Impact of N-Terminal Domains for Corticotropin-Releasing Factor (CRF) Receptor−Ligand Interactions†

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ABSTRACT: The large extracellular N-terminal domains (NTs) of class B G protein-coupled receptors serve as major ligand binding sites. However, little is known about the ligand requirements for interactions with these receptor domains. Recently, we have shown that the most potent CRF receptor agonist urocortin 1 (Ucn1) has two segregated receptor binding sites Ucn1(1−21) and Ucn1(32−40). For locating the receptor domains interacting with these two sites, we have investigated the binding of appropriate Ucn1 analogues to the receptor N-termini compared to the corresponding full-length receptors. For this purpose receptor NTs of CRF(rat) subtypes 1 and 2(α) without their signal sequences were overexpressed in *Escherichia coli* and folded in vitro. For $CRF_{2(a)}$-rNT, which bears five cysteine residues (C2−C6), the disulfide arrangement C2−C5 and C4−C6 was found, leaving C3 free. This is consistent with the disulfide pattern of CRF_1-rNT, which has six cysteines and in which C1 is paired with C3. Binding studies of N-terminally truncated or C-terminally modified Ucn1 analogues demonstrate that it is the C-terminal part, Ucn1(11−40), that binds to receptor NT, indicating a two-domain binding mechanism for Ucn binding to receptor NT. Since the binding of Ucn1 to the juxtamembrane domain has been shown to be segregated from binding to the receptor N-terminus [Hoare et al. (2004) *Biochemistry 43*, 3996−4011], a third binding domain should exist, probably comprising residues 8−10 of Ucn, which particularly contribute to a high-affinity binding to full-length receptors but not to receptor NT.

Class B GPCRs[1] include peptide−hormone receptors such as those for growth hormone releasing factor (GRF) (*2*), glucagon-like peptide (GLP) (*3*), parathyroid hormone (PTH) (*4*), secretin (*5*), calcitonin (*6*), vasoactive intestinal peptide (VIP) (*7*), pituitary adenylate cyclase activating peptide (PACAP) (*8*), and corticotropin-releasing factor (CRF) (*9*−*15*). Since receptors of this class are not yet available in the amounts required for structural analysis, binding and conformational studies of isolated extracellular domains have been intensively investigated during the last years. The diversity in naturally occurring CRF-like ligands makes the CRF receptor an ideal object for the analysis of binding characteristics of the extracellular receptor parts. CRF (*16*) is a 41-amino acid neuropeptide that is involved in the regulation of the central nervous system by controlling stress-induced secretion of adrenocorticotropic hormone (ACTH) from pituitary glands (*17*). Natural occurring analogues include sauvagine (*18*), urotensin I (*19*), urocortin 1 (*20*, *21*), urocortin 2 (*22*), and urocortin 3 (*23*). The latter two ligands affect the cardiovascular, reproductive, and immune systems (*24*, *25*). Two receptor types have been identified for CRF in mammals, the CRF_1 receptor and CRF_2 receptor (*9*−*15*), which exhibit markedly different ligand specificities (*26*). Structurally, the CRF_1 and CRF_2 receptors are highly conserved, the majority of divergence occurring in the putative signal peptide and the extracellular N-terminal receptor domain (Figure 1). Three splice variants of the CRF_2 receptor have been identified, the $CRF_{2(a)}$ receptor being the dominant variant expressed both in the mammalian brain and in peripheral tissues of humans (*27*). $CRF_{2(a)}$ differs from the $CRF_{2(b)}$ receptor mainly in the N-terminal region and most markedly in the lengths of the putative signal peptides. As a consequence, the number of cysteines in the N-terminal extracellular domains (and therefore the arrangement of the

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[1] Abbreviations: ACN, acetonitrile; BSA, bovine serum albumin; CDAP, cyanodimethylaminopyridinium tetrafluoroborate; CRF, corticotropin-releasing factor; DIEA, diisopropylethylamine; DMF, dimethylformamide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetate; EGTA, ethyleneglycolbis(aminoethyl ether)tetraacetate; exp, expressed rNT without the putative signal sequence; Fmoc, *N*-(9-fluorenyl)methoxycarbonyl; GPCR, G protein-coupled receptor; GSH, reduced glutathione; GSSG, oxidized glutathione; GuHCl, guanidinium hydrochloride; HBTU, 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluoroborate; HPLC, high-performance liquid chromatography; IAA, iodoacetamide; IMAC, immobilized metal affinity chromatography; IPTG, isopropyl-D-thiogalactopyranoside; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; rNT, N-terminal receptor domain; RRA, radioreceptor assay; RP, reversed-phase; SDS, sodium dodecyl sulfate; SPA, scintillation proximity assay; TFA, trifluoroacetic acid; TOF, time-of-flight; Tris, tris(hydroxymethyl)aminomethane.

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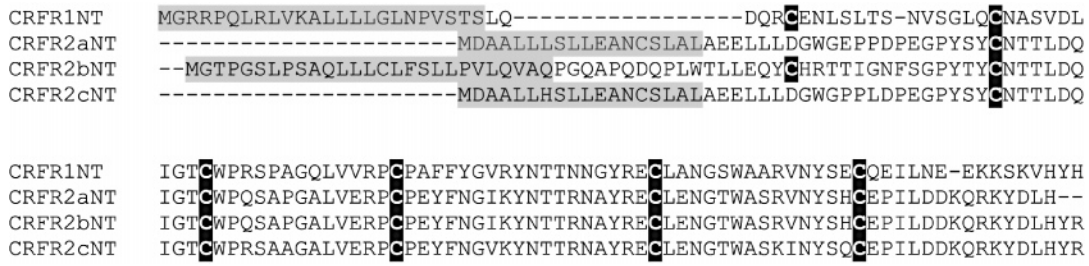

FIGURE 1: Alignment of the extracellular N-terminal domains of CRF_1, $CRF_{2(a)}$, $CRF_{2(b)}$, and $CRF_{2(c)}$ receptors using the program T-Coffee, version 1.41. The putative signal sequences are gray-shaded, whereas cysteines are black-shaded.

potential disulfide bridges) differs between these receptor variants (Figure 1).

Recently, studies of receptor NTs that contain six cysteines such as CRF_1 (*1*, *28*, *29*), $CRF_{2(b)}$ (*30*), PTH-1 (*31*), and GLP-1 receptors (*32*), expressed both in eukaryotic and in prokaryotic systems, have shown that the receptor NT is the main binding domain for peptide ligands. It has also been reported that the specific disulfide pattern of the receptor NT is critical for binding (*33*), whereas the question of whether glycosylation influences the binding behavior of class B GPCRs remains controversial and is treated as such in the current literature (*28*, *34*−*40*). Bearing in mind that the function of glycosylation for GPCRs as well as for other proteins is mainly embraced with regard to their biosynthesis, observed lack of binding of nonglycosylated full-length receptors or domains may be due to incorrect folding or insufficient transport to the cell surface. Moreover, for important members of class B GPCRs (glucagon receptor (*38*), PTH/PTHrP receptor (*39*)) and for the isolated N-terminal domain of another class B GPCR (GLP-1 receptor (*40*)), it has been shown that glycosylation clearly has no impact on the ligand binding.

Here, we investigated the binding behavior of CRF receptor NT and the respective full-length receptors to various and modified ligands. For that purpose, an in vitro folding strategy for preparation of receptor NT produced by heterologous expression in *Escherichia coli* was established. Disulfide arrangement and ligand binding behavior of the products were found to be identical to CRF receptor NT expressed as soluble, in vivo folded proteins (*28*−*30*).

Using ^{125}I-astressin, a nonselective antagonist for CRF receptors, as tracer for binding assays, Hoare et al. reported (*1*) that the peptide agonist affinity for rat CRF_1-receptor NT is only 1.1−3.5-fold lower than the affinity for the whole receptor. In agreement with our findings of two segregated receptor binding sites in the regions from residue 1 to 21 and residue 32 to 40 of the agonist urocortin 1 (*41*), Hoare et al. discuss a two-domain mechanism for receptor agonist interactions in which the receptor NT binds the C-terminus of the agonist and the juxtamembrane domain of the receptor binds the N-terminal portion of the agonist. To elucidate whether the two receptor binding sites found for Ucn1 have counterparts in the CRF receptor, that is, the receptor NT and a juxtamembrane domain, we studied the binding behavior of N-terminally truncated or C-terminally modified Ucn1 analogues to CRF receptor NT and the respective wild-type receptors. On the basis of comprehensive binding studies on isolated N-terminal receptor domains in comparison to the full-length receptors, a new binding model is proposed.

EXPERIMENTAL PROCEDURES

Construction of Expression Plasmids. Signal peptide predictions using a number of different algorithms (SIGFIND (*42*), SIGNALP V2.0 (*43*), PSORT), indicated that the first 24 amino acids of rat CRF_1 receptor consist of a signal peptide sequence. Furthermore, the "DAS" (*44*) and TMAP servers (*45*) predicted the amino acid His^{117} to be linked directly to the first transmembrane helix in the rat CRF_1 receptor. Therefore, a PCR-amplified cDNA coding for Leu^{25}−His^{117} of the CRF_1 receptor was inserted into pET-21a (Novagen, Bad Soden, Germany) yielding pET(CRF_1-NT) to provide CRF_1-receptor NT(exp). Following the same procedure, we amplified a cDNA encoding Ala^{19}−Arg^{114} of the $CRF_{2(a)}$ receptor via PCR and cloned it into a pET-21a vector yielding pET($CRF_{2(a)}$-NT) to provide $CRF_{2(a)}$-receptor NT(exp). The authenticity of the resulting recombinant expression vectors was confirmed by DNA sequence analysis of both strands.

Expression in E. coli and Isolation of Inclusion Bodies. *E. coli* BL21(DE3) (Novagen) were transformed with the plasmids pET(CRF_1-NT) and pET($CRF_{2(a)}$-NT) and grown in LB medium at 37 °C to an optical density of $OD_{600\ nm}$ = 0.5−0.7. Expression of C-terminally $(His)_6$-tagged protein was then induced with 1 mM IPTG for 3.0−3.5 h at 37 °C. Cells were harvested by centrifugation, disrupted, and subjected to lysis, and the inclusion bodies were collected and stored at −20 °C.

Renaturation and Purification. The inclusion body pellet was solubilized in 5 M GuHCl, 20 mM Tris, pH 7.5, by shaking and sonication. After centrifugation, the proteins were purified by IMAC using a chelating Sepharose FF column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) with immobilized Ni^{2+} ions. After reduction of the protein, DTT and Ni^{2+} ions were removed by dialysis at pH 3.0 and 10 °C. Insoluble material was removed by centrifugation, and the pH of the supernatant was readjusted to pH 7.5. Renaturation was achieved by dialysis against 60 volumes of renaturation buffer (0.5 M L-arginine, 100 mM Tris, 1 mM EDTA, 1 mM GSH, 1 mM GSSG, pH 7.5) at a protein concentration of 0.5−0.7 mg/mL for 3 days at 10 °C. For purification via RP-HPLC, a Vydac C4 column (10 mm × 250 mm, 5 μm particle size, 300 Å pore size, number 214TP510) was run in water with increasing concentrations of ACN as mobile phase. An eluent gradient of 10−70% (v/v) ACN/water (0.1% TFA) over 70 min with a flow rate of 4 mL/min was used. The purified protein was lyophilized for 24 h.

Peptides. Astressin was purchased from Bachem AG, Bubendorf, Switzerland. NBI 27914 was bought from

Table 1: Primary Structures and Analytical Data of CRF Analogues[a]

Peptide	Sequence	Calc. M.W.	Found[b] [M+H]$^+$
ovine CRF	SQEPPISLDLTFHLLREVLEMTKADQLAQQAHSNRKLLDIA	4670.4	4667.5
human/rat CRF	SEEPPISLDLTFHLLREVLEMARAEQLAQQAHSNRKLMEII	4757.6	4755.5
rat urocortin 1(1-40)	DDPPLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV	4707.4	4705.5
rat urocortin 1 acid	DDPPLSIDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV	4708.4	4706.4
rat urocortin 1(7-40)	------IDLTFHLLRTLLELARTQSQRERAEQNRIIFDSV	4124.8	4123.2
rat urocortin 1(8-40)	-------DLTFHLLRTLLELARTQSQRERAEQNRIIFDSV	4011.6	4010.2
rat urocortin 1(9-40)	--------LTFHLLRTLLELARTQSQRERAEQNRIIFDSV	3896.5	3895.1
rat urocortin 1(10-40)	---------TFHLLRTLLELARTQSQRERAEQNRIIFDSV	3783.3	3782.1
rat urocortin 1(11-40)	----------FHLLRTLLELARTQSQRERAEQNRIIFDSV	3682.2	3680.8
rat urocortin 1(12-40)	-----------HLLRTLLELARTQSQRERAEQNRIIFDSV	3535.1	3533.9
rat urocortin 1(13-40)	------------LLRTLLELARTQSQRERAEQNRIIFDSV	3397.9	3396.3
rat urocortin 1(14-40)	-------------LRTLLELARTQSQRERAEQNRIIFDSV	3242.7	3241.8
rat urocortin 1(15-40)	--------------RTLLELARTQSQRERAEQNRIIFDSV	3129.6	3128.4
mouse urocortin 2	VILSLDVPIGLLRILLEQARYKAARNQAATNAQILAHV	4153.0	4151.4
urotensin I	NDDPPISIDLTFHLLRNMIEMARNENQREQAGLNRKYLDEV	4869.5	4867.4
sauvagine[c]	ZGPPISIDLSLELLRKMIEIEKQEKEKQQAANNRLLLDTI	4599.4	4597.5
astressin[d]	fHLLREVLEBARAEQLAQEAHKNRKLBEII * *	3563.24	-

[a] Except for the rat urocortin 1 acid, carrying C-terminally the free amino acid residue, all peptides were synthesized as C-terminal amides. The truncated rat urocortin 1 analogues (7−40) to (13−40) are N-terminally acetylated. [b] Observed *m*/*z* of the monoisotope compared with the calculated $[M + H]^+$ monoisotopic mass. [c] Z represents pyroglutamic acid, [d] f represents D-phenylalanine; B represents norleucine; ∗ indicates a lactam bridge connecting the side chains of glutamic acid and lysine.

BIOTREND GmbH, Koeln, Germany. [^{125}I-Tyr^0]-sauvagine (2200 Ci/mmol) and [^{125}I-Tyr^0]-urocortin 1 (2200 Ci/mmol) were obtained from Perkin-Elmer Life and Analytical Sciences (Billeria, MA) and Amersham Biosciences Europe GmbH (Freiburg, Germany), respectively. All other peptides were synthesized automatically by the solid-phase method using standard Fmoc chemistry in continuous flow mode (TentaGel S RAM resin 0.21 mmol/g for peptide amides, TentaGel S PHB resin (Rapp Polymere Tuebingen, Germany) for the free acid of urocortin, HBTU, 2 equiv of DIEA, coupling 20 min, deblocking with 20% piperidine in DMF for 15 min, final cleavage with 95% TFA/5% water for 3 h). Purification of crude peptides was carried out by preparative HPLC on PolyEncap A300 (10 μm particle size, 250 mm × 20 mm i.d., Bischoff Analysentechnik GmbH, Leonberg, Germany) in water with increasing concentrations of ACN as mobile phase. An eluent gradient of 5−70% (v/v) ACN/water (0.1% TFA) over 70 min with a flow rate of 10 mL/min was used. Purified peptides of >95% purity (HPLC) were lyophilized for 24 h. The peptides were characterized by MALDI mass spectroscopy on a Voyager-DE STR BioSpectrometry Workstation MALDI-TOF mass spectrometer (Perseptive Biosystems, Inc., Framingham, MA) using α-cyano-4-hydroxycinnamic acid and sinapinic acid as matrix and gave the expected $[M + H]^+$ mass peaks (Table 1).

Disulfide Pattern Analysis. Disulfide pattern analysis of all heterologously produced CRF receptor NT was carried out by standard procedures of enzymatic digestion using trypsin, chymotrypsin, or Glu-C followed by MALDI-MS. Lyophilized CRF_1-receptor NT(exp) was dissolved in deionized water (Milli-Q, Millipore Corporation) to a concentration of 500 μM. Seventeen microliters of the protein solution (100 μg of protein) was diluted with 100 μL of 50 mM ammonium bicarbonate buffer, pH 7.5. Digestion with 5 μg of trypsin (sequencing grade, Roche Molecular Diagnostics) was performed at 20 °C for 12 h. For further digestion, the cleaved fragment 30−57 and 86−96 of CRF_1-receptor NT-(exp) was purified by analytical HPLC and analyzed (calculated $[M + H]^+$ 4153.90 Da, found 4154.71 Da). Then,

approximately 10 μg of the purified and lyophilized fragment were dissolved in 20 μL of 100 mM ammonium bicarbonate buffer, pH 7.5, for digestion by 1 μg of GluC (sequencing grade, Roche Molecular Diagnostics, 0.5 μg/μL in 1 mM HCl) for 12 h at 20 °C. Lyophilized CRF_1-receptor NT(exp) was dissolved in deionized water to a concentration of 50 μM. The protein solution (75 μL, 42.7 μg of protein) was diluted with 25 μL of 100 mM ammonium bicarbonate buffer, pH 7.5, and the pH was readjusted to pH 7.5. The resulting solution was subjected to endoproteinase digestion by either 2 μg of chymotrypsin (sequencing grade, Roche Molecular Diagnostics, 1 μg/μL in 1 mM HCl) or 2 μg of GluC at 37 and 20 °C, respectively, for 16 h. The reaction was stopped by addition of TFA to a final concentration of 0.3% (v/v) TFA.

For analyzing the disulfide pattern in $CRF_{2(a)}$-receptor NT(exp) bearing five cysteines, the free cysteine, not being involved in a disulfide bond, was blocked by alkylation with IAA (phosphate buffer, pH 5.7, 25 μM protein, 5 mg of IAA, 2 M GuHCL, 12 h at 37 °C) or cyanylation with CDAP (phosphate buffer, pH 5.7, 25 μM protein, 0.25 mM CDAP, 1 mM EDTA, 12 h at 40 °C). Removal of excess reactants was performed by dialysis against 2 M GuHCl, 20 mM Tris, 1 mM EDTA, pH 7.5. For subsequent endoproteinase digestion, the dialyzed and modified protein was rediluted to a concentration of 50 μM. The protein solution (75 μL, 46.5 μg of protein) was diluted with 25 μL of 100 mM ammonium bicarbonate buffer, pH 7.5, and the pH was readjusted to pH 7.5. Digestion of 100 μL of the resulting protein solution was performed by addition of either 1.25 μg of chymotrypsin (1 μg/μL in 1 mM HCl) or 1.25 μg of GluC (0.5 μg/μL in 1 mM HCl) at 37 and 20 °C, respectively, for 16 h. The reaction was stopped by addition of TFA to a final concentration of 0.3% (v/v) TFA in water.

MALDI-MS measurements were performed on a Voyager-DE STR biospectrometry workstation MALDI-TOF mass spectrometer using α-cyano-4-hydroxycinnamic acid and sinapinic acid as matrix for analyses of fragmented proteins. The program SearchXLinks (www.caesar.de/searchxlinks/) was used to analyze the mass spectra of protein digests with regard to the presence of disulfide-linked fragments.

Circular Dichroism Measurements. Circular dichroism (CD) spectra were measured at 25 °C on a J-720 spectropolarimeter (Jasco, Tokyo, Japan) in a quartz cell of 0.2 cm path length over the range 200−260 nm. The instrument was calibrated with an aqueous solution of (+)-10-camphersulfonic acid. CD spectra were the average of a series of six scans made at 0.1 nm intervals. Protein concentrations were 1×10^{-5} M in 15 mM SDS, 15 mM phosphate buffer (pH 7.4). The amount of helix was estimated from the relation $\%_h = ([\Phi]^{222} - [\Phi]^0_{222})/[\Phi]^{100}_{222}$, where $[\Phi]^{222}$ is the determined mean residue ellipticity at 222 nm. For $[\Phi]^0_{222}$ and $[\Phi]^{100}_{222}$, representing 0% and 100% helix content, values of −2340 and 30 300 deg·cm^2/dmol, respectively, were used.

Ligand Binding Assays. After the animals were killed by decapitation (rats, male Wistar rat, Schoenwalde, Germany) or CO_2 (mice, male NMRI BR, Charles River, Sulzfeld, Germany), whole brains of rats and hearts of mice were rapidly dissected and homogenized. After centrifugation steps, the pellets were resuspended in 50 mM Tris, 10 mM $MgCl_2$, 2 mM EGTA, 0.15 mM bacitracin, and 0.0015% aprotinin, pH 7.2 (assay buffer), centrifuged, and again resuspended in assay buffer containing 0.32 M sucrose and stored at −20 °C. All steps were carried out at 4 °C.

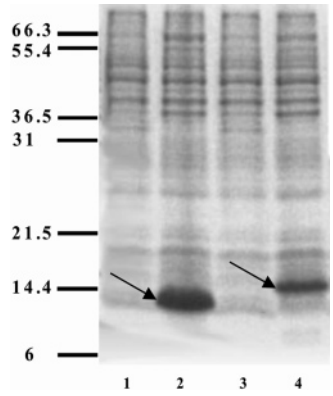

FIGURE 2: Polyacrylamide (15%) SDS gels of the N-terminal CRF receptor domains. *E. coli* lysates from noninduced (lane 1) and induced (lane 2) CRF_1-rNT producing cells and from noninduced (lane 3) and induced (lane 4) $CRF_{2(a)}$-rNT producing cells are resolved.

Radioreceptor Assay. Binding assays for [^{125}I-Tyr^0]-sauvagine were performed as described previously (*46*). Receptor affinities ($K_d = 1/K_{as}$) were estimated using the nonlinear least squares curve fitting program RADLIG (BIOSOFT, Cambridge, U.K.).

Scintillation Proximity Assay. The competitive binding assays were performed in triplicate in 2.0 mL colorless reaction tubes (Biozym Diagnostik GmbH, Oldendorf, Germany) at room temperature using a polyvinyltoluene copper His-tag SPA bead suspension (Amersham Biosciences Europe GmbH, Freiburg, Germany) in assay buffer containing 0.1% BSA. The following reagents, diluted in assay buffer, were added in order: 100 μL of unlabeled peptide with increasing peptide concentrations or buffer, 75 μL of [^{125}I-Tyr^0]-urocortin 1 (120 pM final concentration), and 75 μL of CRF-receptor NT(exp) (20 ng/tube). After incubation of the reaction mixture for 2 h, 50 μL of 10 mg/mL bead suspension was added. The final reaction mixture was shaken and then incubated for 4 h. Finally, the tubes were counted in a Wallac 1410 set up in a ^{3}H cpm-mode (SPA-cpm). Total binding observed and normalized at 100 pM final concentration of tracer was about 6000 SPA-cpm and 13 000 SPA-cpm for CRF_1 and $CRF_{2(a)}$ receptor NT, respectively, with a nonspecific signal of about 3000 SPA-cpm for both NTs determined in the presence of unlabeled 1 μM astressin. Binding data were analyzed by using the GraphPad Prism software.

RESULTS

Expression of Recombinant Proteins. Expression of CRF receptor NT using the pET-21a vectors pET(CRF_1-NT) and pET($CRF_{2(a)}$-NT) allows high-yield production in *E. coli*. SDS−PAGE analysis of cell lysates demonstrate the overexpression of the proteins (Figure 2). Due to a different mobility of the $CRF_{2(a)}$-receptor NT(exp), its band does not correspond to the actual size of 12.5 kDa. CRF_1-receptor NT(exp) was produced with 60−70 mg of inclusion bodies/L of cell culture; expression of $CRF_{2(a)}$-receptor NT(exp) provided yields of 40−60 mg of inclusion bodies/L of cell culture.

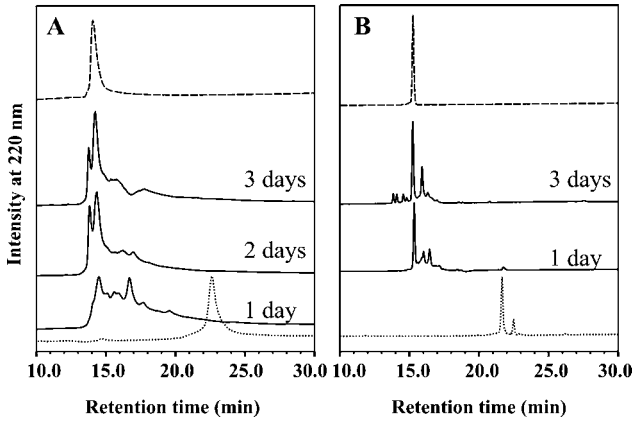

FIGURE 3: HPLC profiles monitoring the progress in folding over 3 days in comparison to reduced protein (dotted line) for CRF_1-NT (A) and $CRF_{2(a)}$-NT (B). The dashed lines (A, B) show the final products after purification.

Refolding and Purification. For renaturation, the solubilized proteins purified by IMAC were dialyzed against the renaturation buffer. The presence of L-arginine in the renaturation buffer was necessary to prevent aggregation of misfolded species (*47*−*49*). The renaturation buffer also contained a redox system to permit reshuffling of incorrect disulfide bonds. The progress of folding was analyzed by RP-HPLC over a time period of several days. As shown in Figure 3A,B, an enrichment of one species could be observed after 3 days of folding for both N-terminal domains. After renaturation, preparative RP-HPLC was chosen for protein purification because of its high-resolution capacity. It should be noted that the folding efficiencies for the $CRF_{2(a)}$ N-terminal domain were better than those of the CRF_1-receptor NT(exp). Thus, a 30% better yield over the entire refolding and purification process was obtained for the refolded $CRF_{2(a)}$-receptor NT(exp) (10 mg from 100 mg of purified inclusion body protein) compared to the refolded CRF_1-receptor NT(exp) (7 mg from 100 mg of purified inclusion body material).

Product Characterization. MALDI-MS confirmed the identity and the molecular masses of the refolded and purified receptor fragments: CRF_1-receptor NT(exp) produced from pET(CRF_1-NT) calculated des-Met^1-$[M + H]^+$ 11 812.62 Da, found 11 813.47 Da; $CRF_{2(a)}$-receptor NT(exp) produced from pET($CRF_{2(a)}$-NT) calculated desMet^1-$[M + H]^+$ 12 154.65 Da, found 12 154.99 Da and 12 465.14 Da (glutathione adduct).

Assignment of Disulfide Bridges. For CRF_1-receptor NT(exp) mass spectrometric analysis of fragments obtained by enzymatic digestion resulted in the same disulfide pattern previously determined for other six-cysteine-containing CRF-receptor NTs, such as CRF_1-receptor NT (*28, 29*) and $CRF_{2(b)}$-receptor NT (*30*), which were expressed as soluble, in vivo folded proteins. After standard digestion, fragments of the in vitro refolded CRF_1-receptor NT showed linkages between Cys^{68} (C4) and Cys^{102} (C6), Cys^{30} (C1) and Cys^{54} (C3), and Cys^{44} (C2) and Cys^{87} (C5) (Table 2).

The $CRF_{2(a)}$-receptor NT(exp) contains only five cysteine residues. Consequently, only two disulfide bridges and one unlinked cysteine can be expected. Moreover, for the $CRF_{2(a)}$-receptor NT(exp), we faced the problem of disulfide scrambling (*50*) when performing standard digestion at pH 7.5, resulting in an inconsistent disulfide pattern deduced from the fragments found after chymotrypsin and GluC digestion. To prevent disulfide scrambling, free cysteine residues were blocked by alkylation or cyanylation at pH 5.7 with IAA (*51*) and CDAP (*52*), respectively, prior to proteolysis. After enzymatic digestion of the alkylated or cyanylated NT using chymotrypsin, the fragments revealed disulfide bridging between Cys^{40} (C2) and Cys^{83} (C5), as well as between Cys^{64} (C4) and Cys^{83} (C6) (Table 2). On the basis of this unambiguous assignment of disulfide bridges, it can be concluded that Cys^{50} (C3) is not involved in forming disulfide bonds. Thus, the $CRF_{2(a)}$-receptor NT(exp) shows the same disulfide pattern described for CRF_1-receptor NT and $CRF_{2(b)}$-receptor NT, respectively (see above).

Ligand Binding. To assess binding characteristics of the N-terminal domains of CRF_1 and $CRF_{2(a)}$ receptors, respectively, the affinity of CRF family members was investigated by competitive displacement studies of [^{125}I-Tyr^0]-urocortin 1 employing the nonseparation technique SPA. For these experiments, C-terminally $(His)_6$-tagged CRF-receptor NTs were incubated with [^{125}I-Tyr^0]-urocortin 1 followed by an additional incubation with copper-loaded SPA beads to capture the protein−ligand complex via copper-chelate interactions. The binding data are summarized in Table 3. CRF_1-receptor NT(exp) and $CRF_{2(a)}$-receptor NT(exp) bound ovine CRF (EC_{50} = 371 and 420 nM) and human/rat CRF (682 and 367 nM) with lower affinity than urocortin 1 (119 and 98 nM). The antagonist astressin exhibited a strong binding to CRF_1-receptor NT(exp) and $CRF_{2(a)}$-receptor NT(exp) (EC_{50} = 1.96 and 6.7 nM) (Figure 4). Here, the affinity of astressin for the full-length CRF_1 receptor (0.55 nM) and CRF_2 receptor (0.34 nM) differs only by about 1 order of magnitude. For the nonpeptidic antagonist NBI 27914 (*53*), no binding to either of the investigated N-terminal CRF receptor domains was found. In contrast to the high affinity of sauvagine for the full-length receptors, no binding was observed to receptor NT up to a concentration of 1 μM (data not shown). The CRF_2 receptor selective agonist mouse urocortin 2 bound with higher affinity to $CRF_{2(a)}$-receptor NT(exp) (76 nM) than to CRF_1-receptor NT(exp) (619 nM), suggesting an influence of receptor NT on subtype selectivity.

To investigate whether modifications of N- or C-terminal receptor binding sites of Ucn1 affect affinities for receptor NTs, respective full-length receptors, or both, we determined affinities for N-terminally truncated Ucn1 analogues and Ucn1-acid, reducing the length of the N-terminal binding site or changing the C-terminal amide to the free acid, respectively (Table 3). Stepwise shortening by six [Ucn1-(7−40)] up to 10 amino acid residues [Ucn1(11−40)] had no significant effect on affinities for receptor NT in comparison to Ucn1. Significant reduction in affinity for receptor NT was observed after further truncation; particularly for Ucn1(15−40), affinities for CRF_1-receptor NT(exp) and $CRF_{2(a)}$-receptor NT(exp) drop dramatically. In case of full-length receptors, analogue results were obtained after N-terminal truncation of 14 amino acid residues; that is Ucn1(15−40) did not show any receptor binding. Elongation by the next five amino acid residues (Ucn1(10−40)) resulted in an strong increase (about 2 orders of magnitude) of affinities for full-length receptors, an effect that has not been seen in the case of receptor NT. Interestingly, there are some differences between the two receptor subtypes; while Ucn1-(10−40) compared with the whole Ucn1 exhibited an 8-fold

Table 2: Assignment of Proteolytic Fragments Determined by MALDI-MS Analysis

fragment	cysteine	cysteine pattern	calcd MW	found $[M + H]^+$
		CRF_1-Receptor NT(exp), Trypsin		
30−57 and 86−96	Cys30,44,54−Cys87	C1,2,3−C5	4154.94	4154.71
58−76 and 97−110	Cys68−Cys102	C4−C6	3758.85	3759.93
		Fragment 30−57 and 86−96 from CRF_1-receptor NT(exp), GluC		
30−31 and 50−57	Cys30−Cys54	C1−C3	1193.56	1194.10
32−49 and 86−96	Cys44−Cys87	C2−C5	2996.40	2998.00
32−49 and 87−96	Cys44−Cys87	C2−C5	2866.34	2868.42
		CRF_1-Receptor NT(exp), Chymotrypsin		
26−33 and 51−63	Cys30−Cys54	C1−C3	2388.11	2389.20
43−50 and 85−93	Cys44−Cys87	C2−C5	1881.82	1881.41
64−72 and 100−106	Cys68−Cys102	C4−C6	1853.89	1854.51
64−71 and 100−106	Cys68−Cys102	C4−C6	1706.82	1707.55
		CRF_1-Receptor NT(exp), GluC		
32−49 and 87−101	Cys44−Cys87	C2−C5	3459.59	3461.00
		$CRF_{2(a)}$-Receptor NT(exp), Chymotrypsin, Cyanylation		
40−44 and 81−89	Cys40−Cys83	C2−C5	1655.71	1655.74
38−59 and 81−84 [a]	Cys40,50−Cys83	C2,3−C5	2868.42	2868.21
45−68 and 96−102 [a]	Cys50,64−Cys98	C3,4−C6	3486.93	3486.90
		$CRF_{2(a)}$-Receptor NT(exp), Chymotrypsin, Alkylation		
39−43 and 80−88	Cys40−Cys83	C2−C5	1655.70	1655.50
59−67 and 95−108	C64−Cys98	C4−C6	2868.31	2868.30
39−58 and 80−88[b]	Cys40,50−Cys83	C2,3−C5	3486.80	3485.48

[a] Cyanylation adduct. [b] Glutathione adduct.

Table 3: Summarized Results of Ligand Binding Studies by SPA

	receptors		receptor N-termini	
ligand	CRF_1 rat brain K_d [nM]	$CRF_{2(b)}$ mouse heart K_d [nM]	CRF_1 EC_{50} [nM]	$CRF_{2(a)}$ EC_{50} [nM]
ovine CRF	2.70 ± 0.34	0.20 ± 9.5	371 ± 82	420 ± 31
human/rat CRF	4.38 ± 0.70	4.39 ± 0.79	682 ± 97	367 ± 26
rat urocortin 1(1−40)	0.89 ± 0.11	0.39 ± 0.05	119 ± 45	97.7 ± 11.9
rat urocortin 1 acid	48.90 ± 6.50	60.90 ± 24.90	*a*	*a*
rat urocortin 1(7−40)	42.70 ± 13.70	1.70 ± 0.62	93.90 ± 33.1	133 ± 20
rat urocortin 1(8−40)	5.84 ± 2.04	0.24 ± 0.07	106 ± 55	45.6 ± 4.9
rat urocortin 1(9−40)	47.20 ± 12.10	1.17 ± 0.42	70.80 ± 12.60	43.6 ± 8.3
rat urocortin 1(10−40)	4.97 ± 0.52	0.05 ± 0.01	85.10 ± 11.5	55.8 ± 8.6
rat urocortin 1(11−40)	55.50 ± 1.10	3.80 ± 1.42	109 ± 26	77.6 ± 10.8
rat urocortin 1(12−40)	29.90 ± 3.70	19.60 ± 7.20	425 ± 109	167 ± 33
rat urocortin 1(13−40)	63.70 ± 10.10	26.50 ± 7.60	>1000	566 ± 62
rat urocortin 1(14−40)	346 ± 67	131 ± 14	*a*	>1000
rat urocortin 1(15−40)	*a*	*a*	*a*	*a*
mouse urocortin 2	>1000	0.10 ± 0.04	619 ± 186	76.3 ± 15.1
sauvagine	1.67 ± 0.21	0.49 ± 0.44	*a*	*a*
astressin	0.55 ± 0.18	0.34 ± 0.12	1.96 ± 0.12	6.75 ± 1.51
NBI 27914	20.90 ± 4.70	*a*	*a*	*a*

[a] No binding.

higher affinity for CRF_2-receptor, it showed a 5-fold lower affinity for CRF_1-receptor, indicating a role for subtype selectivity of amino acid residues at positions 10 and 11. In agreement with this suggestion, we have demonstrated that specific amino acid replacements (VPXX, wherein X represents a hydrophobic amino acid residue) at positions 9−12 in urocortin 1 resulted in CRF_2-receptor-selective agonists (*54*). Moreover, conversion of the C-terminal amide of Ucn1 to the corresponding acid exhibited a strong effect on affinity for receptor NT as well as full-length receptors, demonstrating that the C-terminal binding site Ucn1(32−40) is involved in binding to receptor NT.

Circular Dichroism Measurements. To assess the effect of terminal modification of the ligand Ucn1 on the helical content, CD spectra of Ucn1-amide and Ucn1-acid were recorded in the presence of SDS micelles. As seen in Figure 5, an α-helical content of 28% was determined for Ucn1-amide compared with 24% for the corresponding Ucn1-acid under identical conditions. Similarly, α-helical contents between 25% and 34% were observed for N-terminally truncated Ucn1 analogues used in binding studies.

DISCUSSION

Comprehensive studies of structure−function relationships of class B GPCRs are hampered by the limited availability of purified, functional full-length receptors. Determination of binding behaviors of isolated extracellular receptor domains for specific ligands may offer an alternative approach for elucidation of interacting sites. Here, we compared affinities of urocortin 1 and its analogues, which

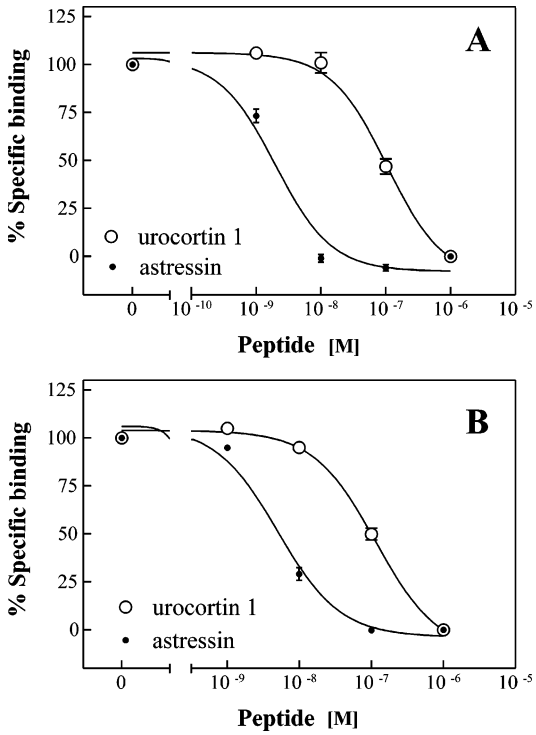

FIGURE 4: Inhibition of specific [^{125}I-Tyr^0]-urocortin 1 binding to CRF-rNT by unlabeled CRF-like ligands urocortin 1 and astressin using the SPA technology: (A) data points for binding to CRF_1-rNT represent pooled data from three independent experiments performed in triplicate for both ligands; (B) data points for binding to $CRF_{2(a)}$-rNT represent pooled data from six independent experiments (astressin) and nine independent experiments (urocortin 1) performed in triplicate. All values are given as mean ± SEM.

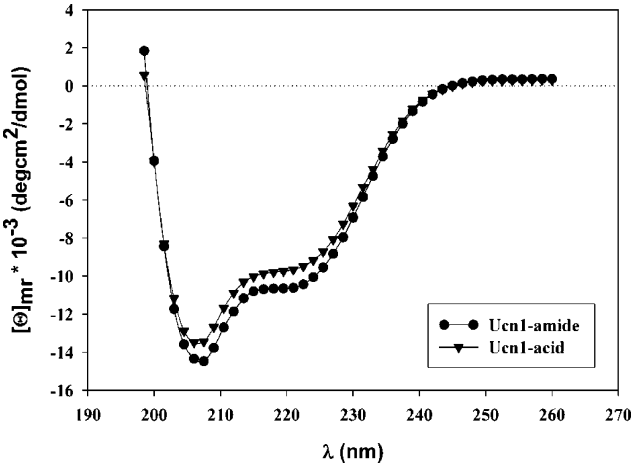

FIGURE 5: CD spectra of Ucn1-amide (●) and Ucn1-acid (▼) in the presence of SDS micelles in 15 mM phosphate buffer (pH 7.4).

have been modified within the two segregated receptor binding sites, for CRF receptor NTs and their respective full-length receptors.

A heterologous expression/in vitro folding system for the production of receptor NT in *E. coli* has been developed, providing nonglycosylated protein. Although the influence of protein glycosylation on the binding behavior of GPCRs or their isolated domains is discussed controversially in the literature, there are several examples for unaffected receptor function of GPCRs class B despite the lack of *N*-glycans (*38*−*40*). The receptor NTs were expressed in the form of inclusion bodies, where the protein is present in large amounts in a highly enriched form. Using liquid/solid separation, we recovered the CRF receptor NTs in yields of 40−70 mg/L. The regeneration of the native disulfide bonds and the decreased solubility of folding intermediates are crucial issues in the renaturation. Several protocols for in vitro protein folding assisted by oxido-shuffling reagents (*55*) and solubilizing additives, which prevent unproductive aggregation, have been developed during recent years. For both CRF_1-receptor NT(exp) and $CRF_{2(a)}$-receptor NT(exp), an optimized folding protocol led to enrichment of one major folded species and large amounts of refolded protein with a renaturation yield of 40%−50% and an overall yield of 10% after purification.

Protein folding with concomitant disulfide bond formation requires both the regeneration of intramolecular noncovalent interactions and the formation of covalent bonds between cysteines. The disulfide patterns in the extracellular N-terminal domains of the CRF_1 and $CRF_{2(a)}$ receptors were determined by enzymatic digestion and MALDI-MS analysis of the resultant fragments. Data obtained for the in vitro folded CRF_1-receptor NT(exp) revealed disulfide bonding (C1−C3, C2−C5, C4−C6) identical to that observed by other groups for in vivo folded CRF_1-receptor NT (*28*, *29*) and $CRF_{2(b)}$-receptor NT (*30*). Our results are also consistent with studies of six-cysteine-containing N-terminal domains of other members of the receptor class B, such as PTHR-1 and GLPR-1 (*31*, *32*). It is worth mentioning that a completely reduced and alkylated CRF_1-receptor NT(exp) did not show any binding activity, indicating the importance of disulfide bridges for ligand interactions. It should be emphasized that $CRF_{2(a)}$-receptor NT(exp) contains only five cysteines in contrast to the six cysteines of CRF_1-receptor NT and $CRF_{2(b)}$-receptor NT. It is the first CRF receptor variant investigated comprising an odd number of cysteines, which results in one nonbonded cysteine. Alkylation or cyanylation of the free cysteine in $CRF_{2(a)}$-receptor NT(exp), subsequent enzymatic digestion, and determination of disulfide fragments showed an analogous C2−C5/C4−C6 disulfide pattern to the CRF_1-receptor and a nonbonded C3.

To evaluate ligand binding characteristics of the in vitro folded proteins, the affinity of CRF-like peptides for CRF_1-receptor NT(exp) and $CRF_{2(a)}$-receptor NT(exp) were estimated by SPA. With [^{125}I-Tyr^0]-urocortin 1 as tracer, binding characteristics of our CRF_1-receptor NT(exp) were found to be similar to data reported for a corresponding soluble receptor NT produced by in vivo folding (*29*), in that a consistent rank order of affinities for the peptidic ligands astressin > urocortin 1 > CRF and no binding in the case of sauvagine were detected.

Considering our two-domain binding mechanism found for urocortin 1, the existence of two segregated domains, Ucn1(1−21) and Ucn1(32−40), for CRF_1 receptor binding (*41*) suggests counterparts in CRF receptors such as the receptor NT and the juxtamembrane domain. Our results show that modification of the C-terminal binding site by conversion of the peptide amide to the corresponding acid results in an analogue that exhibits a dramatic loss in affinity for receptor NT and full-length receptors as well, indicating involvement of the C-terminal Ucn1(32−40) domain in binding to CRF receptor NT. It should be noted that this C-terminal modification does not result in a conformational change of the ligand as determined by CD measurements in

the presence of SDS micelles. This result clearly shows other than conformational reasons for the dramatic loss in affinity upon conversion of the peptide amide to the corresponding acid. N-Terminal truncation by 14 amino acid residues in Ucn1(15−40) showed no binding for receptor NT or for full-length receptors. Its N-terminal elongation by the next three to four amino acids led to analogues that exhibited a high affinity to wild-type receptors as well as to soluble receptor NT, suggesting that at least those three to four amino acid residues of the N-terminal binding site Ucn1(1−21) must also be involved in binding to receptor NT, suggesting a two-domain binding mechanism for the ligand−receptor NT interaction. A two-domain binding model for CRF_1 receptor NT interaction with peptide ligands is also supported by the fact that there are two regions comprising residues at positions 43−50 and 76−84 within the receptor NT that are crucial for their binding (*56*). Further elongation of Ucn1-(11−40) led to a strongly increased affinity for wild-type receptors but did not affect the binding to receptor NT, demonstrating that the very N-terminal amino acid residues, particularly residues 8−10, do not contribute to receptor NT binding but bind probably to receptor juxtamembrane domains. Hoare et al. (*1*) have shown that the peptide ligand binding to the receptor juxtamembrane domain is segregated from binding to the receptor NT. From this follows the existence of three segregated binding domains. The suggestion of a three-domain binding mechanism is seemingly in contrast to recently reported results on binding studies for CRF_1-receptor NT annealed to the transmembrane of the activin IIB receptor (*1*). With [^{125}I-Tyr^0]-astressin as a radioligand, it has been shown that the affinity of urocortin 1 for the CRF_1-receptor NT/activin IIB was only 2.5-fold lower than its affinity for the full-length receptor, suggesting a very weak contribution of the juxtamembrane domain to receptor binding. This conflicting observation may result from the different radioligands used. Formerly reported binding characteristics of CRF_1-receptor NT/activin IIB have demonstrated that urocortin 1 was able to displace astressin as the radioligand, but there was, surprisingly, no detectable specific binding of labeled urocortin 1 (*57*, *58*). Moreover, it has been demonstrated that astressin, in comparison to the competitive antagonist antisauvagine-30, must bind in a different manner (Schild plot analysis (*59*)) and, in contrast to urocortin 1 and CRF, astressin can be N-terminally truncated leading to very short peptides that showed high and selective affinity to the CRF_1 receptor (*60*, *61*). Therefore, astressin cannot be recommended as a radioligand for investigating the mechanism of urocortin 1 binding to CRF receptors. For another class B receptor, parathyroid hormone receptor, photoaffinity cross-linking studies have shown the existence of two segregated binding sites in that the very NT of the PTH bound to the sixth transmembrane domain and part of the third extracellular loop and a photolabel at position 13 of the 34-mer PTH bound to receptor NT close to the membrane region (*62*). This ligand binding close to the membrane may well provide generally for orientation of the ligand N-terminus of this receptor class. Interestingly, the receptor subtypes differ in their affinities to N-terminally elongated Ucn1(12−40) analogues. For example, for $CRF_{2(a)}$ receptor, the truncated analogue Ucn1-(10−40) exhibited a comparably high affinity to that of whole Ucn1, whereas for the CRF_1 receptor, more than the next five amino acid residues are necessary to show such high affinity as that of Ucn1. A similar observation has been made for N-terminally truncated human CRF and sauvagine analogues with respect to their affinities for CRF_1- and $CRF_{2(b)}$-receptors (*63*), suggesting different binding modes of ligand N-termini to receptors. Comparison of affinities between non-subtype-selective urocortin 1 with CRF_2-receptor-selective mouse urocortin 2 for soluble receptor NT demonstrates that a moderate difference is indicative of a contribution of receptor NT to receptor subtype selectivity.

In conclusion, our results show that binding of urocortin 1 to CRF receptors proceeds via a multidomain binding mechanism in that two segregated receptor binding sites of urocortin 1 bind to the receptor NT and N-terminal amino acids of urocortin 1 interact with the juxtamembrane domain of the receptor, forming an additional binding domain.

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