lysates had been preincubated with a monoclonal antibody directed against the N-terminal HA epitope tag (batched) and a secondary anti-mouse IgG antibody, linked to horseradish peroxidase (POD). 3) Peroxidase activity was determined by a simple color reaction (for details, see “Experimental Procedures”).

Non-transfected COS-7 cells were unable to bind significant amounts of the radiolabeled muscarinic antagonist, [3H]NMS. In contrast, radioligand binding studies carried out with membranes prepared from COS-7 cells cotransfected with m3-trunk and m3-tail revealed the appearance of a significant number of high affinity [3H]NMS binding sites ($K_D = 130 \pm 11 \text{ pM}$; $B_{max} = 55 \pm 4 \text{ fmol/10}^6 \text{ cells}$) (the corresponding values for m3’(wt) were: $K_D = 290 \pm 8 \text{ pM}$; $B_{max} = 134 \pm 5 \text{ fmol/10}^6 \text{ cells}$). The most straightforward explanation for this observation is that the m3-trunk and m3-tail polypeptides can directly associate with each other to form a functional receptor protein. To provide more direct evidence for this notion, we employed the sandwich ELISA strategy summarized in Fig. 2 (for further details, see “Experimental Procedures”).

The results of a typical sandwich ELISA assay are shown in Fig. 3. Cell lysates prepared from COS-7 cells that were transfected with the m3-trunk polypeptide alone gave only a very weak ELISA signal (optical density (OD) measured at 490 nm: 0.018 ± 0.002), which was similarly low as the signal found with vector-transfected cells (0.014 ± 0.002). However, lysates prepared from cells cotransfected with m3-trunk and m3-tail resulted in a 9.5-fold increase in OD readings (above background), indicative of fragment association (Fig. 3). The ELISA signal observed in the m3-trunk/m3-tail coexpression experiments amounted to approximately 70% of the signal found with lysates prepared from cells expressing the m3’(wt) receptor.

**Correlation of ELISA Signals with Receptor/Receptor Fragment Expression Levels**—We next wanted to verify that the magnitude of the ELISA signals observed in the coexpression experiments directly correlated with the amount of expressed receptor polypeptides. Toward this goal, COS-7 cells were cotransfected with increasing amounts of m3-trunk and m3-tail DNA (0.25–4 $\mu$g for each fragment). Transfection mixtures were supplemented with vector DNA (pED-FS) to keep the amount of transfected plasmid DNA constant at 8 $\mu$g. Analogous experiments were carried out with the m3’(wt) receptor.

Initially, protein expression was studied via Western blotting analysis (Fig. 4). For these studies, the m3-trunk and m3-tail fragments were detected with the 12CA5 anti-HA monoclonal and the anti-C-m3 polyclonal antibodies, respectively (expression of the m3’(wt) receptor was probed with both antibodies). All polypeptides yielded immunoreactive bands of the expected molecular mass (Fig. 4). To quantitate the intensities of these bands, blots were subjected to scanning densitometry. The results of these measurements are summarized in Fig. 4, in which protein expression levels are expressed in arbitrary units. Fig. 4 illustrates that the expression levels of m3-trunk and m3-tail as well as of m3’(wt) increased in an almost linear fashion with the amount of transfected receptor/polypeptide DNA.

Similarly, OD values determined via sandwich ELISA strictly correlated with the amount of transfected receptor/
fragment DNA, as found with m3(wt)-expressing cells as well as cells cotransfected with m3-trunk and m3-tail (Fig. 5A). Transfection mixtures were supplemented with vector DNA to keep the amount of transfected plasmid DNA constant at 4 μg (m3(wt)) or 8 μg (m3-trunk/m3-tail), respectively. Immunoblotting experiments were carried out as described under “Experimental Procedures.” Membrane proteins were separated by 10% (A) or 15% (B) SDS-PAGE, followed by Western blotting and scanning densitometry of immunoreactive bands (see insets). A, the full-length receptor (m3(wt); filled circles) and the m3-trunk fragment (open circles) were visualized using the 12CA5 anti-HA monoclonal antibody. B, the m3-tail polypeptide (open circles) and the m3(wt) receptor (filled circles) were detected with the anti-C-m3 polyclonal antibody. The estimated molecular masses of the immunoreactive bands were (in kDa): m3(wt) (48–52), m3-trunk (26–28), and m3-tail (18–20). The presented data are taken from one representative experiment; two additional experiments gave similar results.

FIG. 4. Correlation between m3 receptor fragment expression levels and amount of transfected plasmid DNA as studied via Western blot analysis. COS-7 cells were transfected with increasing amounts of m3(wt) (0.25–4 μg) (filled circles) or cotransfected with m3-trunk and m3-tail DNA (0.25–4 μg for each fragment) (open circles). Transfection mixtures were supplemented with vector DNA to keep the amount of transfected plasmid DNA constant at 4 μg (m3(wt)) or 8 μg (m3-trunk/m3-tail), respectively. Immunoblotting experiments were carried out as described under “Experimental Procedures.” Membrane proteins were separated by 10% (A) or 15% (B) SDS-PAGE, followed by Western blotting and scanning densitometry of immunoreactive bands (see insets). A, the full-length receptor (m3(wt); filled circles) and the m3-trunk fragment (open circles) were visualized using the 12CA5 anti-HA monoclonal antibody. B, the m3-tail polypeptide (open circles) and the m3(wt) receptor (filled circles) were detected with the anti-C-m3 polyclonal antibody. The estimated molecular masses of the immunoreactive bands were (in kDa): m3(wt) (48–52), m3-trunk (26–28), and m3-tail (18–20). The presented data are taken from one representative experiment; two additional experiments gave similar results.

FIG. 5. Assembly of m3 receptor fragments studied via sandwich ELISA and radioligand binding studies. COS-7 cells were transfected with increasing amounts of m3(wt) (filled circles) or cotransfected with m3-trunk and m3-tail DNA (open circles), as described in the legend to Fig. 4. A, sandwich ELISA measurements were carried out as described under “Experimental Procedures” (see also Fig. 2). OD readings were taken at 490 nm. Data are presented as means (S.E. < 5% for all data points) of three independent experiments, each performed in triplicate. B, B_{max} values were determined in [3H]NMS saturation binding studies as described under “Experimental Procedures.” Data are presented as means (S.E. < 5% for all data points) of three or four independent experiments, each performed in triplicate.

terminus. For this first set of experiments, ELISA plates coated with the anti-C-m3 antibody were used. As shown in Fig. 3, coexpression of m3-tail with V2-trunk or GnRH-trunk led to marked reductions in OD readings (OD = 0.040 ± 0.002 and 0.043 ± 0.003, respectively) in the sandwich ELISA, as compared with cells coexpressing m3-trunk and m3-tail. Interestingly, when the muscarinic agonist carbachol (1 mM) was added to lysates prepared from cells cotransfected with m3-trunk and m3-tail, a small (about 10%) but significant (p < 0.01) increase in ELISA signals was observed (Fig. 3). This effect was not observed in the presence of the vasopressin receptor agonist, arginine-vasopressin (AVP; 10 μM). Similarly, addition of carbachol or AVP to lysates prepared from cells coexpressing m3-tail and V2-trunk (or GnRH-trunk) did not lead to a significant increase in OD readings (Fig. 3).

To exclude the possibility that the pronounced reduction in ELISA signals observed with cells coexpressing m3-tail and V2-trunk (or GnRH-trunk) was due to poor expression of V2-trunk (as compared with m3-trunk) and to further examine the
Importance of Conserved Proline Residues for \textit{m}3 Muscarinic Receptor Assembly—It is generally believed (though not proven directly experimentally) that some of the residues that are highly conserved among GPCRs are important for proper receptor assembly. We therefore speculated that mutational modification of these residues should interfere with the efficient association between N- and C-terminal receptor fragments, as probed via sandwich ELISA.

To test this hypothesis, we initially focused our attention on a set of three proline residues that are present not only in the \textit{m}3 muscarinic receptor but in almost all GPCRs of the rhodopsin family (4, 5). As shown in Fig. 1, these proline residues are located in TM V (Pro$^{242}$), TM VI (Pro$^{505}$), and TM VII (Pro$^{540}$). We first created three \textit{m}3 receptor fragments in which these proline residues were individually replaced with alanine, resulting in P242A-m3-trunk, P505A-m3-tail, and P540A-m3-tail. We then coexpressed these mutant fragments with m3-tail (P242A-m3-trunk) or m3-trunk (P505A-m3-tail and P540A-m3-tail) and monitored fragment association via ELISA. As outlined above, lysates prepared from cells coexpressing the m3-trunk and m3-tail polypeptides resulted in a robust increase in OD readings (9.5-fold above values determined for cells expressing m3-trunk alone; Fig. 7). In contrast, ELISA signals were found to be drastically reduced when one of the cotransfected receptor fragments contained a Pro$\rightarrow$ Ala point mutation. As shown in Fig. 7, the remaining responses were only about 2-fold greater than background values.

Radioligand binding studies yielded results that were in good agreement with the ELISA data. Fig. 8 shows that the number of detectable $[^3]$H]NMS binding sites ($B_{\text{max}}$) was strongly reduced (by 3.3–8.7-fold, as compared with m3-trunk/m3-tail-expressing cells) in coexpression experiments that included fragments containing a Pro$\rightarrow$ Ala mutation. These remaining $[^3]$H]NMS binding sites were characterized by $K_D$ values similar to those obtained with cells cotransfected with m3-trunk and m3-tail (m3-trunk/m3-tail, 130 $\pm$ 11 pm; m3-trunk/P505A-m3-tail, 171 $\pm$ 16 pm; m3-trunk/P540A-m3-tail, 148 $\pm$ 13 pm; P242A-m3-trunk/m3-tail, 205 $\pm$ 23 pm).

In order to exclude the possibility that the reduced signals seen in the coexpression experiments that included \textit{m}3 receptor fragments containing Pro$\rightarrow$ Ala point mutations were due to lowered expression of the mutant polypeptides, expression of the different mutant \textit{m}3 receptor fragments was monitored via Western blotting analysis. Polypeptides were visualized by immunoblotting using either the 12CA5 anti-HA monoclonal antibody (m3-trunk fragments) or the anti-C-m3 polyclonal antibody (m3-tail fragments). As shown in Fig. 9, all polypeptides yielded immunoreactive bands of the expected molecular mass. Moreover, the receptor fragments containing the indicated Pro$\rightarrow$ Ala mutations were found to be expressed at similar levels as the corresponding non-mutated fragments (Fig. 9). Scanning densitometry of the immunoreactive bands revealed no significant differences in the expression levels of the mutated versus the non-mutated receptor polypeptides (data not shown).

Effect of Ligands on the Assembly of \textit{m}3 Receptor Fragments

![Figure 7](image7.png)

![Figure 6](image6.png)
**Muscarinic Receptor Assembly**

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**Fig. 8.** [H]NMS binding studies using cells coexpressing m3 receptor fragments carrying Pro → Ala point mutations. COS-7 cells were cotransfected with the indicated m3-trunk and m3-tail fragments (4 μg of DNA/plate/plasmid). $B_{\text{max}}$ values were determined in [H]NMS saturation binding studies as described under “Experimental Procedures.” Data are presented as means ± S.E. of three independent experiments, each performed in triplicate.

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**Fig. 9.** Expression of m3 receptor fragments carrying Pro → Ala point mutations studied via Western blotting. COS-7 cells were cotransfected with the following plasmids (4 μg each): 1 (m3’(wt)), 2 (m3-trunk + m3-tail), 3 (m3-trunk + P540A-m3-tail), 4 (m3-trunk + P505A-m3-tail), 5 (P242A-m3-trunk + m3-tail), 6 (m3-tail), and 7 (empty vector; pcD-PS). Membrane extracts were prepared about 70 h later and subjected to 10% (A) or 15% (B) SDS-PAGE. Polypeptides were visualized via immunoblotting using the 12CA5 anti-HA monoclonal antibody (A) or the anti-C-m3 polyclonal antibody (B) (the receptor epitopes recognized by the two antibodies are indicated in Fig. 1). Protein molecular size standards (in kDa) are indicated. Two additional experiments gave similar results.

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**Fig. 10.** Interaction of coexpressed mutant m3 receptor fragments studied via ELISA. COS-7 cells were cotransfected with non-mutated m3-trunk and m3-tail fragments (A, control) or the indicated mutant versions of m3-tail and m3-trunk (B–D) (4 μg of DNA/plate/plasmid). Sandwich ELISA measurements were carried out as described under “Experimental Procedures” (using 96-well plates coated with the anti-C-m3 polyclonal antibody), either in the presence or absence of the agonist carbachol (1 mM) or the antagonist NMS (0.1 mM). Three types of mutations were studied: B, mutations known to disrupt receptor/G protein interactions in the full-length m3 receptor (in I253Y/Y254I-m3-tail, the positions of Ile$^{253}$ and Tyr$^{254}$ were reversed (23); in T231A-m3-trunk, an extra alanine residue was inserted directly after Ser$^{493}$ (24)); C, point mutations that reduce agonist binding affinities in the full-length m3 receptor (20, 21); D, point mutations that have no effect on the ligand binding and functional properties of the full-length receptor (20). Data are presented as means ± S.E. of three or four independent experiments, each carried out in quadruplicate. *, $p < 0.01$ (paired t test).

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**Controlling Pro → Ala Mutations**—We next wanted to examine whether ligands were able to promote fragment association in coexpression experiments that included m3 receptor fragments containing Pro → Ala point mutations. For these studies, four different types of ligands were used: the full agonist carbachol (1 mM), the partial agonist tetramethylammonium (TMA, 10 mM), the antagonists NMS (0.1 μM) and QNB (0.1 μM), and the allosteric ligands iberiotoxin (0.1 mM), gillamine (10 μM), and eburnamonine (0.1 mM; Ref. 28). When added to lysates prepared from cells cotransfected with m3-trunk and m3-tail, all ligands, except TMA, gave small (approximately 10%) but significant ($p < 0.01$) increases in OD readings in the sandwich ELISA (Fig. 7). Interestingly, this ligand-dependent increase in ELISA signals was greatly enhanced when one of the two cotransfected m3 receptor fragments contained a Pro → Ala point mutation (P242A, P505A, or P540A). In this case, all ligands (except TMA) led to OD readings that, when corrected for background “noise” (determined with m3-trunk-expressing cells), were increased by about 300–500%, as compared with the values determined in the absence of ligands (Fig. 7).

**Effect of Other Point Mutations on the Assembly of N- and C-terminal m3 Receptor Fragments**—To examine the specificity of the detrimental effects of the different Pro → Ala point mutations on proper fragment assembly, we next introduced a variety of other point mutations into either m3-trunk or m3-tail. Subsequently, we measured the ability of the resulting mutant polypeptides to associate with m3-tail (in the case of mutant m3-trunk fragments) or m3-trunk (in the case of mutant m3-tail fragments) via sandwich ELISA. Three different types of mutations were included in this analysis, based on their effects on the function of the full-length m3 receptor. Two of the mutations (I253Y/Y254I and 493+1A-m3-trunk) were found to completely disrupt m3 receptor/G protein coupling while...
having little effect on ligand binding (23, 24). The second group comprises mutations that are known to reduce agonist binding affinities but still allow receptor/G protein coupling (Y148F, T231A, T234A, Y506F, Y529F, and Y533F; Refs. 20 and 21). Finally, two additional point mutations (T502A and T537A) were included (for control purposes) that have virtually no effect on the ligand binding and functional properties of the full-length m3 receptor (20).

To examine the effect of the different mutations on m3 receptor assembly, sandwich ELISA measurements were carried out using lysates prepared from cells coexpressing a mutant m3-trunk or m3-tail polypeptide and the corresponding non-mutated N- or C-terminal m3 receptor fragment. These studies showed that the majority of the mutations had little or no effect on proper fragment assembly (Fig. 10). All mutant fragments (with the exception of Y529F- and Y533F-m3-tail, which led to 32% and 45% reductions in ELISA signals, respectively) gave OD readings that were similar to those found with lysates prepared from m3-trunk/m3-tail-expressing cells. Moreover, addition of the agonist, carbachol (1 mM), or the antagonist, NMS (0.1 μM), led to small increases in ELISA signals (by approximately 10%; p < 0.01) in all coexpression experiments (again with the exception of Y529F- and Y533F-m3-tail) (Fig. 10).

**DISCUSSION**

Studies with split GPCRs have shown that GPCRs can be assembled from two or more coexpressed receptor fragments (11–19). In this study, we have examined the association between two m3 muscarinic receptor fragments, referred to as m3-trunk and m3-tail (Fig. 1), as a model system. Consistent with previous studies (12, 14), coexpression of these two polypeptides in COS-7 cells resulted in the “reconstitution” of a significant number of high affinity binding sites for the muscarinic radioligand, [3H]NMS.

To examine the interaction between the m3-trunk and m3-tail fragments in a more direct fashion, we employed a sandwich ELISA using three different antibodies. The first antibody, a rabbit polyclonal antibody referred to as anti-C-m3, was used for the coating of microtiter plates and allowed the capture of the m3-tail fragment from cell lysates prepared from cotransfected COS-7 cells. The second antibody was a mouse monoclonal antibody (12CA5) that was able to detect the HA epitope tag that had been added to the N terminus of the m3-trunk polypeptide. Addition of the third antibody, an antihuman IgG antibody linked to horseradish peroxidase, allowed the detection of the m3-trunk polypeptide by a simple enzymatic reaction.

Cell lysates prepared from cells transfected with the m3-trunk polypeptide or vector DNA alone gave only residual ELISA signals (OD measured at 490 nm). However, a robust increase (9.5-fold) in the magnitude of ELISA signals was observed when cells coexpressing m3-trunk and m3-tail were analyzed (Fig. 3). These observations indicate that the employed sandwich ELISA provides a sensitive and convenient experimental system to study the association between the m3-trunk and m3-tail fragments.

To further investigate the potential usefulness of the sandwich ELISA for studying mechanisms of GPCR assembly, we next examined whether the strengths of the ELISA signals correlated with actual amounts of coexpressed receptor fragments. For these studies, COS-7 cells were transfected with increasing amounts of m3-trunk and m3-tail DNA, followed by the preparation of cell lysates and Western blotting analysis to monitor polypeptide expression levels. These studies showed that increasing the amount of transfected fragment DNA led to almost linear increases in the expression levels of the m3-trunk and m3-tail fragments (Fig. 4).

Sandwich ELISA measurements showed that the observed increases in polypeptide expression levels led to gradual increases in the number of detectable m3-trunk/m3-tail complexes (Fig. 5A). A similar correlation was also found in [3H]NMS saturation binding studies (Fig. 5B). Taken together, these findings indicate that the sandwich ELISA employed here provides a highly sensitive method to monitor the efficiency of fragment (GPCR) assembly.

Another question that we addressed in a set of initial experiments was related to the receptor specificity of fragment assembly. For these studies, COS-7 cells were cotransfected with the m3-tail polypeptide and two different N-terminal GPCR fragments referred to as V2-trunk and GnRH-trunk. These latter two fragments were derived from the human V2 vasopressin (16) and GnRH receptors (17), respectively, and are structurally homologous to m3-trunk (note that all “trunk fragments” contained an N-terminal HA tag). ELISA measurements showed that the m3-tail polypeptide was unable to interact with V2-trunk and GnRH-trunk in an efficient manner (Fig. 6). To exclude the possibility that the low ELISA signals seen in these experiments was due to reduced expression levels of V2-trunk (or GnRH-trunk), measurements were also carried out with cells coexpressing V2-trunk and V2-tail (a fragment that is structurally homologous to m3-tail; Ref. 16). For these experiments, plates were coated with a rabbit polyclonal antibody (anti-C-V2; Ref. 16) directed against the C terminus of the V2 receptor. These studies demonstrated that V2-trunk and V2-tail were able to interact with each other with high efficiency. In contrast, ELISA signals were very weak when cells cotransfected with V2-tail and m3-trunk or GnRH-trunk were analyzed. In sum, these findings convincingly show that efficient association of receptor fragments is highly receptor-specific.

Currently, little is known about the structural mechanisms required to maintain GPCRs in a functionally competent fold. We therefore decided to use the sandwich ELISA outlined above to identify amino acids in the m3 muscarinic receptor that are critical for proper receptor assembly. Initially, we focused our attention on a set of three conserved proline residues (Pro242, Pro505, and Pro540) that are present not only in the m3 muscarinic receptor but in almost all GPCRs of the rhodopsin family (4, 5). A previous study (22) had shown that individual replacement of these prolines with alanine residues in the full-length m3 receptor resulted in a pronounced reduction in specific radioligand binding sites (Bmax). Based on these results, we speculated that the three conserved proline residues might be required for the proper assembly of the transmembrane receptor core.

To test this hypothesis, mutant m3-tail or m3-trunk fragments were created in which the three conserved prolines were individually substituted with alanine residues. Cell lysates prepared from cells cotransfected with a mutant form of m3-trunk and the non-mutated m3-tail polypeptide (or vice versa) were then analyzed via sandwich ELISA. These studies showed that each of the three Pro → Ala mutations led to pronounced reductions in ELISA signals (by 6–19-fold as compared with cells cotransfected with non-mutated m3-trunk and m3-tail fragments) (Fig. 7). This decrease in the magnitude of ELISA signals was accompanied by strong reductions in the number of detectable radioligand binding sites (Bmax) (Fig. 8). Western analysis indicated that the polypeptides containing the different Pro → Ala point mutations were expressed at similar levels as the non-mutated m3-trunk and m3-tail fragments (Fig. 9). Taken together, these results provide the first direct evidence
that the three conserved proline residues play key roles in m3 muscarinic receptor assembly.

Based on the unique structure and geometry of the proline side chain, proline residues are predicted to introduce “kinks” into TM helices, resulting in an altered direction and/or orientation of the helical backbones (29–31). It is therefore likely that the mutated proline residues (Pro242, Pro905, and Pro450) do not directly participate in establishing interhelical contact sites but that their mutational modification results in impaired helix-helix interactions due to indirect structural effects.

To examine the specificity of the detrimental effects of the different Pro→Ala point mutations on the interaction between m3-trunk and m3-tail, we subsequently screened a larger number of m3 receptor point mutations for their ability to interfere with proper fragment assembly. These mutations were chosen based on their known effects on the function of the full-length m3 receptor (20–24). As expected, mutations that had no effect on the ligand binding and G protein-coupling properties of the wild type receptor (T502A and T537A) also did not interfere with the proper assembly between m3-trunk and m3-tail fragments (as studied via ELISA; Fig. 10). Similar results were obtained when fragments were used for coexpression studies that contained mutations known to selectively disrupt receptor/G protein coupling (1253Y/T2541 and 493+1A; for details regarding the structure of these mutant receptors, see Fig. 1 and Refs. 23 and 24). This latter observation excludes the possibility that fragment assembly requires association of the fragment complex with G proteins. Finally, we examined the effects of point mutations known to selectively reduce the binding affinities of the neurotransmitter acetylcholine and other muscarinic agonists (Y148F, T231A, T234A, Y506F, Y529F, and Y533F; Refs. 20 and 21). ELISA measurements showed that these mutations (except for Y529F and Y533F, which caused 32% and 45% reductions in OD readings, respectively), had little or no effect on the efficiency of assembly between the m3-trunk and m3-tail polypeptides (Fig. 10), indicating that these mutations do not interfere with the stable assembly of the TM receptor core. This observation is consistent with the view that these residues are predicted to project into the interior of the TM helical bundle (where they can interact with agonist ligands) and are not engaged in direct helix-helix interactions (4, 5, 20, 21). In sum, these studies strongly support the notion that the ability of the P242A, P505A, and P540A substitutions to interfere with m3 muscarinic receptor assembly is highly specific for these mutations. More generally, our data indicate that the sandwich ELISA employed here can be used to assess whether or not a specific residue plays a critical role in proper receptor assembly.

In another set of experiments, we examined the effect of different classes of muscarinic ligands (agonists, antagonists, or allosteric ligands) on the efficiency of association between the m3-trunk and m3-tail polypeptides. Sandwich ELISA measurements using cells cotransfected with m3-trunk and m3-tail showed that all ligands investigated (except TMA, which left ELISA signals virtually unaffected) led to small but significant increase in OD readings (by approximately 10%). This effect was considerably more pronounced (increase in specific ELISA signals by approximately 300–500%) when one of the cotransfected fragments carried a Pro→Ala mutation (see above) that were shown to interfere with efficient fragment assembly (Fig. 7). A likely explanation for this observation is that muscarinic ligands can act as anchors to stabilize fragment association by contacting residues that are located on both the m3-trunk and m3-tail fragments and that this stabilizing effect becomes more pronounced when receptor assembly is impaired due to “destabilizing” mutations. This view is consistent with a great number of mutagenesis studies suggesting that classical muscarinic agonists and antagonists bind to their target receptors by contacting many different amino acids located on different TM domains (primarily TM III, V, VI, and VII; see Ref. 32 for a review).

Interestingly, allosteric ligands such as alcuronium (33, 34), gallamine (35, 36), and eburnamonine (28) promoted m3 receptor fragment assembly in a fashion similar to classical muscarinic agonists and antagonists. Such ligands can modulate the binding of classical muscarinic agents to the primary muscarinic binding site (located within the TM helical bundle) via interaction with a secondary (allosteric) binding site (37). This allosteric site is predicted to be located “extracellularly” of the primary (classical) ligand recognition site and is thought to involve residues located on the second and third extracellular loops and exofacial portions of adjacent TM helices (38–40). Our observation that all studied allosteric ligands were able to facilitate the interaction between the m3-trunk and m3-tail polypeptides, especially when fragment association was impaired due to Pro→Ala mutations, provides more direct evidence for the notion that multiple extracellular epitopes are involved in the recognition of this class of compounds.

It should be noted that the partial muscarinic agonist TMA, a rather small positively charged ammonium compound, failed to promote the interaction between the m3-trunk and m3-tail polypeptides, even in the presence of the different Pro→Ala point mutations. Mutagenesis studies (21) suggest that TMA primarily interacts with a conserved TM III aspartate residue on the m3 receptor protein (Asp447 in m3-trunk; Fig. 1) and that TMA binding does not critically depend on residues located on TM VI and VII (which are contained in m3-tail). This observation provides an explanation for the inability of TMA to stabilize the interaction between the m3-trunk and m3-tail fragments.

In conclusion, our results provide novel insight into the structural basis of GPCR assembly. Given the fact that all GPCRs share a similar molecular architecture, the experimental approach outlined here should be generally applicable.

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