Probing the Structure and Function of the Tachykinin Neurokinin-2 Receptor through Biosynthetic Incorporation of Fluorescent Amino Acids at Specific Sites*

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A general method for understanding the mechanisms of ligand recognition and activation of G protein-coupled receptors has been developed. A study of ligand-receptor interactions in the prototypic seven-transmembrane neurokinin-2 receptor (NK2) using this fluorescence-based approach is presented. A fluorescent unnatural amino acid was introduced at known sites into NK2 by suppression of UAG nonsense codons with the aid of a chemically misacylated synthetic tRNA specifically designed for the incorporation of unnatural amino acids during heterologous expression in Xenopus oocytes. Fluorescence-labeled NK2 mutants containing an unique 3-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-2,3-diaminopropionic acid (NBD-Dap) residue at either site 103, in the first extracellular loop, or 248, in the third cytoplasmic loop, were functionally active. The fluorescent NK2 mutants were investigated by microspectrofluorimetry in a native membrane environment. Intermolecular distances were determined by measuring the fluorescence resonance energy transfer (FRET) between the fluorescent unnatural amino acid and a fluorescently labeled NK2 heptapeptide antagonist. These distances, calculated by the theory of Förster, permit to fix the ligand in space and define the structure of the receptor in a molecular model for NK2 ligand-receptor interactions. Our data are the first report of the incorporation of a fluorescent unnatural amino acid into a membrane protein in intact cells by the method of nonsense codon suppression, as well as the first measurement of experimental distances between a G protein-coupled receptor and its ligand by FRET. The method presented here can be generally applied to the analysis of spatial relationships in integral membrane proteins such as receptors or channels.

Neurokinin (tachykinin)-2 receptor (NK2)1 is a member of the G protein-coupled receptors family (1). This class of receptors comprises integral membrane proteins that transmit signals into cells in response to a variety of extracellular stimuli such as hormones of a variety of structures, from monoamines to large peptides, and even light or ions. The particular receptors are predicted to have seven transmembrane regions and are believed to interchange between a number of different conformations that can selectively bind agonist or antagonist ligands and activate G proteins with important functional consequences (2, 3). G protein-coupled receptors exploit diverse strategies for ligand recognition, using either the transmembrane domain, the extracellular surface, or even the N-terminal segment. Detailed structural information is important to the understanding of the mechanisms of ligand recognition and activation of G protein-coupled receptors and in the design of therapeutic agents. Obtaining insight into the three-dimensional structure of these receptors represents a formidable task because of the difficulties of obtaining and purifying sufficiently large amounts of protein to produce crystals for high resolution x-ray or electron diffraction. Novel biochemical and biophysical methods are needed not only to overcome the hurdle to explore the molecular architecture of receptors (4, 5) but also for investigating the dynamics of their interaction with ligands.

One powerful approach is the investigation of extrinsic fluorophores incorporated at known sites in the receptor or ligand by fluorescence spectroscopy. Fluorescent labels serve as sensitive indicators of the microenvironment at a given residue as well as structural changes in receptor (6–8). Time-resolved techniques will permit the study of the structural changes to which a given residue might be submitted during receptor activation in a native membrane environment. Finally, fluorescence resonance energy transfer between a donor and acceptor pair located in the receptor and the ligand will permit intermolecular distances between these labels to be estimated.

Introducing fluorescent groups at specific sites in G protein-coupled receptors presents a major challenge. Classical methods such as solid-phase synthesis or chemical modification of reactive side chains have been used in vitro to incorporate unnatural biophysical probes into proteins but few of these approaches are suitable for large membrane proteins (9). In the recent years, expansion of the genetic code by suppression of the nonsense stop codon UAG has been developed for the site-directed incorporation of a variety of unnatural amino acids with novel properties into proteins but few of these approaches are suitable for large membrane proteins (9). In the recent years, expansion of the genetic code by suppression of the nonsense stop codon UAG has been developed for the site-directed incorporation of a variety of unnatural amino acids with novel properties into proteins in vitro in cell-free systems (10–12). Only recently has this been extended to heterologous expression of membrane proteins in an intact cell system: the Xenopus oocyte (13).

Here we report a novel biophysical approach for understanding the structure and function of a representative G protein-
coupled receptor, the tachykinin NK2 receptor. This approach combines the biosynthetic incorporation of extrinsic fluorescent reporter groups into proteins in intact cells with the widely employed technique of fluorescence resonance energy transfer (FRET) (14, 15) for distance measurements. Here we use these experimentally determined distances to build a model for the interaction of a peptide ligand with NK2 in a native membrane environment.

**EXPERIMENTAL PROCEDURES**

**Materials—**SP6 polymerase and T7 polymerase were from Promega (Madison, WI). FokI and BstNI endonucleases were from New England Biolabs (Beverly, MA). Chemicals used in buffers were from Sigma.

**Preparation of DNA Constructs—**To prepare the nulmin30(22) plasmid, the nulmin30 polymerase initiation site, in a transcription vector (pGEMNK2) (18). The plasmid pUCTRNA1 was linearized by BstNI and used as a template for the run-off transcription with T7 RNA polymerase to yield approximately 300 μg of capped mRNA from 100 μg of template. Plasmid pUCTRNA2 encoding yeast suppressor tRNA was prepared according to the procedure described previously (6). Sequences prepared from all the constructs were sequenced.

**Oocytes—**Oocytes were obtained from the ovaries of mature New York White rabbits and were aspirated by glass micropipettes. Whole cell voltage-clamp recordings were made from the isolated oocytes using a conventional two-microelectrode whole-cell configuration. All solutions contained 145 mM NaCl, 1.8 mM CaCl₂, 10 mM HEPES-KOH (pH 7.4, 295 mOsm), and were continuously gassed with 95% O₂-5% CO₂.

**Radioligand Binding Assays—**Membranes from 5 oocytes (about 50 μg of protein) were used in 0.25 ml of reaction volume. Saturation binding experiments with NBD-labeled peptides were performed in 100 ng of suppressor RNA, and incubated for 24 h at 20°C. Oocyte membrane fractions were prepared as described previously in Nemeth and Chollet (24). Electrophysiological recordings were carried out as described in Nemeth and Chollet (24).

**Fluorescence Measurements—**An argon gas laser beam (514 nm, 10 milliwatts) was focused to the sample by a Zeiss Achroplan 10/0.25 microscope objective, then directed by fiber optics to the entrance slit of a monochromator (Jobin Yvon-CPI40). Whole fluorescence spectra were detected by a CCD camera (model 576 UV, Princeton Instruments) linked to a computer (Apple, Mac Spectrum ST 135 software, Rhea Corporation) for data collection and analysis.

**FRET—**The distance (R) between the NBD and tetramethylrhodamine (TMR) groups was calculated by Förster’s equation: R₃₃₃ = K₃₃₃(λ₅₆₅) / Φ₃₃₃(λ₅₆₅), where R₃₃₃ is the distance for 50% transfer efficiency. Φ₃₃₃ was calculated from R₃₃₃ = 9.786 × 10⁻⁶ (λᵣ₃₃₃ / Φᵣ₃₃₃) / D₃₃₃, where λᵣ₃₃₃ is the wavelength of the donor and λ₃₃₃ is the wavelength of the acceptor where

**Chromatography—**Chromatography was performed on a Vydac C₄ (24×250 mm, 5 μm) column eluted at a flow rate of 1 ml/min, gradient of acetonitrile in 100 mM triethylammonium acetate, pH 7.0.

**Acetylation—**Acetylation was performed by acetylation for 1 h with a solution of acetic anhydride (50 μl) and dimethylformamide (200 μl) at room temperature. The resulting deprotected acyl-tRNA was dried in vacuo for 5 min and stored at −20°C.

**Small Angle X-ray Scattering—**Small angle X-ray scattering measurements were carried out on a wavelength of 1.4 Å at 298 K with 180 watts, power 115 watts (P/N: 61041040).

**RESULTS—**The yield was approximately 10 μg of capped mRNA from 10 μg of plasmid DNA. The mRNA was used directly for injection into oocytes.

**Construction of Suppressor RNA—**Suppressor RNA was prepared by T7 polymerase run-off transcription from a linearized plasmid template by a modification of the method of Noren et al. (22). A 120-base pair synthetic template fragment encoding yeast suppressor tRNA was prepared by annealing and ligation of four synthetic oligonucleotides. This fragment contains KpnI and HindIII sticky ends, a T7 promoter site, and two nucleotides upstream from the 3'-end of the yeast tRNA and a BstNI site that cut at the 3'-end of the T7RNA. This 120-base pair fragment was subcloned into KpnI and HindIII sites of pUC18. The new plasmid pUCRNA1 was checked by polymerase chain reaction of the cloned fragment, sequencing, and restriction mapping. pUCRNA1 was linearized by FokI and BstNI and used as a template for the run-off transcription with T7 RNA polymerase to yield approximately 300 μg of tetramethylrhodamine (TMR) constructs was calculated to be 0.74 for proteins.

**Chemical Acylation of Suppressor RNA—**A representative protocol for the chemical acylation of SuppressRNA with NBD-Dap was performed. T7 RNA polymerase was used to transcribe a linearized plasmid encoding NBD-labeled RNA from the suppressor site. The products were purified by high performance liquid chromatography (column Vydac C₄, 214TP54, 1 ml/min, gradient of acetonitrile in 100 mM triethylammonium acetate, pH 7.0, and lyophilized.

**Characterization of NBD-labeled RNA—**Characterization of NBD-labeled RNA was carried out by small angle X-ray scattering (SAXS) measurements.

**Conclusions—**The results of this study indicate that the NBD-labeled RNA can be used as a probe for the structural characterization of the NK2 receptor and its mutants.

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Structure and Function of NK2 Receptor by Fluorescence

RESULTS

Design and Construction of a Suppressor tRNA—An UAG nonsense suppressor tRNA (Sup-tRNA, Fig. 1) was designed, constructed, and used for the introduction of unnatural amino acids in membrane receptor proteins expressed in Xenopus oocytes. Sup-tRNA was derived from yeast tRNA<sup>Phc</sup> by changing the anticodon to CUA to recognize UAG nonsense codon (28) and by introducing G20U and A73G mutations (29). Replacement of G at position 20 in the D stem loop by U was introduced to reduce the rate of aminoaacylation by Phe-tRNA synthetase, thus preventing the introduction of natural amino acid at the UAG site. Mutation of the discriminator base at position 73 from A to G was made to further decrease the ability of Xenopus endogenous tRNA synthetases to reacylate Sup-tRNA. These changes were introduced by redesigning a vector described by Noren et al. (22) that allows for the synthesis of tRNA of defined length by run-off transcription. Both the 76-nucleotide full-length Sup-tRNA and a 74-nucleotide truncated form lacking the terminal pCpA in the 3′-end were used for the incorporation of unnatural amino acids in membrane receptor proteins expressed in Xenopus oocytes. 

control of the fluorescence of the donor in the presence and absence of the acceptor, respectively.

Molecular Modeling—The model was built using Promod (25) and automated modeling technique as described previously (33). This method has been shown to produce bacteriorhodopsin models in which Cu atom positions differed from the experimental EM structure by only 1.9 Å root mean square deviation. The sequences of the seven helices were determined by multiple sequence alignment of the NK2 receptor with other members of the G protein-coupled receptor family. The relative positions and orientation of the helices were derived from the study of Baldwin (26) and were based on the projection map of rhodopsin determined by electron diffraction (27).

![Fig. 1. Structure of suppressor tRNA aminoacylated with NBD-Dap. Cloverleaf structure of the semi-synthetic UAG suppressor tRNA derived from yeast tRNA<sup>Phc</sup> and chemically aminoacylated with the fluorescent amino acid analogue NBD-Dap (Sup-tRNA-NBD). Circles indicate mutations that reduce the rate of acylation by tRNA synthetase. The box indicates the anticodon.](image)

![Fig. 2. Functional characterization of NK2 receptor and NBD-labeled mutants obtained by controlled suppression of UAG stop codons in Xenopus oocytes.](image)

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of yeast UAG suppressor tRNA	extsuperscript{Ph}CUA (28) for the biosynthetic incorporation of nonnatural amino acids. Surprisingly, co-injection of synthetic, nonacylated suppressor yeast tRNA	extsuperscript{Ph}CUA and cRNA transcript for F248st gave functional NK2 receptor (Fig. 2). Furthermore, we found that yeast tRNA	extsuperscript{Ph}CUA was aminoacylated with [14C]phenylalanine in oocytes, thus retaining its identity (data not shown). However, minor acylation with other amino acids cannot be totally excluded. This indicated that synthetic suppressor tRNA from yeast, containing hypermodified base, was recognized and aminoacylated by Xenopus tRNA synthetase (31). The method was therefore not suitable for controlled suppression of UAG stop mutants. This problem was circumvented by preparing Sup-tRNA, a synthetic analogue containing two base mutations that reduce the rate of aminoacylation while preserving the structure (see above). To test that Sup-tRNA has conserved its ability to transfer chemically acylated amino acids during protein biosynthesis in intact cells, we first regenerated wild-type NK2 from F248st mutant. This was achieved by co-injection in oocytes of 25 ng of F248st cRNA and 100 ng of Sup-tRNA chemically acylated with phenylalanine (Sup-tRNA-Phe). Currents indistinguishable from the true wild type receptor were observed (Fig. 2). In contrast, there was no detectable NK2 activity when F248st was co-injected with either nonacylated Sup-tRNA or the shorter 74-nt form Sup-tRNA(-CA) (Fig. 2). Also, there was no current elicited by NKA when Sup-tRNA-Phe was injected in the absence of cRNA. We then investigated the incorporation of unnatural, fluorescent amino acid analogues into the sites 103 or 248 in NK2. When oocytes were co-injected with cRNA encoding the stop mutants R103st or F248st, and Sup-tRNA misacylated with NBD-Dap (Sup-tRNA-NBD), currents similar in shape and amplitude to those for wild-type NK2 were measured by voltage clamping (Fig. 2). Several controls were performed to demonstrate the fidelity of unnatural mutagenesis. Co-injection of stop mutant cRNAs and nonacylated Sup-tRNA, in either the truncated (-CA) 74 nucleotide form or the full-length 76 nucleotide form, gave no responses to 0.1 mM NKA in at least 10 separate experiments. In addition, injection of stop mutant cRNA alone, or injection of Sup-tRNA-NBD alone, also failed to give any response (data not shown). Fig. 3 shows that there was a slight delay of onset for chloride currents from NK2 suppression mutants compared to wild type receptors. Taken together, these data indicate that functional mutant receptors containing the unnatural NBD-Dap amino acid at either position 103 or 248 had been synthesized by oocytes.

Ligand Binding and Evaluation of Suppression Efficiency—The K\textsubscript{d} values for binding of the radiolabeled NK2 antagonist PhCO-(\(\delta\)-125I-phenol-3-propionyl)-Orn-Ala-D-Trp-Phe-Pro-Pro-Nle-NH\textsubscript{2} to membrane fractions from oocytes were similar in the nanomolar range for wild-type NK2 and fluorescent mutan receptor R103NBD or F248NBD (Table I). In addition, these values were comparable to the K\textsubscript{d} for binding to NK2 expressed in CHO cells. Representative saturation binding curves and Scatchard analysis of the data are shown in Fig. 4. Oocyte batch to batch variability was important; this was leveled off in binding assays that used membrane fractions averaged from 100–200 oocytes. Voltage-clamping also showed some oocyte to oocyte variability within the same batch. The efficiency of suppression, calculated from the expression levels (B\textsubscript{max}) for wild type NK2 and suppression mutants was 18 ± 10% (Table I). There was no specific binding in oocytes injected with either stop mutan cRNA alone or misacylated Sup-tRNA alone.

Characterization of NBD Fluorescent-labeled NK2 Receptors—Fluorescence-labeled NK2 receptors were investigated by epifluorescence microspectrofluorimetry. Fig. 5 shows the specific fluorescence of NK2 mutants R103NBD and F248NBD in oocyte membrane fractions immobilized on a quartz microscope slide using an excitation wavelength of 476 nm. Typically, spectra were recorded with membranes from 10 oocytes. There was no detectable change in the intensity or emission maximum of the fluorescence spectrum of the environment-sensitive NBD group at position 248 upon binding of either the heptapeptide antagonist GR94800 (20 nM) or the natural agonist NKA (20 nM) to the receptor (Fig. 5).
Fluorescent Properties of TMR-labeled NK2 Ligand—The heptapeptide PhCO-Lys(ε-TMR)-Ala-D-Trp-Phe-D-Pro-Pro-Nle-NH₂ labeled with the fluorescent TMR was prepared as described previously (16). This fluorescent peptide was assayed for NK2 binding affinity by competitive displacement of the NK2 selective antagonist ³H-GR100679 (37) using CHO cells stably transfected with NK2 receptors as described elsewhere (16). The pKᵣ was 8.80. The excitation and emission maxima of PhCO-Lys(ε-TMR)-Ala-O-Trp-Phe-O-Pro-Nle-NH₂ showed some variation with the polarity of the solvent as shown in Table II and Fig. 5. In Tris-HCl, pH 7.4, dioxane, 95:5, a solvent whose polarity mimics the microenvironment of the fluorophore when bound to the receptor (6), the absorption and emission maxima were 548 nm (ε = 129,000 M⁻¹ cm⁻¹) and 572 nm, respectively. We also characterized this ligand using the techniques of fluorescence anisotropy and collisional quenching as described earlier (6). When bound to NK2 receptor in stably transfected CHO cells, PhCO-Lys(ε-TMR)-Ala-O-Trp-Phe-O-Pro-Nle-NH₂ was indistinguishable from the well characterized NBD analogue PhCO-Lys(ε-NBD)-Ala-O-Trp-Phe-O-Pro-Nle-NH₂ that we recently described (data not shown) (6). In particular, steady-state fluorescence anisotropy which directly reflects the molecular mobility of the fluorescent reporter group, indicated that the receptor-bound ligand was nearly totally immobilized in the nanosecond time range. The Stern-Volmer constant for quenching of TMR fluorescence of NK2-bound ligand by collision with iodide ions was 5.0 ± 1.0 M⁻¹ compared to 16.5 ± 1.5 M⁻¹ for the ligand free in solution (Fig. 6). The partial quenching of the TMR group indicated that the fluorophore is located at the membrane-water interface (6).

Fluorescence Energy Transfer between TMR-labeled Ligand and NBD-labeled NK2 Receptors—When the antagonist ligand PhCO-Lys(ε-TMR)-Ala-O-Trp-Phe-O-Pro-Nle-NH₂ labeled with the fluorescence acceptor TMR (16) was bound to the NK2 ligands and NK2 receptors as described previously (16). The pKᵣ was 8.80. The excitation and emission maxima of PhCO-Lys(ε-TMR)-Ala-O-Trp-Phe-O-Pro-Nle-NH₂ showed some variation with the polarity of the solvent as shown in Table II and Fig. 5. In Tris-HCl, pH 7.4, dioxane, 95:5, a solvent whose polarity mimics the microenvironment of the fluorophore when bound to the receptor (6), the absorption and emission maxima were 548 nm (ε = 129,000 M⁻¹ cm⁻¹) and 572 nm, respectively. We also characterized this ligand using the techniques of fluorescence anisotropy and collisional quenching as described earlier (6). When bound to NK2 receptor in stably transfected CHO cells, PhCO-Lys(ε-TMR)-Ala-O-Trp-Phe-O-Pro-Nle-NH₂ was indistinguishable from the well characterized NBD analogue PhCO-Lys(ε-NBD)-Ala-O-Trp-Phe-O-Pro-Nle-NH₂ that we recently described (data not shown) (6). In particular, steady-state fluorescence anisotropy which directly reflects the molecular mobility of the fluorescent reporter group, indicated that the receptor-bound ligand was nearly totally immobilized in the nanosecond time range. The Stern-Volmer constant for quenching of TMR fluorescence of NK2-bound ligand by collision with iodide ions was 5.0 ± 1.0 M⁻¹ compared to 16.5 ± 1.5 M⁻¹ for the ligand free in solution (Fig. 6). The partial quenching of the TMR group indicates that the fluorophore is located at the membrane-water interface (6).

Table II

| Solvent          | Absorption maximum in nm (ε (M⁻¹ cm⁻¹)) | Relative fluorescence |
|------------------|-----------------------------------------|-----------------------|
| Methanol         | 540 (145,000)                           | 9240                  |
| 50 mM Tris-HCl, pH 7.4 | 554 (73,000)                           | 1524                  |
| 50 mM Tris-HCl, pH 7.4 | 548 (129,000)                           | 7715                  |
| Dioxane, 95:5 (v/v) |                                        |                       |

**Fig. 5. Spectrofluorimetric analysis of NBD-labeled NK2 receptors and TMR-labeled NK2 heptapeptide ligand.** Specific fluorescence spectra of NBD-labeled mutants R103NBD (A) and F248NBD (B) in immobilized oocyte membranes are shown. Excitation was at 476 nm. This specific fluorescence spectrum is the difference between the total fluorescence spectra of membranes from oocytes injected with NK2 F248t cRNA plus Sup-tRNA-NBD and the background fluorescence from membranes of oocytes injected with wild-type NK2 cRNA plus Sup-tRNA-NBD. The emission signal was stable to photobleaching under measuring conditions. C, effect of agonist and antagonist on fluorescence from NK2 F248NBD mutant. Superimposition of three traces corresponding to spectra of F248NBD recorded immediately before and 10 min after addition of 20 mM NKA or 20 mM PhCO-Ala-Ala-O-Trp-Phe-O-Pro-Nle-NH₂. Representative data of three independent experiments is shown. D, excitation (dashed line) and emission (solid line) spectra of the antagonist PhCO-Lys(ε-TMR)-Ala-O-Trp-Phe-O-Pro-Nle-NH₂ in 50 mM Tris-HCl, pH 7.4, dioxane, 95:5 (v/v).
DISCUSSION

In this study we present a novel fluorescence spectroscopic approach to obtain structural information on integral membrane proteins as demonstrated for the NK2 neurokinin G protein-coupled receptor. The aim was to use FRET techniques between a fluorescent donor group engineered in the NK2 receptor at known sites, and an acceptor fluorophore in the ligand, for the determination of a structural model for interaction of a peptide ligand with NK2.

Site-specific fluorescent labeling of NK2 was accomplished in intact cells by suppression of TAG nonsense codon placed at known sites in NK2 using a suppressor tRNA aminoacylated with the fluorescent L-amino acid analogue NBD-Dap. The key steps in this approach are: 1) the mutation of a selected site in NK2 cDNA to TAG termination codon and the preparation of the cognate cRNA transcript containing the UAG codon; 2) the construction by chemical and enzymatic methods of a misacylated suppressor tRNA that recognize the UAG stop codon and functions biosynthetically as a source of unnatural amino acid; and 3) the heterologous expression of receptor containing unnatural amino acids in Xenopus oocytes. The oocyte cell translation machinery recognizes the exogeneous aminoacylated suppressor tRNA and incorporates the fluorescent residue into the nascent protein at sites preselected by creation of a nonsense codon in the gene. To improve the efficiency of the incorporation, we designed and constructed a synthetic UAG suppressor tRNA (Sup-tRNA-NBD, Fig. 1) derived from yeast tRNA(Phe). This contained the anticodon CUA that recognizes the UAG stop mutation in the cRNA. The fluorescent amino acid NBD-Dap was acylated chemically to the 3'-end and two mutations (G20U and A73G) were introduced to reduce the rate of reacylation with natural amino acids by endogeneous aminoclyl-tRNA synthetases (28, 29). UAG was chosen as a stop codon for controlled suppression because of its low usage as a natural termination codon in oocytes (17%), compared to UAA (36%) or UGA (47%) (32). The yields of suppression were in the 10–30% range. We never detected NBD fluorescence in membranes prepared from oocytes injected with the fluorescent Sup-tRNA-NBD only. This suggests that suppression of UAG stop codons naturally occurring in oocytes by the exogeneously added suppressor tRNA was insignificant in the context of this study.

The functional activity of NK2 and fluorescent suppression mutants (Fig. 2) was assayed by activation of Ca2+-dependent chloride currents (24, 30). The minor differences observed among the chloride currents profiles represent the degree of variability of response that is normally seen in oocytes. Similar oocyte to oocyte variations were observed for wild type NK2 in the same batch. The data demonstrate that functional NK2 mutant receptors, containing the unnatural NBD-Dap amino acid at either position 103 or 248, were synthesized by oocytes. The only detectable difference between suppression mutants and wild type receptor was the latency of onset of chloride channel opening (Fig. 3) which probably reflects the differences in expression level.

Fluorescent NK2 receptors in oocyte membranes were further characterized by saturation binding analysis with the specific NK2 antagonist PhCO-Lys(6,125I-phenol-3-propionyl)-Orn-Ala-Trp-Phe-Pro-Pro-Orn-Nle-NH2. Kd values for binding of this ligand to oocyte membrane fractions were comparable for wild-type NK2 and R103NBD and F248NBD mutants. Moreover, these Kd values were comparable to those obtained for NK2 receptor stably expressed in CHO cells (Table I), thus indicating that the structural integrity of the mutants was conserved.

Fluorescent labels placed at known sites can monitor confor-

**FIG. 6.** Stern-Volmer plots for collisional quenching of TMR-labeled NK2 heptapeptide ligand fluorescence by iodide. The quenching of fluorescence emission of PhCO-Lys(6,125I-phenol-3-propionyl)-Orn-Ala-Trp-Phe-Pro-Pro-Orn-Nle-NH2 at 5 nM was calculated with the Stern-Volmer equation (36): $F/F_0 = 1 + K_{SV} [I]$ where $F/F_0$ is the ratio of fluorescence intensities in the absence and presence of iodide. The Stern-Volmer quenching constant $K_{SV}$ was determined from the slope of $F/F_0$ as a function of the iodide concentration [I]. ● = free ligand in solution; ○ = ligand specifically bound to NK2. A representative plot of three independent experiments is shown.

**FIG. 7.** FRET between NBD-labeled NK2 mutants and TMR-labeled peptide antagonist. Representative FRET between receptor-bound tetramethylrhodamine-labeled antagonist PhCO-Lys(6,125I-phenol-3-propionyl)-Orn-Ala-Trp-Phe-Pro-Pro-Orn-Nle-NH2 and mutants R103NBD (left panel) or F248NBD (right panel), respectively. The 476-nm line of the argon laser was used for NBD excitation in order to reduce the direct excitation of the tetramethylrhodamine fluorophore. In each panel, traces 1 represent specific fluorescence emission spectra of NBD-labeled NK2 mutants as described in Fig. 5. Traces 2 represent fluorescence emission spectra in the presence of bound TMR-antagonist peptide. The distance (R) between the NBD and TMR groups was calculated by Förster's equation $R = R_0 (1/E - 1)^{1/6}$ where $R_0$ is the distance for 50% transfer efficiency E as described under “Experimental Procedures.”
mational changes in receptors in response to ligand binding. In that respect, position 248 in the C-terminal part of the third intracellular loop of NK2 is particularly interesting as this region has been shown to be involved in receptor activation and G protein coupling for other 7-transmembrane receptors (38, 39). We observed no change in the fluorescence of mutant F248NBD during binding of either agonist or antagonist (Fig. 5). Without totally ruling out that this region undergoes conformational change during receptor functioning, this however indicates that there is no detectable change in the hydrophobicity around site 248 because NBD fluorescence depends strongly on the polarity of its environment.

Evidence for fluorescence energy transfer was provided by quenching of the NBD-labeled receptor (donor) fluorescence. This quenching was used to quantify the energy transfer because, under the conditions of measurement, there was no evidence for other deactivation process of NBD fluorescence. An important factor influencing the FRET experiments and their interpretation is the dipole orientation factor $k_0$. In theory, this factor can take any value between 0 and 4. $k_0$ can be precisely determined only if the positions of the two fluorescent molecules is fixed and known. When the energy donor is free to rotate and the energy acceptor is fixed, $k_0$ can range from $1/3$ compared to 4/3 depending on the angle between the transition moment of the acceptor and the donor-acceptor separation vector (15). The error on the calculated $R_0$ due to the uncertainty in the value of $k_0$ in this range is $\pm 11\%$. We are making this assumption in the experiments described in this study. From the steady state fluorescence anisotropy experiments, we can deduce that the TMR-labeled ligand bound to NK2 is immobilized. Also it is reasonable to assume that residues 103 and 248 located in loops connecting membrane-spanning domains have some freedom to rotate.

In previous spectrofluorometric observations and collisional quenching experiments from our laboratory (6) we have shown that the antagonist heptapeptide ligand binds the wild type receptor in a hydrophobic pocket on the extracellular side of the transmembrane segments and that the fluorophore is located at the membrane-water interface. The distance between the bound TMR ligand and position 248 estimated by FRET is consistent with a seven transmembrane topology for the NK2 receptor and provides an experimental validation of this model. The membrane bilayer can be viewed as a hydrophobic core region of about 30 Å flanked by two interfacial regions of about 15 Å each (40). The acceptor and donor fluorophores are predicted in the interfacial areas on opposite sides of the membrane. Residues Gln109, Cys167, His198, and Gly273 around the extracellular side of the third, fourth, fifth, and sixth transmembrane segments, respectively, were found to be critical for peptide antagonist binding to NK2. By placing the NK2 residue 248 on the cytoplasmic face, residue 103 in the first extracellular loop, and taking into account the measured FRET distances between the TMR peptide, the position of the ligand becomes defined in space. This consideration resulted in the three-dimensional model of NK2 in Fig. 8 using the projection structure of rhodopsin as a general basis for the G protein-coupled receptors folding (27). To refine this model, we integrated the FRET experimental distances with the other constraints. Fig. 8 shows the most probable orientation for the fluorescent heptapeptide antagonist bound to NK2, in the context of this model. The fluorescent TMR group lies outside of the bundle formed by the seven membrane spanning domain. Insertion of the peptide ligand between the fifth and sixth transmembrane domains suggests that antagonism may be caused by preventing correct packing of the helices required for receptor activation. Future measurement of FRET distances between the fluorescent ligand, or an analogue labeled at a different position, and other labeled sites in NK2 will permit further refining of this model.

In conclusion, we presented the first report of the introduction of fluorescent probes at specific sites in a membrane receptor in intact cells by unnatural suppression mutagenesis. We also showed that these labeled receptors can be used to measure intermolecular distances between these sites and bound ligands. By placing the fluorescent probe to different sites in the receptor, it will be possible to improve the structural model of the receptor and its ligands in a native membrane environment. In the future, time-resolved fluorescence measurements will permit the study of rapid dynamic changes in the receptor structure (34, 35). These methods can now be generally applied to understand the molecular architecture and conformational changes of integral membrane proteins such as

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channels or receptors as well as to protein-protein or ligand-protein interactions.

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