Juxtamembrane Region of the Amino Terminus of the Corticotropin Releasing Factor Receptor Type 1 Is Important for Ligand Interaction

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Received July 27, 2000; Revised Manuscript Received October 25, 2000

ABSTRACT: The functional properties of the amino terminus (NT) of the corticotropin releasing factor (CRF) receptor type 1 (R1) were studied by use of murine (m) CRFR1 and rat (r) parathyroid hormone (PTH)/parathyroid hormone-related peptide receptor (PTH1R) chimeras. The chimeric receptor CXP, in which the NT of mCRFR1 was annealed to the TMs of PTH1R, and the reciprocal hybrid, PXC, bound radiolabeled analogues of sauvagine and PTH(3−34), respectively. Neither hybrid bound radiolabeled CRF or PTH(1−34). CRF and PTH(1−34) weakly stimulated intracellular cAMP accumulation in COS-7 cells transfected with PXC and CXP, respectively. Thus the NT is required for ligand binding and the TMs are required for agonist-stimulated cAMP accumulation. Replacing individual intercysteine segments of PXC with their mCRFR1 counterparts did not rescue CRF or sauvagine radioligand binding or stimulation of cAMP accumulation. Replacement of residues 1−31 of mCRFR1 with their PTH1R counterparts resulted in a chimeric receptor, PEC, which had normal CRFR1 functional properties. In addition, a series of chimeras (F1PEC−F6PEC) were generated by replacement of the NT intercysteine residues of PEC with their PTH1R counterparts. Only F1PEC, F2PEC, and F3PEC showed detectable CRF and sauvagine radioligand binding. All of the PEC chimeras except F5PEC increased cAMP accumulation. These data indicate that the Cys^{68}−Glu^{109} domain is important for binding and that the Cys^{87}−Cys^{102} region plays an important role in CRFR1 activation.

The neuropeptide corticotropin releasing factor (CRF)[1] binds to specific CRF receptors in the brain and anterior pituitary and consequently regulates behavioral and endocrine responses to stress. CRF exerts its action through specific G protein-coupled receptors that consist of an extracellular amino terminus (NT), seven transmembrane domains (TM), three extracellular loops (EC), three intracellular loops (IC), and an intracellular carboxy terminus (CT). The CRF receptors share structural similarity with the PTH family of receptors. The NT of these receptors contains six cysteine residues and is intermediate in length when compared to the extensive NT of glycoprotein hormone receptors or to the short NT of the adrenergic receptors. Two CRF receptor genes, CRFR1 and CRFR2, which share 70% sequence identity, have been identified. CRFR1 was cloned from human, mouse, sheep, chicken, *Xenopus*, rat, and shrew (*1*−*7*). Three alternatively spliced variants of CRFR2, all within the NT region, have been identified (*8*). Activation of the CRF receptors results in stimulation of adenylate cyclase (*9*−*14*) and phospholipase C (*15*).

The CRF receptors interact with multiple CRF-related peptides. Three CRF-related peptides have been described in different species. Sauvagine is a 40 amino acid peptide isolated from the skin of the frog *Phylomedosa sauvgei* (*16*); urotensin I is a 41 amino acid peptide isolated from the caudal neuorosecretory organ of teleost fish (*17*); and urocortin, a mammalian homologue of urotensin I, was isolated from rat and human brain (*18*). Mammalian CRFR1 shows equal binding affinities for ovine, rat/human (r/h), and bovine CRF, as well as urotensin I, sauvagine, and urocortin (*9*−*13*). Although urotensin I, urocortin, and sauvagine bind with high affinity to CRFR1, they have a much higher affinity for CRFR2 (*9*, *13*, *18*, *19*).

CRF and CRF-related ligands and their receptors mediate complex endocrine and behavioral functions. Neuroendocrine abnormalities involving the CRF system have been implicated in a number of disorders, including anxiety, depression, eating disorders, Alzheimer's disease, and stroke (*20*, *21*). Targeting these diseases with specific pharmacologic agents, which interact with CRF receptors, is underway (*22*); however, our understanding of the receptor's structural properties that determine ligand binding specificity and receptor activation is incomplete. Receptor chimeras have been informative for mapping ligand recognition domains for several members of this receptor family (*23*−*26*). These chimeric receptor studies have provided evidence for the role

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[1] Abbreviations: NT, amino terminus; CRF, corticotropin releasing factor; R1, receptor type 1; m, murine; r, rat; PTH, parathyroid hormone; PTHrP, parathyroid hormone-related peptide; PTH1R, parathyroid hormone/parathyroid hormone-related peptide receptor chimeras; CRF, corticotropin-releasing factor; TM, transmembrane domains; EC, extracellular loops; IC, intracellular loops; CT, carboxy terminus; r/h, rat/human; ECR, c-*myc*-tagged murine corticotropin releasing factor receptor type 1; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; ATTC, American Type Tissue Culture; YQLS, [Gln^0,Tyr^1,Leu^{17}]-substituted sauvagine; PTH(3−34), [Nle^{8-18},Tyr^{34}]-substituted parathyroid hormone, residues 3−34, ending in NH_2; PTH-(1−34), [Nle^{8-18},Tyr^{34}]-substituted parathyroid hormone residues 1−34, ending in NH_2; TFA, trifluoroacetic acid; ACN, acetonitrile; IBMX, 3-isobutyl-1-methylxanthine. See Figure 1 for nomeclature of receptor chimeras.

10.1021/bi001758y CCC: $20.00
Published on Web 01/10/2001

of the NT domains in ligand binding and of the TM domains in receptor activation.

Several studies have recently characterized important ligand-binding domains within the CRF receptors. The EC3 loop of CRFR1 was implicated in CRF and urocortin binding (*27*, *28*). Additionally, the NT of CRFR1 is important for the binding of urocortin and astressin to a chimeric rCRFR1/rat growth hormone releasing factor receptor chimera (*29*). Studies with chimeric CRFR1/CRFR2 have determined that residues within the EC1 and EC2 domains are important for r/hCRF, urocortin, and sauvagine binding (*30*, *31*). Chimeras between the ligand-selective *Xenopus* CRFR1 and the nonselective hCRFR1 mapped the sauvagine-selective binding domain in hCRFR1 to residues Arg^{76}, Asn^{81}, and Gly^{93} within the NT (*26*).

The reviewed data elucidate some important residues for CRF and CRF-related peptides binding within the extracellular domain of CRFR1. The present study is designed to identify the ligand interaction domains within the entire NT of the CRFR1. One important feature of the NT is the presence of disulfide bridges that may form a ligand-binding pocket (*32*). We examined the role of the segments flanked by the highly conserved cysteine residues in ligand interaction by constructing CRFR1 and PTH1R hybrids in which the corresponding intercysteine residues were exchanged. The murine CRFR1 and PTH1R are 57% similar in amino acid sequence but possess distinct ligand binding selectivity. This high level of sequence homology increases the likelihood that the chimeras would maintain a functional conformation. The distinct ligand specificity of these two receptors allows mapping of the domains required for ligand selectivity in reciprocal hybrids.

EXPERIMENTAL PROCEDURES

Construction of Epitope-Tagged Mouse CRFR-1 and Epitope-Tagged PTH1Rs. A c-*myc*-tagged CRFR1 was constructed by inserting eight amino acids, QKLSEEDL, from the c-*myc* epitope within the NT of mCRFR1 between Glu^{31} and Ser^{32}. The resulting epitope-tagged CRFR1 (ECR) had similar functional properties to the wild type (*32*). The PTH1R was tagged with either the human influenza hemagglutinin (HA) tag (YPYDVPDYA) or the c-*myc* tag (QQKLISEEDL) by replacing the amino acid sequences ENKDVPTGS and SGKFYPESKENKDV, respectively (*33*). This region is known to be tolerant to mutations in the PTH1R (*33*).

Construction of the Chimeric Mutants. An *Xma*I site was introduced by site-directed mutagenesis at the junction of NT and TM1 of HA-PTH1R and ECR cloned in pcDNA1 (Invitrogen, Carlsbad CA). Insertion of the restriction site resulted in three point mutations within the ECR sequence, Lys^{110}, Lys^{111}, and Ser^{112} to Thr, Arg, and Glu, respectively, which is the corresponding sequence found in PTH1R. These mutations did not change the functional properties of the receptor. Insertion of the *Xma*I site in HA-PTH1R did not change the amino acid sequence. The amino termini of both receptors were exchanged at the *Xma*I site resulting in two chimeric receptors, CXP [ECR (1−112), PTH1R (178−591)] and PXC [PTH1R (1−180), ECR (113−415)].

A silent *Eco*RV restriction site, which was included within the c-*myc* epitope of both ECR and c-*myc*-PTH1R, and a unique *Mlu*I restriction site, which exists within the pcDNAI vector, was utilized to exchange receptor fragments within the amino termini of ECR and c-*myc*-PTH1R. The *Eco*RV/*Mlu*I fragments of both ECR and c-*myc*-PTH1R were exchanged, resulting in the PEC chimeric mutant [c-*myc*-PTH1R (1−97), ECR (32−415)]. Both PEC and PXC were used as templates for the construction of the other chimeric mutants (Figure 1).

Table 1: Cell Surface Expression of the PEC and PXC Chimeric Receptors in Transiently Transfected COS-7 Cells[a]

PXC series[b]	expression (% of control)	PEC series[c]	expression (% of control)
PXC	76 ± 8.4	PEC	133 ± 1.4
F1PXC	150 ± 5.4	F1PEC	110 ± 1.4
F2PXC	65 ± 7.2	F2PEC	145 ± 4.0
F3PXC	69 ± 1.1	F3PEC	130 ± 3.5
F4PXC	38 ± 2.7	F4PEC	100 ± 7.5
F5PXC	51 ± 1.5	F5PEC	110 ± 2.7
F6PXC	81 ± 8.4	F6PEC	128 ± 4.0
F5−6PXC	91 ± 11.0		

[a] Cell surface expression was measured by the 9E10 and G48 antibody binding directed against the c-*myc*-and HA epitopes inserted in the amino termini of PEC and PXC (Figure 1). The data are means of two experiments. Each experiments was performed in triplicate wells. The data are means ± SD. [b] Expression is normalized to the expression of HA-tagged PTH1R except for F1PXC, which is normalized to the c-*myc*-tagged mouse CRFR1. [c] Expression is normalized to the c-*myc*-tagged mouse CRFR1 (ECR).

Homologue-scanning mutagenesis was used to exchange segments between CRFR1 and PTH1R. These segments correspond to the residues flanked by Cys^{30}−Cys^{44}, Cys^{44}−Cys^{54}, Cys^{54}−Cys^{68}, Cys^{68}−Cys^{87}, Cys^{87}−Cys^{102}, and Cys^{102}−Glu^{109} in c-*myc*-tagged mCRFR1 and by Cys^{48}−Cys^{108}, Cys^{108}−Cys^{117}, Cys^{117}−Cys^{131}, Cys^{131}−Cys^{148}, Cys^{148}−Cys^{170}, and Cys^{170}−Glu^{177} in PTH1R, respectively. The intercysteine blocks were exchanged to preserve the integrity of the disulfide bonds. The chimeric receptors F1PEC, F2PEC, F3PEC, F4PEC, F5PEC, and F6PEC correspond to replacement of intercysteine sequences from PEC with the corresponding sequences from PTH1R (Figure 1). The other series of chimeric receptors F1PXC, F2PXC, F3PXC, F4PXC, F5PXC, and F6PXC correspond to replacement of intercysteine sequences from PXC with the corresponding sequences from mCRFR1 (Figure 1). These chimeras were constructed by site-directed mutagenesis or PCR. All mutations were confirmed by sequencing.

COS-7 Cell Transfection. COS-7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin and streptomycin. The cells were transfected at 90% confluency by the DEAE-dextran method (*15*). Cells were harvested at 72 h and measurements of receptor expression on the cell surface, cAMP accumulation, and radioligand binding assays were performed.

Measurement of Receptor Expression on the Cell Surface. Ascites fluid enriched with the anti-c-*myc* monoclonal antibody 9E10 was produced by injecting hybridoma cells, obtained from American Type Tissue Culture (ATTC), in the peritoneal cavity of pristane-primed Balb-c mice (Charles River Laboratories, Wilmington, MA). The anti-HA monoclonal antibody 12CA5 was purchased from Boehringer Mannheim (Indianapolis, IN). Transfected COS-7 cells in 24-well plates were rinsed with phosphate-buffered saline

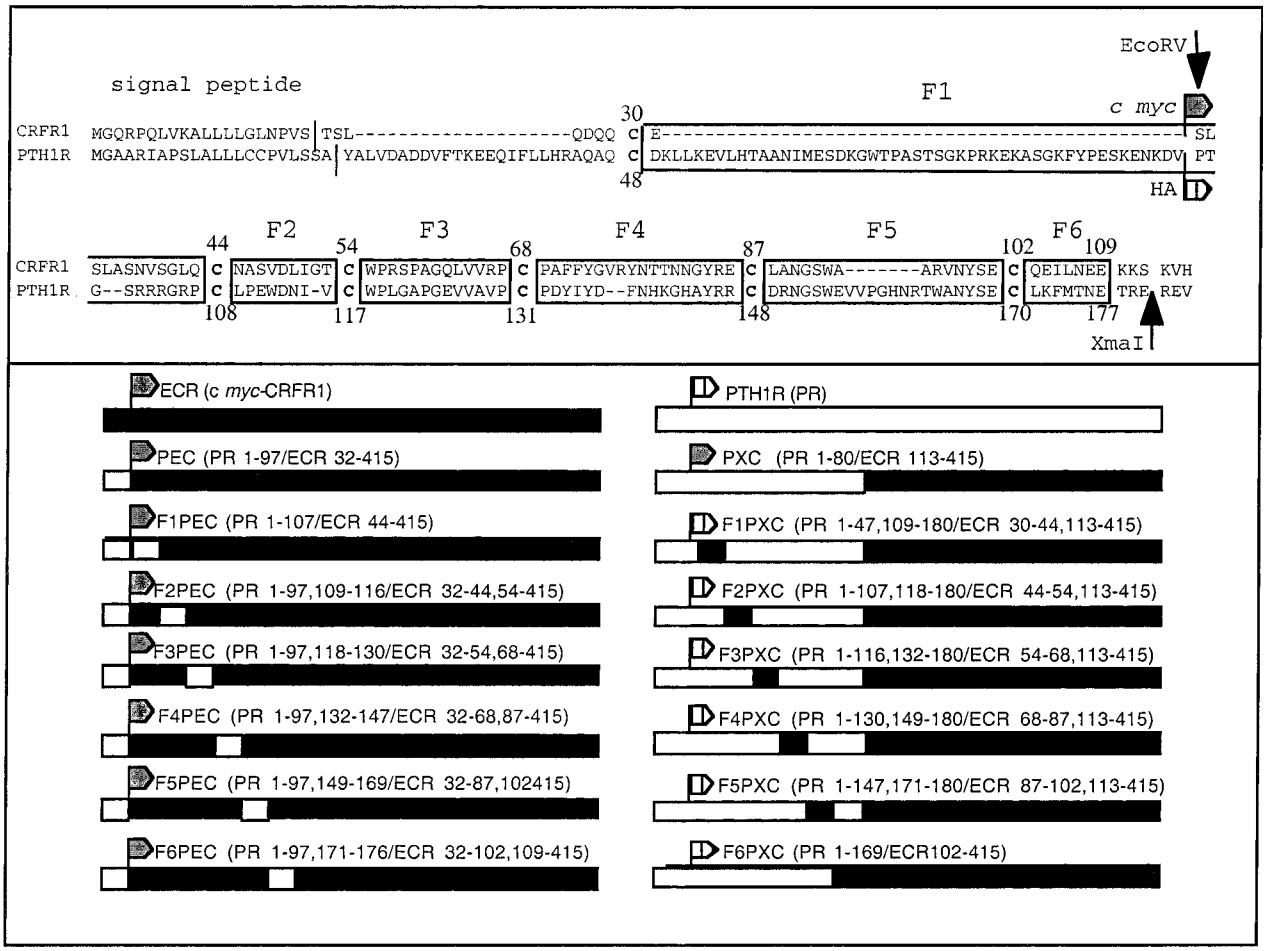

FIGURE 1: Amino acid alignment of CRFR1 and PTH1R amino termini (NT) and a schematic representation of the chimeric mutants. (Upper panel) Cysteine residues in mCRFR1 and PTH1R are aligned and the exchanged residues representing the F1−F6 regions are boxed. The arrows indicate the position of the c-*myc* and HA epitope tag. The *Xma*I site represents the positions of the exchanged NT and TMs in CXP and PXC. (Lower panel) Schematic representation of PEC and PXC chimeras. The numbers represent the amino acid residues of each chimera.

(PBS) containing 5% heat-inactivated fetal bovine serum (FBS) and incubated with the monoclonal antibody 9E10 or 12CA5 at 1:1000 dilution. The cells were incubated for 2 h at room temperature, rinsed with PBS, and incubated for another 2 h with ^{125}I-labeled sheep anti-mouse immunoglobulin G diluted in PBS/5% FBS (200 000 cpm/well). The supernatant was removed, and the cells were washed (3×) and then lysed with 1 N NaOH. The cell lysates were collected and the radioactivity was counted in a micromedic γ counter.

Radioligand Binding Assay. Radioligand binding assays were performed as previously described (*15*). Intact COS-7 cells in 24-well plates were rinsed with a binding buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 5% heat-inactivated horse serum, 0.5% heat-inactivated fetal bovine serum) and incubated with ^{125}I-ligand (100 000 cpm/well) in the presence of increasing concentrations of unlabeled ligand (0, 0.01, 1.0, 3.0, 10, 30, 100, and 1000 nM) at room temperature for 2 h. At the end of the incubation period, the cells were rinsed three times with binding buffer and lysed with 1 N NaOH. The lysates were collected and counted for radioactivity.

Peptide Radiolabeling. The sauvagine analogue [Gln^0,-Tyr^1,Leu^{17}]sauvagine (YQLS) and the PTH analogues [Nle$^{8-18}$,Tyr^{34}]bPTH(3−34)NH_2 [PTH(3−34)] or [Nle$^{8-18}$,Tyr^{34}]bPTH(1−34)NH_2 [PTH(1−34)] (5 μg each) were radiolabeled by use of chloramine T (14 μg) and Na^{125}I (1.5 mCi of 2200 Ci/mmol; NEN Life Science Products). The reaction was terminated by adding metabisulfate (35 μg). Excess Na^{125}I and other salts were removed by a SepPak C18 cartridge that was equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with 70% acetonitrile (ACN) and 0.1% TFA. The radiolabeled peptides were purified on a C18 column by high-performance liquid chromatography. The radiolabeled peptides were eluted with a 30−50% ACN gradient in 0.1% TFA for PTH(3−34) or PTH(1−34) and a 40−70% ACN gradient in 0.1% TFA for YQLS.

Agonist-Stimulated cAMP Accumulation. Intact COS-7 cells in 24-well plates, transfected with the CRF receptor constructs, were chilled on ice for 30 min, rinsed with ice-cold PBS, and challenged with r/hCRF in DMEM containing 2 mM 3-isobutyl-1-methylxanthine (IBMX), 1 mg/mL BSA, and 35 mM HEPES, pH 7.4. The cells were then incubated at 37 °C for 15 min. The supernatant was removed and the plates were frozen on dry ice for 10 min. Intracellular cAMP was extracted by thawing the cells in 50 mM HCl (1.0 mL). An aliquot of the acid extract was diluted (1:100) in sodium acetate buffer (50 mM, pH 5.5) and the cAMP content was determined by radioimmunoassay (*34*).

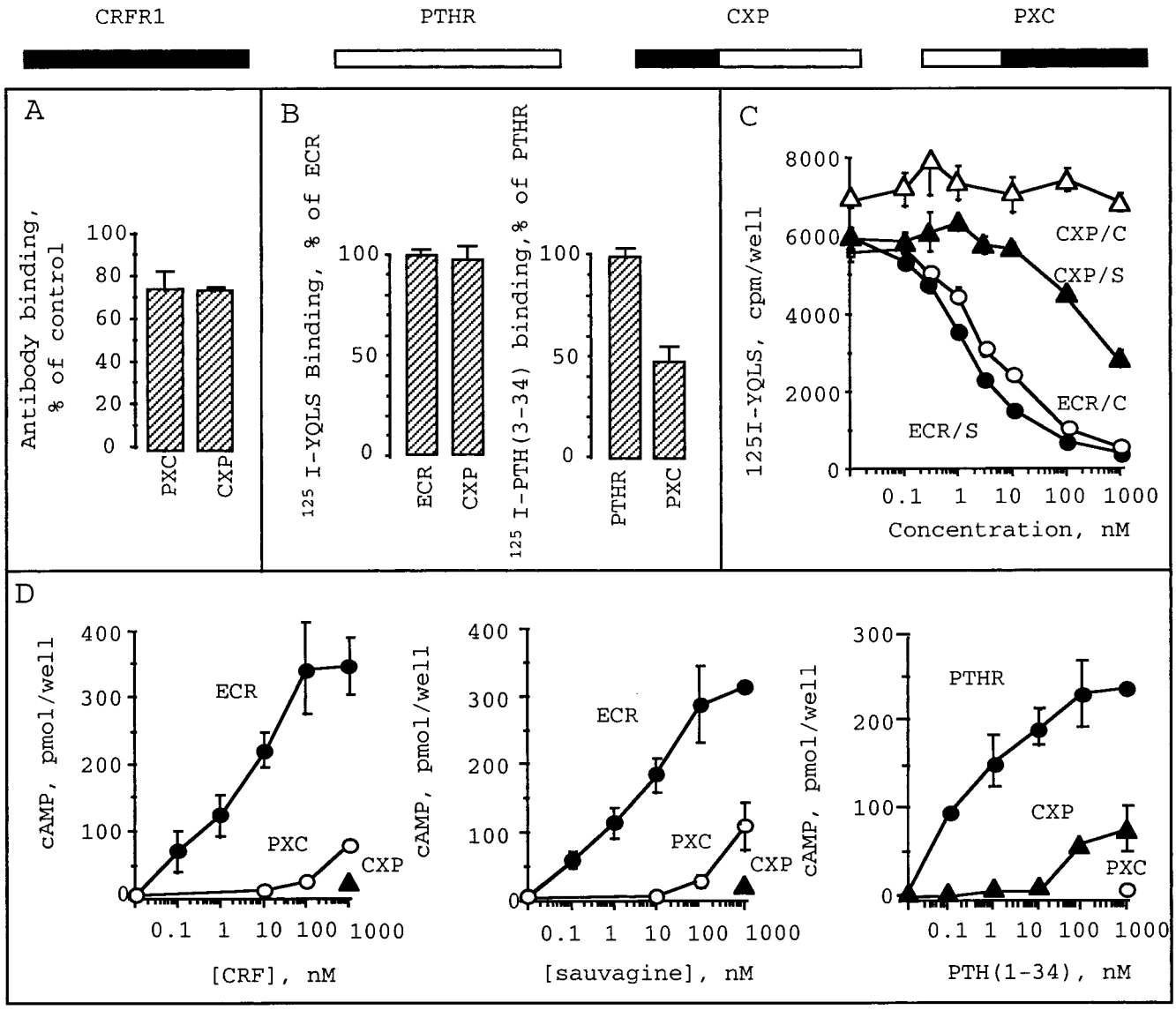

FIGURE 2: Cell surface expression, agonist binding, and agonist-induced stimulation of cAMP accumulation in COS-7 cells transiently expressing the wild type and the PXC and CXP chimeric receptors. (A) Cell surface expression was measured by 9E10 and 12CA5 antibody binding directed against the c-*myc* and the HA epitopes inserted in the NT of CXP and PXC, respectively. The data represent percent expression of PXC and CXP as compared to HA-tagged PTH1R and c-*myc*-tagged CRFR1 (ECR), respectively. (B) Specific binding of ^{125}I-YQLS and ^{125}I-PTH(3−34) to epitope-tagged PTH1R, ECR, PXC, and CXP. The cells were incubated with 100 000 cpm/well of the radioligand and nonspecific binding was measured in the presence of 1000 nM unlabeled ligands and was subtracted from total binding. (C) Competition of ^{125}I-YQLS binding to CXP and ECR with increasing concentrations of unlabeled sauvagine (S) and CRF (C). (D) Stimulation of cAMP accumulation (15 min, 37 °C, 2 mM IBMX) by CRF, sauvagine, and PTH(1−34) in COS-7 cells transfected with PTH1R, ECR, PXC, and CXP. All panels are representative figures of two experiments performed in triplicate wells with similar results.

RESULTS

Functional Importance of the CRFR1 Amino-Terminal Domain. Insertion of the c-*myc* epitope tag into the mouse CRFR1 (Figure 1) has been shown not to alter its CRF binding or signaling properties (*32*). The c-*myc* tag enables assessment of cell surface expression of the receptor by a double antibody binding assay in intact cells (Figure 2A). Stimulation of cAMP accumulation, cell surface expression, and radioligand binding was assessed for both CXP and PXC and was compared to those of the c-*myc*-tagged mCRFR1 (ECR) and HA-PTH1R. Expression of both CXP and PXC was 73% of control (Figure 2A). Sauvagine but not CRF competed with ^{125}I-YQLS for binding to CXP ($K_i \approx 500$ nM) (Figure 2B,C). A radiolabeled truncated PTH analogue, ^{125}I-PTH(3−34), specifically bound to PXC (Figure 2B), whereas the intact PTH analogue, ^{125}I-PTH(1−34), did not bind to PXC (data not shown). Weak cAMP stimulation was observed when PXC was challenged with CRF and sauvagine (Figure 2D), and no stimulation was observed when PXC was challenged with PTH(1−34). Similarly, a weak cAMP stimulation was observed in CXP challenged with PTH(1−34) (Figure 2D); however, CRF and sauvagine did not increase cAMP accumulation with this chimera (Figure 2D). These data indicate that the amino-terminal domains of both receptors are important for ligand binding and that the region containing the TM domains, the connecting EC and IC loops, and CT are sufficient for signaling. However, efficient ligand−receptor interaction requires both the extracellular domain and the transmembrane domain regions.

Properties of the PEC Chimeric Mutants. To further characterize the domains required for ligand interactions within the NT of CRFR1 we used another chimera, PEC, in

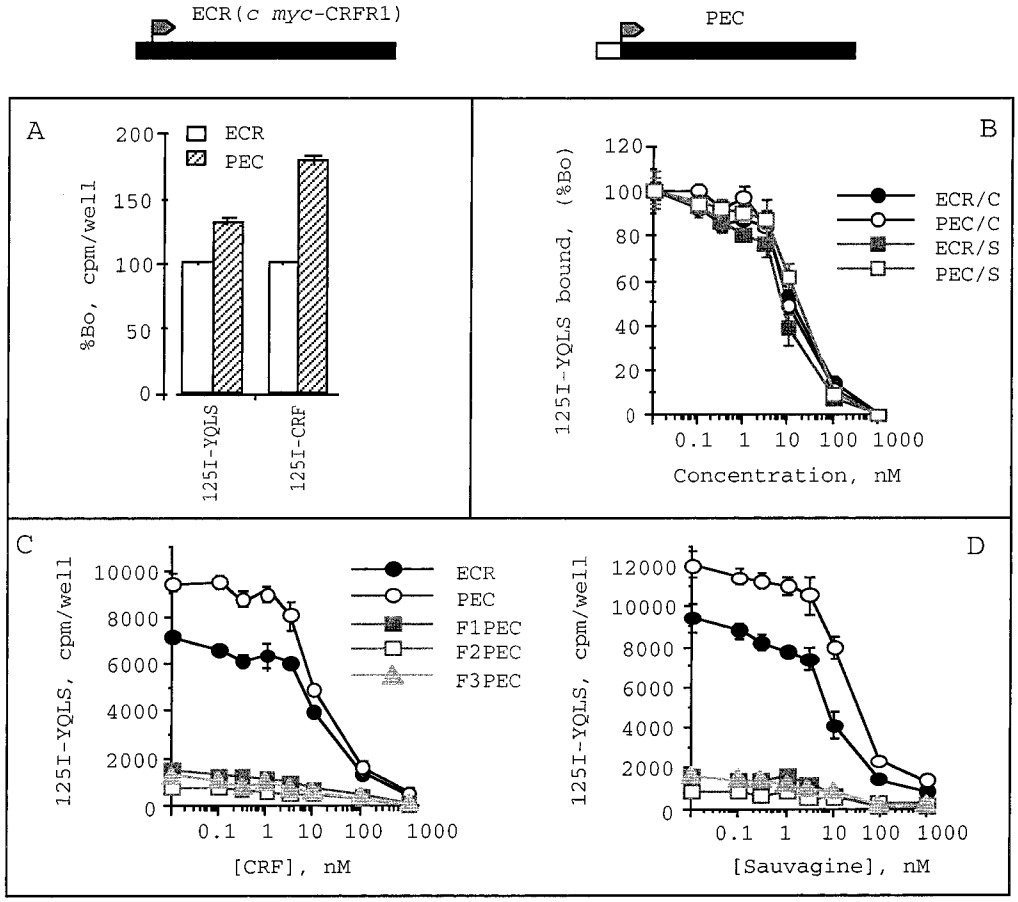

FIGURE 3: Ligand binding to ECR and the PEC chimeras: Competition of ^{125}I-YQLS binding to PEC, ECR, and F1−F3PEC chimeras with increasing concentrations of unlabeled CRF (A, C) and sauvagine (B, D). All panels are representative figures of 2−5 experiments performed in triplicate wells.

which the first 31 residues of mCRFR1 were replaced by the first 97 residues of the c-*myc*-PTH1R (Figure 1). Cell surface expression of PEC was 133% of ECR (Table 1) while specific binding of ^{125}I-YQLS and ^{125}I-CRF to PEC was 130% and 180% of control, respectively (Figure 3A). The apparent binding affinities of sauvagine and CRF to PEC and ECR were similar (Figure 3B, Table 1); this suggests that the increased ligand binding is secondary to increased expression. CRF and sauvagine increased cAMP accumulation similarly and dose-dependently in PEC and ECR (Figure 4A,B, Table 1). These data indicate that the first 31 residues of CRFR1 are not critical for CRF receptor binding specificity and that the corresponding residues from PTH1R can functionally substitute for them without impairing affinity for CRF or sauvagine or altering the receptor's signaling properties stimulated by both ligands. Downstream domains of the NT were then investigated with a series of chimeric mutants based on PEC; these chimeras were designed to add more PTH1R sequences to the PEC background by a homologue-scanning approach. The following chimeras were constructed, F1PEC, F2PEC, F3PEC, F4PEC, F5PEC, and F6PEC, by replacing amino-terminal regions flanked by consecutive cysteine residues in CRFR1 with corresponding residues from PTH1R (Figure 1). All of the PEC chimeras were expressed at levels similar to or higher than that of ECR (Table 1). Only F1PEC, F2PEC, and F3PEC bound ^{125}I-YQLS (Figure 3C,D) at detectable levels (~2% of total cpm added). The apparent binding K_is for CRF and sauvagine in F1PEC, F2PEC, and F3PEC, are similar (Table 2). No detectable radioligand binding was observed with the other chimeras (F4PEC−F6PEC). Additionally, F1PEC, F2PEC, and F3PEC showed binding to ^{125}I-CRF that was similar to that observed for ^{125}I-YQLS (data not shown), while F4PEC, F5PEC, and F6PEC had no detectable binding. F1PEC, F2PEC, and F3PEC increased cAMP accumulation when challenged with CRF or sauvagine (Figure 4C,D), and their EC_{50} values for CRF and sauvagine were not significantly different from those of ECR or PEC (Table 2). As predicted from the diminished binding, F4PEC had a relatively decreased response to CRF (Figure 4C, Table 2) and to sauvagine (Figure 4D, Table 2) compared to PEC or ECR (Table 2). F5PEC was weakly stimulated by CRF, whereas F6PEC had a relatively good stimulation by CRF (Figure 4C, Table 2). In contrast, both F5PEC and F6PEC had weak responses to sauvagine (Figure 4D, Table 2). These data indicate that the region flanked by Cys^{87} and Glu^{109} (F5−F6) is important for the interaction of CRFR1 with both CRF and sauvagine.

Properties of the PXC Chimeric Mutants. Neither CRF nor sauvagine radioligands bound PXC, although these agonists did weakly increase cAMP accumulation (Figure 2D). To examine whether any of the CRFR1 intercysteine segments improves the PXC responsiveness to CRF or sauvagine, we exchanged each intercysteine segment in PXC with the corresponding segment from mCRFR1. Six chimeric receptors, F1PXC, F2PXC, F3PXC, F4PXC, F5PXC, and F6PXC, were constructed, each of which had an HA tag, except for F1PXC, which had a c-*myc* epitope. PXC, F1PXC,

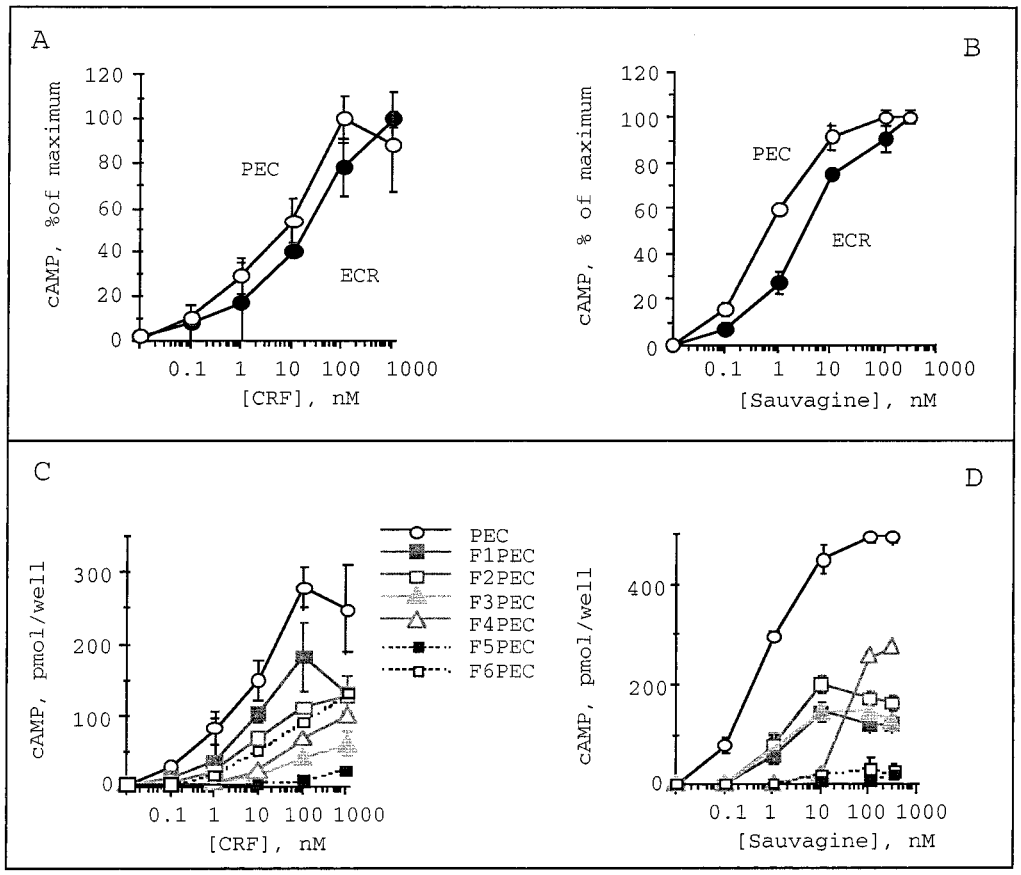

FIGURE 4: Agonist-induced stimulation of cAMP in COS-7 cells transiently expressing the wild-type and PEC chimeric receptors: Stimulation of cAMP accumulation (15 min, 37 °C, 2 mM IBMX) by CRF (A, C) and sauvagine (B, D) in COS-7 cells transfected with PEC and ECR. All panels are representative figures of 2−4 experiments performed in triplicate wells.

Table 2: Apparent Binding Affinities (K_is) and cAMP Accumulation EC_{50}s of PEC Chimeric Mutants[a]

mutant	K_i(sauvagine) (nM)	K_i(CRF) (nM)	EC_{50}(sauvagine) (nM)	EC_{50}(CRF) (nM)
ECR	3.8 ± 2.4	11.2 ± 10.9	4.5 ± 0.4	6.3 ± 4
PEC	5.6 ± 4.3	17.6 ± 14.5	1.25 ± 0.2	3.0 ± 2.5
F1PEC	3.0 ± 0.6	12.0 ± 7.2	1.3 ± 0.5	8.3 ± 2.9
F2PEC	12.4 ± 3.3	18.2 ± 4.6	3.2 ± 3.3	13.1 ± 7.0
F3PEC	9.5 ± 3.7	6.5 ± 4.3	1.2 ± 0.3	17.7 ± 6.8
F4PEC	NB[b]	NB	27 ± 7.6	18.0 ± 7.6
F5PEC	NB	NB	>100	>100
F6PEC	NB	NB	>100	11.3 ± 8.0

[a] Each experiment was performed in triplicate wells. The data are means ± SD of 2−5 experiments. [b] NB, no radioligand binding was detected.

F2PXC, F3PXC, F5PXC, and F6PXC expression levels were at least 50% of control (Table 1); only F4PXC has a relatively diminished expression level, less than 50% of control (Table 1). None of the PXC chimeras bound ^{125}I-CRF or ^{125}I-YQLS. These data indicate that individual intercysteine segments do not restore CRF binding to PXC.

The PXC chimeric mutants were then tested for CRF and sauvagine-stimulated cAMP accumulation. CRF and sauvagine increased cAMP accumulation in all the PXC mutants; however, stimulation required high concentrations of CRF and sauvagine. Maximum stimulation was not achieved with a 1000 nM concentration of CRF or sauvagine (Figure 5); these data further confirm that single intercysteine domains do not restore CRF or sauvagine responsiveness to PXC.

Since F5PEC and F6PEC have a decreased cAMP stimulation compared to PEC, we examined whether replacement of both the F5 and the F6 domains in the PXC background improved cAMP simulation and/or radioligand binding. Thus a combined F5−6PXC mutant was constructed that showed an expression level of 91% (Table 1). However, F5−6PXC did not show detectable radioligand binding (data not shown) and CRF and sauvagine-stimulated cAMP accumulation was not different from those obtained in PXC, F5PXC or F6PXC (Figure 5). These data suggest that high-affinity interaction may involve additional domains within the NT region.

DISCUSSION

The roles of the amino terminus, the transmembrane regions, and the connecting loop(s) in binding and activation of G protein-coupled receptors have been the subject of extensive investigation in multiple hormonal systems. Unlike other G protein-coupled receptors, the CRF and PTH family of receptors is characterized by unique features that include six highly conserved cysteine residues in an intermediate-sized amino-terminal domain (*23*, *24*, *35*, *36*). Other G protein-coupled receptors, such as the glycoprotein hormone receptors (*37*, *38*), have extensive amino termini that are sufficient for high-affinity binding, whereas the minimal amino termini of other receptors, such as the catecholamine receptors, may have little function in ligand recognition (*39*, *40*). Our study demonstrates that, for CRFR1, the region within the amino terminus flanked by Cys^{68} and Glu^{109}

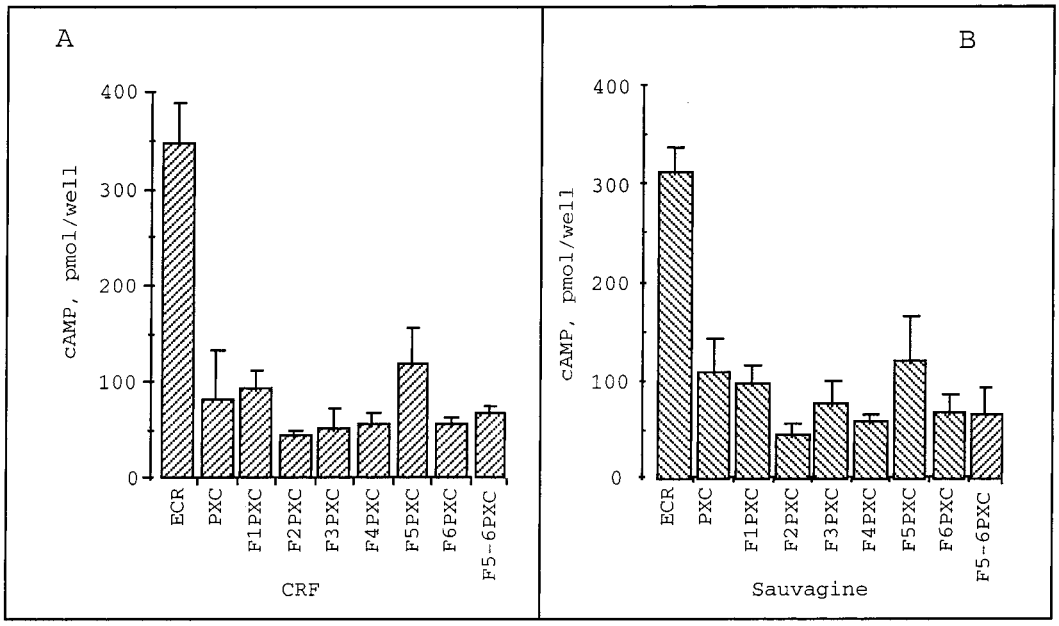

FIGURE 5: Agonist-induced stimulation of cAMP accumulation in COS-7 cells transiently expressing the wild-type and PEC chimeric receptors: Stimulation of cAMP accumulation (15 min, 37 °C, 2 mM IBMX) by 1000 nM CRF (A) and 1000 nM sauvagine (B) in COS-7 cells transfected with ECR and F1−F6PXC. All panels are representative figures of 4 experiments performed in triplicate wells with similar results.

(F4−F6), which is proximal to TM1, is required for high-affinity binding and for recognition of CRF and sauvagine by CRFR1.

The two chimeric receptors, CXP and PXC, in which the entire amino-terminal domains of CRFR1 and PTH1R were exchanged, were well expressed and both chimeric receptors bound analogues of their cognate ligands with a specificity determined by the NT region. However, CXP bound a radiolabeled sauvagine analogue (YQLS) but did not bind the radiolabeled CRF. These data indicate that, in contrast to the sauvagine analogue, CRF binding involves interactions with the EC and/or TM domains of CRFR1 for high-affinity binding. This interpretation is consistent with the reported data, in which astressin (a peptidic antagonist based on a N-terminally truncated CRF molecule) but not intact urocortin (a CRF-related peptide) bound to a CRFR1/activin IIB chimeric receptor in which the NT of CRFR1 was annealed to the TM of the activin IIB receptor (*29*). Interestingly, PXC bound a radiolabeled amino-terminally truncated PTH analogue [PTH(3−34)] (∼50% of PTH1R total binding) but did not bind intact PTH(1−34) or PTHrP-(1−36) radioligand. The heterologous TMs and connecting loops in the reciprocal hybrids, CXP and PXC, may hinder the binding of CRF and PTH(1−34) to CXP and PXC but not the binding of the sauvagine and PTH(3−34) analogues to these hybrids. It is possible that additional domains may be required for the binding of CRF and PTH(1−34) to CXP and PXC, respectively. In this regard, the EC3 of CRFR1 was shown to contain important residues for CRF interaction (*28*). Similarly, the EC3 of PTH1R contains residues that are important for PTH(1−34) but not PTH(3−34) interaction (*24*,*41*). Taken together, these data suggest poor molecular complementarity between the NT and/or connecting loops in the PXC and CXP chimera, resulting in molecular hindrance of CRF agonist binding, whereas the N-terminally truncated antagonists appear to be less dependent on such complementarity. Our data that the sauvagine analogue binds to CXP also suggest that sauvagine binding is less dependent on the TMs and the EC loops of CRFR1.

Replacement of the first 31 residues in CRFR1 with the corresponding amino-terminal region from PTH1R resulted in a chimeric receptor that displayed wild-type CRFR1 functional characteristics; this suggests that the first 31 residues are not essential for CRF or sauvagine binding and activation specificity and that the PTH1R sequences in PEC adequately substituted for the replaced CRFR1 sequences. Interestingly, this region was found to be important for PTH/PTH1R interaction (*24*, *42*, *43*). Thus this most amino-terminal domain appears to play different roles in CRFR1 and PTH1R.

The highly conserved six extracellular cysteine residues within the NT are of particular interest in the CRF/PTH family of receptors. We have previously shown that four of these cysteines are essential for CRFR1 function (*32*). In the present study we used a homologue-scanning approach to address the role of the residues between the six cysteines (F1−F6 domains). Reciprocal replacement of F1, F2, or F3 regions in PXC or PEC with the corresponding amino acid residues from CRFR1 and PTH1R, respectively, did not improve the responsiveness of PXC to CRF or sauvagine, nor did they abolish the function of PEC. These data indicate that the NT region up to the fourth conserved cysteine (Cys^{68}) in CRFR1 is not critical for ligand binding, although it may play a less critical role in receptor conformation and/or binding affinity. For instance, F1, F2, and F3PEC had normal expression levels but decreased maximum radioligand binding capacity; this decrease could result mostly from a decreased binding affinity, however, no substantial decrease in apparent binding affinity was observed. It is therefore possible that a large fraction of the surface-expressed receptors is in a low-affinity or a nonfunctional conformation and a relatively smaller fraction exists in a conformation that displays normal binding and signaling properties. Our data concur with published data in which deletion of a segment

in hCRFR1 that corresponds to the F1 region did not affect receptor function (*44*). However, the F2 region was reported to be critical for CRF interaction in hCRFR1 and hVIP−R2 hybrids (*44*). Alternatively, the F2 region of PTH1R may compensate for the absence of the authentic CRFR1 residues. Taken together, our data indicate that compared to the F4, F5, and F6 regions the F1, F2, and F3 domains play a less important role in CRFR1 ligand interaction.

Replacement of either of F4, F5, or F6 regions in PEC with the corresponding amino acid residues from PTH1R fully abolished ligand binding of the PEC chimeras, although all of these chimeras had good expression levels. These data suggest that the F4, F5, and F6 regions are required for CRF and sauvagine binding specificity and are consistent with published reports showing that the F4 region of hCRFR1 is important for sauvagine binding (*44*). Replacement of the F4, F5, or F6 regions in PXC with the corresponding sequences from CRFR1 did not cause a gain in radioligand binding. Thus, none of these intercysteine CRFR1 domains alone is sufficient for radioligand binding.

The F5PEC chimera had a dramatic decrease in CRF and sauvagine-stimulated cAMP accumulation, suggesting that the F5 region is particularly important for stabilizing the interaction of both ligands with CRFR1. In contrast, F6PEC had a blunted response to sauvagine but not to CRF, suggesting that the residues flanked by Cys^{102}−Glu^{109} are particularly important for sauvagine interaction. Similarly, the dramatically decreased sauvagine potency in F4PEC when compared to that of CRF suggests that the residues flanked by Cys^{68}−Cys^{87} are more important for sauvagine than for CRF interactions. Taken together these data indicate an important role for the F4, F5, and F6 regions in ligand interaction and show that there is a variable degree of specificity for CRF and sauvagine, with F4 and F6 being particularly important for the latter peptide.

In conclusion, the NT domain is required for the interaction of CRF and sauvagine with CRFR1. Productive interactions in terms of receptor activation and stimulation of cAMP accumulation require the presence of the TM regions. Within the NT of CRFR1, the residues flanked by Cys^{68}−Glu^{109} are particularly important for ligand−receptor interactions. Differences between the interactions of various CRF-related ligands may be exploited for drug development when the physiologic and pathologic roles are better understood.

ACKNOWLEDGMENT

We acknowledge Dr. Thomas Gardella and Dr. Louise Williams for their critical reading and comments.

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BI001758Y