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Evidence That Interspecies Polymorphism in the Human and Rat Cholecystokinin Receptor-2 Affects Structure of the Binding Site for the Endogenous Agonist Cholecystokinin*

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The cholecystokinin (CCK) receptor-2 exerts very important central and peripheral functions by binding the neuropeptides cholecystokinin or gastrin. Because this receptor is a potential therapeutic target, great interest has been devoted to the identification of efficient antagonists. However, interspecies genetic polymorphism that does not alter cholecystokinin-induced signaling was shown to markedly affect activity of synthetic ligands. In this context, precise structural study of the agonist binding site on the human cholecystokinin receptor-2 is a prerequisite to elucidating the molecular basis for its activation and to optimizing properties of synthetic ligands. In this study, using site-directed mutagenesis and molecular modeling, we delineated the binding site for CCK on the human cholecystokinin receptor-2 by mutating amino acids corresponding to that of the rat homolog. By doing so, we demonstrated that, although resembling that of rat homolog, the human cholecystokinin receptor-2 binding site also displays important distinct structural features that were demonstrated by susceptibility to several point mutations (F120A, Y189A, H207A). Furthermore, docking of CCK in the human and rat cholecystokinin receptor-2, followed by dynamic simulations, allowed us to propose a plausible structural explanation of the experimentally observed difference between rat and human cholecystokinin-2 receptors.

Although both receptors recognize sulfated CCK with comparable high affinity, the CCK2R has high affinity for both sulfated and non-sulfated gastrin (1, 2). CCK1R and CCK2R are seven-transmembrane spanning receptors that belong to the superfamily of G protein-coupled receptors and have ~50% homology (3–5). CCK1R are mainly found in the peripheral organs, where they regulate pancreatic secretion and gallbladder and gastrointestinal motility, but are also found in some areas of the central nervous system, where they regulate satiety and analgesia. CCK2Rs are predominantly present throughout the central nervous system, where they regulate anxiety/panic attacks and dopamine release, implicated in the pathogenesis of dopaminergic related movement and behavioral disorders in humans. In the gut, CCK2Rs regulate acid and histamine secretions, gastrointestinal motility, as well as growth in the gastric mucosa (1, 2). The important physiological functions mediated by CCK receptors, and therefore their possible implication in associated disorders, have generated considerable interest in the identification of ligands that selectively activate or block CCK1R and CCK2R (6). To date, a large panel of such molecules has been designed covering a wide range of functional activities, such as full, partial, and inverse agonists, as well as antagonists (6). Pharmacological characterization of selective synthetic CCK2R ligands among different species pointed out an interspecies genetic polymorphism that does not alter endogenous hormone-induced signaling but markedly affects both the affinity and activity of synthetic ligands (7). This is the case for L-365,260 and L-364,718, two reference non-peptidic CCK2R antagonists that present reversible in the affinity rank order on canine or human recombinant CCK2R despite a 93% identity in their protein sequence (8). Furthermore, efficacy of PD-135,158, a CCK2R partial agonist, is 60% of CCK-induced maximal production of inositol phosphates on mouse CCK2R but only 20% on human CCK2R, despite 90% identity (9). These interspecies differences toward synthetic ligands were shown to be caused by sequence variations within the binding site of the CCK2R (7). Such data pointed out the high importance of in vivo and in vitro tests with human protein targets.

The aims of the present study, using site-directed mutagenesis and molecular modeling, were to delineate the binding site for CCK on the human CCK2R and to investigate whether insensitivity of endogenous ligands to interspecies polymorphism between rat and human CCK2R can be explained by differences in their positioning into the CCK2R binding site or whether they are related to distinct susceptibilities to mutations. For that purpose, we took advantage of our previous data, which demonstrated an interaction between His101 of rat...
CCK2R and Asp9 of CCK, between the Tyr145 and Asn158 residues of rat CCK2R and the carboxyl-terminal amide of CCK (10), as well as the contribution of Arg57 and Phe120 residues in CCK high affinity binding (11, 12). These key amino acids of the CCK2R binding site were mutated in the human CCK2R, and the mutant receptors were characterized for CCK affinity, as well as efficacy and potency, to stimulate inositol phosphates accumulation. By doing so, we demonstrated that, although resembling that of rat CCK2R, the human CCK2R binding site also displays important distinct structural features that are confirmed by susceptibility to point mutations and docking, followed by dynamic simulations.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sulfated ([Trh-Thr-Glu-Thr-Lys-Thr-Thr-Tyr(125)NH2]CCK25–33 (H-Arg-Asp-Tyr/SO2-H-Thr-Gly-Tyr-Nle-Asp-Phe-NH2, CCK9S), was synthesized as described previously (13). 125I-Sodium (2000 Ci/mmol) was obtained from Amersham Biosciences. Sulfated [Thr28, Nle31]CCK25–33 was conjugated with Bolton–Hunter reagent, purified, and radiiodinatated as described previously (14) and is referred to as [125I]CCK9S.

**Site-directed Mutagenesis and Transfization of COS-7 cells**—All mutants were constructed by oligonucleotide-directed mutagenesis (QuickChanger™ site-directed mutagenesis kit, Stratagene) using the human CCK2R cDNAs cloned into pRFENeo vector as a template. The presence of the desired and the absence of undesired mutations were confirmed by automated sequencing of the complete CCK2R coding sequence (Applied Biosystems).

**COS-7 cells** (1.5 × 10^5) were plated onto 10-cm culture dishes and grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum in a 5% CO2 atmosphere at 37 °C. After overnight incubation, the cells were transfected with 0.5 μg/plasmid of pRFENeo vectors containing the cDNA for the wild-type or mutated CCK2 receptor, using a modified DEAR-dextran method as described previously (10). The cells were transfected to 24-well plates at a density of 20,000–150,000 cells/well 24 h after transfection, depending on the transfected mutant and transient expression to be performed.

**Binding Experiments**—Approximately 24 h after the transfection of the transfected cells to 24-well plates, the cells were washed with phosphate-buffered saline, pH 7.4, containing 0.1% bovine serum albumin and then incubated for 60 min at 37°C in 0.3 ml of DMEM containing 0.1% bovine serum albumin, with either 50 μM (wild-type CCK2R, R356K), 100 μM (R57A, F120A, Y189F), 250 μM (H207A, N353A, Y189A, R356D) or 600 μM (R356D) [125I]CCK9S in the presence or in the absence of unlabeled CCK9S. The cells were washed twice with cold phosphate-buffered saline, pH 7.4, containing 2% bovine serum albumin, and cell-associated radioligand was collected by cell lysis with NaOH 0.1 N. The radioactivity was directly counted in a γ counter (Auto-γ, Packard, Downers Grove, IL). Receptor density and Kd were calculated from the binding data and used for the human CCK2 receptor CCK9S competition binding experiments using Ligand software (Kell, Cambridge, UK). K values were calculated using the non-linear curve fitting software GraphPad Prism (San Diego, CA).

**Inositol Phosphate Assay**—Approximately 24 h after the transfer to 24-well plates and following overnight incubation in DMEM containing 2 μCi/ml inositol-2-[3H] (Nisotiol (Amersham Biosciences), the transfected cells were washed with DMEM and then incubated for 30 min in 1 ml/well DMEM containing 20 μM LiCl at 37 °C. The cells were washed with inositol phosphate buffer, pH 7.45, phosphate-buffered saline containing 135 mM NaCl, 20 mM Hepes, 2 mM CaCl2, 1.2 mM MgSO4, 1 mM EGTA, 10 mM LiCl, 11.1 mM glucose, and 0.5% bovine serum albumin. The cells were then incubated for 60 min at 37°C in 0.3 ml of inositol phosphate buffer with or without increasing CCK9S concentration. The reaction was stopped by adding 1 ml of methanol/HCl to each well, and the content was transferred to a column (Dowex AG 1-8X, formate form, Bio-Rad, Hercules, CA) for the determination of inositol phosphates. The columns were washed twice with 3 ml of distilled water and twice with 2 ml of 5 mM sodium tetraborate/60 mM sodium formate. The content of each column was eluted by the addition of 2 ml of 1 M ammonium formate/0.5 M ammonium acetate in water, at radiolucivity of 1 ml of eluted fraction was fractionated using a liquid scintillation counter (Packard Instrument Co.). EC50 values were calculated from dose effect curves by non-linear regression curve fitting using GraphPad Prism software (San Diego, CA).

**Molecular Modeling of Human CCK2R-CCK Complex and Dynamic Simulations**—The human CCK2R model was built by homology modeling from the rat CCK2R-CCK complex model described by Gales et al. (11) using Insight II modules (Homology, Discover 3, Biopolymer, Accelrys, San Diego, CA). Alignment of the human CCK2R sequence on the rat CCK2R sequence was performed using the alignment procedure with all the manual refinements that make contact to all G protein coupled receptors and conserved amino acids between the two sequences precisely matched. The coordinates of the human CCK2R were then assigned to those of the rat CCK2R three-dimensional model, based on this alignment. The resulting model was finally subjected to energy minimization by the steepest descent (until convergence to 2 kcal/mol/Å) and conjugated gradient (until convergence to 0.01 kcal/mol) to remove steric clashes. During minimization, Cα trace was tethered using a quadratic potential. This was performed using the Discover 3 module with the consistent force field. The non-bond cutoff distance and the dielectric constant were set up to cell multipoles and were distance-dependent, respectively. The minimized model of human CCK2 receptor was used to perform manual docking of CCK. The conformation of CCK was taken from the optimized complex of rat CCK2R-CCK (11), and docking was performed taking into account the position of CCK in that receptor. The obtained human CCK2R-CCK complex was minimized again according to the previously described minimization protocol, with no Cα restraints, and was further subjected to molecular dynamics in a two-step procedure. In the first step, the complex was heated from 10 K to 300 K during 5000 fs, and in the second step, it was kept at 300 K until equilibration. The tethering force on the Cα atoms was weakened to 280 Kcal/mol/Å2, and Cα was decreased by reducing the force constant value of the quadratic potential from 100 to 80, 50, 30, 10, and 0 every 1000 ps in the second step. The integration time step was set up to 1 fs, and the calculations were performed at constant volume and temperature. A snapshot of the system was saved every 100 fs. Once the system was equilibrated, the coordinates of 20 snapshots were averaged and submitted again to the previously mentioned minimization protocol, with no Cα restraints.

**RESULTS**

**Effects of R57A, F120A, H207A, Y189F, Y189A, and N353A Mutations in the Human CCK2R on CCK Affinity and Potency**—Human R57A, F120A, H207A, Y189F, Y189A, and N353A mutants were constructed and transiently expressed in COS-7 cells. Scatchard analysis of [125I]CCK9S binding to the wild-type human CCK2R indicated that CCK9S binds to high affinity sites with K values of 0.75 ± 0.03 nM (Bmax 3.2 ± 0.3 pmol/106 cells) (Tables I and II). Mutation of Arg57 and Phe120 in alanine or mutation of Tyr189 in phenylalanine slightly reduced, between 4- and 6-fold, CCK9S affinity with K values of 3.4 ± 0.4 nM (Bmax 0.85 ± 0.09 pmol/106 cells), 3.1 ± 0.4 nM (Bmax 0.79 ± 0.08 pmol/106 cells), and 4.5 ± 0.4 nM (Bmax 1.2 ± 0.2 pmol/106 cells), respectively (Tables I and II). Y189A, H207A, and N353A mutants displayed between 25- and 73-fold decrease in CCK9S affinity for these respective K values of 55 ± 8 nM (Bmax 2.7 ± 0.5 pmol/106 cells), 19 ± 2 nM (Bmax 1.1 ± 0.1 pmol/106 cells), and 20 ± 2 nM (Bmax 0.8 ± 0.1 pmol/106 cells) (Tables I and II). In agreement with binding data, the potency of CCK9S to stimulate inositol phosphate accumulation was reduced for all mutants (ECmax) and directly correlated with the loss of affinity for CCK9. The mutants displayed CCK9S efficacy (Emax, Table I) comparable with that of wild-type CCK2R, except for the Y189A mutant, which presented a 44% decrease of CCK9S maximal effect despite similar receptor density (Table I). Comparison of these results with those from our previous studies performed in exactly the same experimental conditions on rat CCK2R mutants reveals that, except for N353A mutation (the human corresponding mutant of rat N358A), all of the mutants carried out on the human CCK2R less markedly affected CCK affinity and activity, supporting the view that the corresponding residues are differentially involved in the binding sites of the two CCK2Rs (Table II, Fmax).

**Effects of R356A, R356H, R356K, and R356D Mutations in Human CCK2R on CCK Affinity and Potency**—Our previous studies (15) on the binding site of the human CCK1R demonstrates that Arg136, located at the top of TM 6, highly contributes to the binding of CCK. Because the homologous amino acid...
in the human CCK2R is Arg356, we determined whether this residue could also be involved in the binding site of the human CCK2R. For this purpose, R356A, R356H, R356K, and R356D mutants were constructed and analyzed. These mutants were designed to remove partially or completely the positive charges of this arginine (R356A, R356H, and R356K mutants) or to introduce a negative charge at that position (R356D mutant).

Mutation of Arg356 in lysine did not modify CCK9S affinity ($K_a = 0.50 \pm 0.05$ nM, $B_{max} = 3.9 \pm 0.4$ pmol/10⁶ cells) nor the potency and efficacy of CCK9S to stimulate inositol phosphate accumulation ($EC_{50} = 0.54 \pm 0.06$ nM). Replacement of Arg356 by a histidine or an alanine reduced 10- and 76-fold, respectively, CCK9S affinity, whereas R356D displayed a 922-fold lower affinity relative to the high affinity component of the wild-type human CCK2R (Table I and Fig. 1). In agreement with binding data, R356H, R356A, and R356D mutants displayed reduced CCK9S potencies to stimulate inositol phosphate accumulation but unchanged efficacy (Table I and Fig. 1). These results indicate that positive charges of the amino acid side chain at position 356 of the CCK2R are important for high affinity binding of CCK.

**Structural Study of Human CCK2R Binding Site Using Docking and Dynamic Simulations**—Results from the current site-directed mutagenesis study support the view that homologous residues in the human CCK2R binding sites are differentially involved in the binding site of CCK compared with rat CCK2R, which we previously characterized. Because human and rat CCK2R present non-conserved amino acids in extra-cellular domains and transmembrane helices (Fig. 2), in a first attempt to explain distinct involvement of His207, we exchanged the neighboring amino acid, namely Val206, for the corresponding amino acid of the rat sequence, Met. However, the resulting CCK2R mutant (V206MH207A) did not display an altered pharmacological profile, as compared with the human H207A mutant (not shown). We also considered the possibility that His207, which can exist in different tautomeric forms, could be differently protonated in human and rat CCK2R. However, pKₐ calculations using the Poisson-Boltzmann equation and the generalized Born approach (embodied in the website chekhov.cs.vt.edu/completion/index.php) yielded similar values (pKₐ for rat 3.3 and 4.2, and for human 3.5 and 4.6) thus ruling out this hypothesis. This led us to hypothesize that non-conserved amino acids, including those in transmembrane helices, likely act collectively to position differently CCK in the binding site of the human CCK2R relative to the rat CCK2R. We therefore performed in silico experiments by building a three-dimensional structure of the human CCK2R–CCK complex based on the coordinates of the rat CCK2R–CCK complex that we previously experimentally validated (11). Thereafter, we applied molecular dynamics to assess whether variations in the CCK2R sequence could result in a distinct location of CCK that could explain pharmacological results. A simplified view of the obtained human CCK2R–CCK complex is presented in Fig. 3, A and C. The docking, followed by dynamic simula-

### Table I

| Binding | Inositol phosphate production |
|---------|------------------------------|
| $K_a$   | $F_{WT}$                     |
| $K_d$   | $F_{WT}$                     |

### Table II

| Human CCK2R | Rat CCK2R |
|-------------|-----------|
| Binding     | IP accumulation | Ref. |
| $K_i$       | $F_{WT}$     | $EC_{50}$ | $F_{WT}$ |

* $p < 0.05$ evaluated by Mann-Whitney test as compared to wild-type receptor value.

*NA* No accumulation of inositol phosphates.
tions, revealed that Arg$^1$ and Asp$^2$ of CCK form internal ionic bonds; sulfated Tyr$^3$ of CCK is in ionic interaction with Arg$^{57}$ and makes a hydrogen bond with Tyr$^{61}$; Thr$^4$ forms a hydrogen bond with Arg$^{356}$; Trp$^6$ forms both a hydrogen bond with His$^{376}$ and aryl sulfur interactions with Met$^{67}$ and Cys$^{107}$ and hydrophobic interactions with Phe$^{120}$ and Phe$^{110}$; Nle$^7$ lies in an hydrophobic pocket composed of Pro$^{371}$, Trp$^{355}$, and Leu$^{367}$; the carboxyl group of Asp$^8$ interacts with Arg$^{356}$ and Ser$^{131}$; the phenyl group of Phe$^9$ is sited in an aromatic pocket made of Tyr$^{189}$, Tyr$^{192}$, Tyr$^{350}$, Trp$^{346}$, Phe$^{342}$, and Phe$^{227}$; and the carbonyl group of CCK amide forms hydrogen bound with the hydroxyl group of Tyr$^{189}$, whereas $\text{NH}_2$ of the CCK amide interacts with Asn$^{353}$ and Thr$^{193}$ (Fig. 3A).

According to these results, the binding site of the human CCK2R resembles that of the rat CCK2R. However, and importantly, CCK drifts deeper in the human CCK2R, representing a difference of $\approx 1.5$ Å, and the C-terminal part of CCK between Trp$^6$ and Phe$^9$ displays a deviation of the backbone of $\approx 2.5$ Å, as compared with rat CCK2R-CCK complex, which was subjected to the same procedure of dynamic simulations (see Fig. 3C for comparison). In addition, the phenyl group of Phe$^9$ has an affected orientation, as compared with the rat complex, where it approximated Tyr$^{189}$. In the human complex, Phe$^9$ seems more inserted in a cavity composed of Tyr$^{189}$, Tyr$^{192}$, Tyr$^{350}$, Trp$^{346}$, Phe$^{342}$, and Phe$^{227}$ (Fig. 3, A and B). Moreover, Asp$^8$ forms stable ionic interactions mostly with Arg$^{356}$ but less with His, which dominated in the rat complex.

**DISCUSSION**

Despite the high degree of homology between species homologs of the CCK2 receptor (ranging from 84 to 93%), pharmacological studies have demonstrated that interspecies polymorphism can significantly alter both the affinity and activity of synthetic ligands (7). Other examples describing polymorphism-induced alteration can be found in the literature for neurokinin, serotonin, and VIP receptors (16–18), but in those cases, both endogenous and synthetic ligands are affected. However, in the case of CCK2R, comparison of CCK or gastrin affinity and activity in cells that express canine, murine, or human recombinant CCK2R revealed similar profiles. It is suggested that the apparent insensitivity of endogenous ligand to receptor polymorphism might be explained by evolutionary selection, which resulted in the elimination of receptor variants that compromised endogenous ligand function (7). In this context, the aim of the present study was to delineate the binding site for CCK on the human CCK2R and to investigate whether...
insensitivity of endogenous ligands to interspecies polymorphism between rat and human CCK2R can be explained by differences in their location into the CCK2R binding site or whether there are related to distinct susceptibilities to mutations.

Using site-directed mutagenesis and pharmacological studies, we found that some mutations were less unfavorable when performed on the human CCK2R than on the rat receptor. The magnitude of the differences between rat and human mutants varied depending on the amino acid studied. Mutation of Arg57 (located at the top of the first transmembrane domain) in alanine reduced 5-fold the CCK affinity in the human receptor versus 21-fold in rat receptor (12). Similarly, Kopin et al. (19) have shown that the human R57Q mutant displayed a 6-fold decreased affinity for CCK, confirming the slight contribution of Arg57 in the human CCK2R for high affinity binding of CCK. Moreover, photoaffinity labeling experiments, using a CCK ligand having its photosensitive moiety attached to its N terminus, identified amino acids at the top of helix I, which agrees with current results supporting that the sulfate of CCK slightly interacts with Arg57 (20). Surprisingly, Phe120, located in the first extracellular loop and which is essential for the high affinity binding of CCK to the rat CCK2R, does not appear to be as essential in human CCK2R. Indeed, mutation F120A in the rat receptor resulted in complete loss of radioligand binding (12), whereas in the current study, the human F120A mutant

![Diagram of CCK2R binding site](image_url)
only displayed a 4-fold lower affinity as compared with wild-type CCK2R. Similar results were obtained with the H207A mutant. Our previous results with the rat receptor clearly demonstrated a direct interaction between His\(^{207}\), located in the second extracellular loop, and penultimate aspartic acid of CCK (10). Here, the contribution of His\(^{207}\) in the human CCK2R binding pocket is quantitatively different. Indeed, the human H207A mutant displayed a 25-fold lower CCK affinity versus 456-fold in the rat (10). Taken together, all of the results thus suggest that the contribution of extracellular residues of human CCK2R is less important than in the rat CCK2R, and most of the residues involved in high affinity binding of CCK are probably located in transmembrane domains.

We next evaluated, in human CCK2R, the contribution of Tyr\(^{189}\) and Asn\(^{353}\), the corresponding amino acids of rat Tyr\(^{189}\) and Asn\(^{358}\) known to make direct interaction with the C-terminus of the CCK2R binding pocket. Furthermore, the human H185A mutant displayed a 25-fold lower CCK affinity versus 456-fold in the rat (10). Taken together, all of the results thus suggest that the contribution of extracellular residues of human CCK2R is less important than in the rat CCK2R, and most of the residues involved in high affinity binding of CCK are probably located in transmembrane domains.

Concerning the lower contribution of Tyr\(^{189}\) in the human CCK2R binding site (as compared with rat CCK2R), in both cases, two types of interactions are seen in the modeled structure: a hydrogen bond with the carbonyl group of Phe\(^9\) of CCK and \(\pi-\pi\) electronic interactions between the two rings. However, the relative positions of the two aromatic rings (Tyr\(^{189}\) and Phe\(^9\) of CCK) are sufficiently different in the human versus the rat complexes to explain the mutagenesis results. In fact, according to dynamic simulation experiments, the relative position of Tyr\(^{189}\) and Phe\(^9\) of CCK, and therefore the location of CCK, within the binding pocket appears to be at least partially governed by the presence of Met\(^{234}\) (TM V) in the human CCK2R instead of an isoleucine in the rat receptor, and conversely, the presence of Met\(^{194}\) (TM IV) in the rat CCK2R instead of a valine in human receptor. In the modeled human CCK2R, Met\(^{234}\) forms a sulfur-\(\pi\) interaction with Phe\(^{342}\), which in turn can influence the aromatic network comprising residues Phe\(^{342}\), Trp\(^{346}\), Phe\(^{327}\), and Tyr\(^{350}\) (Fig. 3, B and C). In the rat CCK2R, it is Met\(^{194}\) that can influence this aromatic network but in an opposite way. Interactions between methionine and aromatic residues, such as Phe and Tyr, are recognized to play an important role in the three-dimensional structure and function of many proteins. For instance, in the CCK1R, a Met residue located in the second extracellular loop was demonstrated to play a key role for correct positioning of Tyr(SO\(_3\)H) of CCK toward its interacting partner, Arg\(^{197}\) (22, 23). In this receptor, another methionine in helix III was demonstrated to govern receptor coupling to G protein by influencing the hydrophobic pocket, which positions Phe of CCK relative to an aromatic residue of helix VI (24). The physicochemical basis of interactions between a sulfur atom and aromatic ring has been investigated and was claimed to explain why, in the protein data base, ~50% of the sulfur atoms are contacting aromatic rings (25).

Analysis of data from studies of other groups reinforces our current findings, showing that conserved residues in the CCK2R from different species contribute differently in CCK high affinity and CCK2R activation. First, although residue Trp\(^{346}\) of the human CCK2R belongs to the aromatic network (Fig. 3, A and B), its exchange for an alanine did not affect pharmacological properties of the receptor (26). In contrast, the corresponding mutation in the rat receptor caused a 34% decrease of inositol phosphate accumulation and a 10-fold decrease of both CCK affinity and potency (27). This is consistent with dynamic simulation data predicting that the C-terminal phenylalanine of CCK makes stacking ring-ring interactions with Tyr\(^{189}\) and Trp\(^{351}\) (corresponding to human Trp\(^{346}\)) located in the sixth transmembrane domain. This is also in line with the key role of Tyr\(^{189}\) in the activation of the rat CCK2R that we documented using site-directed mutagenesis (11). Second, the key role of Phe\(^{342}\) (TM VI) in the aromatic network and the fact that in the human CCK2R this residue interacts with Met\(^{234}\) (TM V) must be compared with findings that the corresponding residue in the rat sequence (Phe\(^{342}\)) is crucial for activation of phospholipase-C by the CCK2R (27, 28). Third, the works of Kopin and colleagues (19, 26) points out the importance of Tyr\(^{61}\) (junction between TM I and N-terminal
region), Met$^{186}$ (TM IV), and Thr$^{193}$ for CCK binding to the human CCK2R. Their data are fully compatible with the refined human CCK2R-CCK complex, which shows hydrogen bonds involving Tyr$^{111}$ and Tyr of CCK as well as Thr$^{193}$ and the amide of CCK (Fig. 3A). On the other hand, the role of Met$^{186}$ is likely indirect and linked to the aromatic network within the binding pocket. Using NMR nuclear Overhauser enhancement transfer, Mierke and co-worker (29) proposed interactions between Trp and Met of CCK with Pro$^{371}$ and Phe$^{374}$ (TM VII). In our modeled structure, Nle, which mimics Met of CCK, approximates Pro$^{371}$. It is interesting to note that there is a good overlapping between the binding site for the C-terminal of CCK and the suggested binding site of non-peptide ligands, as indicated by the rescue/approach approach in which deleterious mutations within the receptor were rescued by subsequent modification of the ligand (30).

To conclude, delineation of the CCK binding site of the human CCK2R, using site-directed mutagenesis and docking followed by dynamic simulations, allowed us to demonstrate that, although roughly resembling that of the rat CCK2R, this binding site also displays important distinct structural features that are confirmed by susceptibility to point mutations. These new important data, together with our experience with the CCK1R (31), will be used to understand how some of the so-called non-peptide antagonists present partial agonist activities (7). This goal is of timely importance due to the fact that CCK2R is a biological target of therapeutic interest (6).

REFERENCES
1. Silvente-Poirot, S., Dufresne, M., Vaysse, N., and Fourmy, D. (1993) Eur. J. Biochem. 215, 513–529
2. Noble, F., Wank, S. A., Crawley, J. N., Bradwejn, J., Sernogy, K. B., Hamon, M., and Roques, B. P. (1999) Pharmacol. Rev. 51, 745–781
3. de Weerth, A., Piseuga, J. R., Huppi, K., and Wank, S. A. (1993) Biochem. Biophys. Res. Commun. 194, 811–818
4. Wank, S. A., Piseuga, J. R., and de Weerth, A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8601–8605
5. Lee, Y. M., Beinborn, M., McBride, E. W., Lu, M., Kolakowski, L. F., Jr., and Kopin, A. S. (1993) J. Biol. Chem. 268, 8164–8169
6. Herranz, R. (2003) Med. Res. Rev. 23, 559–605
7. Kopin, A. S., McBride, E. W., Schaffer, K., and Beinborn, M. (2000) Trends Pharmacol. Sci. 21, 346–353
8. Beinborn, M., Lee, Y. M., McBride, E. W., Quinn, S. M., and Kopin, A. S. (1993) Nature 362, 348–350
9. Kopin, A. S., McBride, E. W., Gordon, M. C., Quinn, S. M., and Beinborn, M. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11043–11048
10. Silvente-Poirot, S., Escrieu, C., Gleys, C., Fehrentz, J. A., Escherich, A., Wank, S. A., Martinez, J., Moroder, L., Maigret, B., Bouisson, M., Vaysse, N., and Fourmy, D. (1999) J. Biol. Chem. 274, 23191–23197
11. Gales, C., Poirot, M., Taillefer, J., Maigret, B., Martinez, J., Moroder, L., Escrieu, C., Pradayrol, L., Fourmy, D., and Silvente-Poirot, S. (2003) Mol. Pharmacol. 63, 973–982
12. Silvente-Poirot, S., Escrieu, C., and Wank, S. A. (1998) Mol. Pharmacol. 54, 364–371
13. Moroder, L., Wilschowitz, L., Gemeiner, M., Gohring, W., Knof, S., Scharf, R., Thamm, P., Gardner, J. D., Solomon, T. E., and Wunsch, E. (1981) Hoppe-Seyler’s Z. Physiol. Chem. 362, 929–942
14. Fourmy, D., Lopez, P., Poirot, S., Jimenez, J., Dufresne, M., Moroder, L., Powers, S. F., and Vaysse, N. (1989) Eur. J. Biochem. 183, 397–403
15. Gigoux, V., Escrieu, C., Fehrentz, J. A., Poirot, S., Maigret, B., Moroder, L., Gully, D., Martinez, J., Vaysse, N., and Fourmy, D. (1999) J. Biol. Chem. 274, 20457–20464
16. Feng, T. M., Yu, H., and Strader, C. D. (1992) J. Biol. Chem. 267, 25668–25671
17. Causier, M. A., Huang, R. R., Feng, T. M., Cheung, A. H., Sadowski, S., Ber, E., and Strader, C. D. (1992) Mol. Pharmacol. 41, 1096–1099
18. Okesenberg, D., Marsters, S. A., O’Dowd, B. F., Jin, H., Havlik, S., Peroutka, S. J., and Ashkenazi, A. (1992) Nature 359, 161–163
19. Kopin, A. S., McBride, E. W., Quinn, S. M., Kolakowski, L. F., Jr., and Beinborn, M. (1995) J. Biol. Chem. 270, 5019–5023
20. Anders, J., Bluggel, M., Meyer, H. E., Kuhne, R., ter Laak, A. M., Kojro, E., and Fahrenholz, F. (1999) Biochemistry 38, 6045–6055
21. Jaggerschmidt, A., Guillaume-Rousselet, N., Vikland, M. L., Goudreau, N., Maigret, B., and Roques, B. P. (1996) Eur. J. Pharmacol. 296, 97–106
22. Gigoux, V., Escrieu, C., Silvente-Poirot, S., Maigret, B., Goulieux, L., Fehrentz, J. A., Gully, D., Moroder, L., Vaysse, N., and Fourmy, D. (1999) J. Biol. Chem. 274, 14380–14386
23. Gigoux, V., Maigret, B., Escrieu, C., Silvente-Poirot, S., Bouisson, M., Fehrentz, J. A., Moroder, L., Gully, D., Martinez, J., Vaysse, N., and Fourmy, D. (1999) Protein Sci. 8, 2347–2354
24. Escrieu, C., Gigoux, V., Archer, E., Verrier, S., Maigret, B., Behrendt, R., Moroder, L., Bignon, E., Silvente-Poirot, S., Pradayrol, L., and Fourmy, D. (2002) J. Biol. Chem. 277, 7546–7555
25. Viguera, A. R., and Serrano, L. (1995) Biochemistry 34, 8771–8779
26. Blaker, M., Ren, Y., Gordon, M. C., Hsu, J. E., Beinborn, M., and Kopin, A. S. (1990) Mol. Pharmacol. 38, 857–863
27. Jaggerschmidt, A., Guillaume, N., Roques, B. P., and Noble, F. (1998) Mol. Pharmacol. 53, 878–885
28. Pommer, B., Marie-Claire, C., Da Nascimento, S., Wang, H. L., Roques, B. P., and Noble, F. (2003) J. Neurochem. 85, 454–461
29. Giragosian, C., and Mierke, D. F. (2002) Biochemistry 41, 4560–4566
30. Kopin, A. S., McBride, E. W., Chen, C., Freidinger, R. M., Chen, D., Zhao, C. M., and Beinborn, M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5925–5930
31. Archer-Lahlou, E., Escrieu, C., Clerc, P., Martinez, J., Moroder, L., Logsdon, C., Kopin, A., Seva, C., Dufresne, M., Pradayrol, L., Maigret, B., and Fourmy, D. (2005) J. Biol. Chem. 280, 10664–10674