Role of the N-Terminal Domain of the Calcitonin Receptor-like Receptor in Ligand Binding†

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ABSTRACT: Calcitonin receptor-like receptor (CRLR) is a seven-transmembrane (7-TM) domain class B G protein-coupled receptor (GPCR) which requires coexpression of different receptor activity modifying proteins (RAMP) to become a functional calcitonin gene-related peptide (CGRP) receptor or an adrenomedullin (AM) receptor. The N-terminal (Nt) extracellular region of class B GPCRs in ligand binding has been reported for receptors such as glucagon and parathyroid hormone. We hypothesize that the Nt-domain of CRLR (Nt-CRLR) is an autonomously folded unit possessing a well-defined structure and is involved in ligand binding and specificity. To obtain structural and functional information on the Nt-CRLR, we cloned and expressed the Nt-CRLR as a fusion protein in *Escherichia coli*. Overexpressed protein formed an inclusion body, which was refolded and purified, resulting in a soluble monomeric protein. Far-UV CD and fluorescence spectra of Nt-CRLR showed characteristics of a folded protein. The ability of Nt-CRLR to bind CGRP and AM independent of RAMPs was determined by studying inhibition of ^{125}I-CGRP and ^{125}I-AM binding to pregnant rat uterine membrane in the presence of Nt-CRLR protein. We observe that Nt-CRLR inhibits ^{125}I-CGRP and ^{125}I-AM binding to rat uterus in a dose-dependent fashion ($IC_{50} = 0.25$ and 0.29 μM, respectively). Taken together, our data provide evidence that Nt-CRLR is structured and further that a significant part of the binding affinity comes from binding to the Nt-domain.

Calcitonin gene-related peptide (CGRP),[1] adrenomedullin (AM), amylin, and calcitonin (CT) belong to the calcitonin family of peptides, distributed in peripheral tissues and nervous system, and induce a wide variety of biological effects (*6*, *7*, *12*, *21*, *23*−*25*, *34*, *37*, *38*). They exhibit overlapping biological functions, suggesting that they interact with similar G protein-coupled receptors (GPCRs). Heterodimer formation of recently discovered single transmembrane domain receptor activity modifying proteins (RAMPs) and calcitonin receptor-like receptor (CRLR) or calcitonin receptor (CTR) define CGRP, AM, or amylin receptors. CRLR is a seven-transmembrane (7-TM) domain class B GPCR and functions as a receptor for CGRP or AM depending on coexpression of RAMPs. CRLR association with $RAMP_1$ results in CGRP function, whereas association with $RAMP_2$ or $RAMP_3$ results in AM function (*20*). CT receptor binds CT in the absence of RAMPs and binds amylin in the presence of $RAMP_1$. Three biological functions for RAMPs have been defined as an accessory protein for CRLR: (1) transport CRLR to the cell surface, (2) determine the glycosylation state of CRLR, and (3) define CRLR pharmacology by allosterically modifying its conformation (*11*, *20*). Although initial reports indicated that glycosylation of CRLR correlates with receptor phenotype (*11*, *13*), recent studies indicate otherwise (*2*, *17*, *22*). Several studies in HEK293 cells have suggested that heterodimerization of CRLR and $RAMP_1$ is a prerequisite for the receptor function. However, the structural basis of heterodimerization and the importance of dimerization for binding, selectivity, and affinity is not known (*9*, *13*). An insight into the molecular structure of the receptor and the receptor−ligand complex is of great pharmaceutical interest but requires large amounts of protein. Like other members of class B GPCRs, CRLR contains a large N-terminal (Nt) domain and six highly conserved cysteine residues that form disulfide bonds (*14*). As the N-terminal domain of class B GPCRs such as receptors for parathyroid hormone, glucagon, and secretin are involved in ligand binding, we hypothesize that the Nt-domain of CRLR (Nt-CRLR) is an autonomously folded unit possessing a well-defined secondary structure and is involved in ligand binding, selectivity, and function.

CRLR, being a member of the GPCR class, is difficult to overexpress as a full-length receptor in any existing expression system for structural studies (*16*). In this study, we have

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[1] Abbreviations: CGRP, calcitonin gene-related peptide; CRLR, calcitonin receptor-like receptor; RAMP, receptor activity modifying protein; AM, adrenomedullin; GPCR, G protein-coupled receptor; TM, transmembrane; Nt, N-terminal; CT, calcitonin; CTR, calcitonin receptor; IGFBP, insulin-like growth factor binding protein; Ni-NTA, nickel nitrilotriacetic acid; EDC, 1-ethyl-3-(dimethylaminopropyl)-carbodiamide; GSH, reduced glutathione; GSSG, oxidized glutathione; GdnHCl, guanidine hydrochloride; BSA, bovine serum albumin; PMSF, phenylmethanesulfonyl fluoride; CD, circular dichroism.

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cloned and expressed Nt-CRLR in *Escherichia coli* to initiate structural and functional studies on receptor−ligand interaction. We have characterized the structural and functional properties of the Nt-CRLR using circular dichroism spectroscopy (CD), analytical ultracentrifugation, fluorescence spectroscopy, competition binding assay, and cross-linking studies. Our data provide evidence for the first time that the Nt-domain of CRLR is structured and is capable of binding CGRP and AM with appreciable affinity in the absence of RAMPS. CGRP and AM bind their receptors with an IC_{50} of ~2 and 9 nM, respectively. The inhibition of this binding by Nt-CRLR is only ~30−80-fold less, indicating that most of the ligand affinity comes from the Nt domain. These results together indicate that the CRLR Nt-domain does not play a role in ligand selectivity but plays a role in ligand affinity and that interaction with exoloops and/or RAMPS is responsible for ligand selectivity.

EXPERIMENTAL PROCEDURES

Plasmid expression vectors were from Invitrogen (Carlsbad, CA). Restriction enzymes were from Gibco BRL (Gaithersberg, MD). Nickel nitrilotriacetic acid (Ni-NTA) metal affinity resin was the product of Qiagen, Inc. (Valencia, CA). ^{125}I-CGRP was from Pharmacia (Amersham, NJ). ^{125}I-AM was purchased from Phoenix (Phoenix Pharmaceuticals, Inc., Belmont, CA). IGFBP-3 was an Oncogene research product from ACN Bioscience Co. (Cambridge, MA). Amylin was purchased from Alpha Diagnostics (San Antonio, TX). 1-Ethyl-3-(dimethylaminopropyl)carbodimide (EDC) was obtained from Sigma (St. Louis, MO). All other chemicals were from reputed commercial suppliers.

Cloning and Expression of the Nt-Domain of Human CRLR. Secondary structure prediction programs for 7-TM proteins indicated that the first TM of CRLR starts from residues between 141 and146. A 333 bp gene fragment coding for amino acids 23−133 of human CRLR (the first 22 residues correspond to the signal sequence) was amplified by the standard PCR technique from the cDNA template encoding the complete receptor with CRLR primer 1, 5′-GAATTAGAAGAGAGTCCTGAG-3′, and CRLR primer 2, 5′-TAGTGCTCACTTCGGATCCTCGTGGGT-3′. The amplified PCR product was directly cloned in frame with the TA cloning site in the pCRT7/NT-TOPO expression vector from Invitrogen as per the manufacturer's protocol (Carlsbad, CA). The resulting expression plasmid pCRLR-NT codes for the extracellular Nt-domain of CRLR along with amino acids incorporated from the expression vector starting from the translation site up to TA cloning site, which includes a His tag and an express epitope at the N-terminus. Further, the expressed protein has three additional residues (Asp-Pro-Lys) at the C-terminus as a result of introducing a restriction site and should not interfere in either structural or functional studies. Gene inserts with the right orientation were screened by PCR with a T7 forward primer and a gene-specific reverse primer. Accuracy of the cloned sequence was confirmed by DNA sequence analysis of both of the DNA strands on an ABI Prism automated DNA sequencer from Applied Biosystems (Branchburg, NJ).

For protein expression, pCRLR-NT plasmid was transformed into an *E. coli* strain BL21λ(DE3) LysS. For shake flask growth, 1 L of Luria broth medium supplemented with 34 μg/mL chloramphenicol and 100 μg/mL ampicillin was inoculated with 10 mL of overnight culture, and growth was maintained at 37 °C on a shaker at 250 rpm. Protein expression was induced at an optical density (OD) of 0.45 with 1 mM isopropyl thiogalactosidase (IPTG). After an additional 3 h incubation, cells were harvested by centrifugation at 4000*g* for 15 min.

Protein Purification. Twelve grams of cell pellet was resuspended in 100 mL of 100 mM Tris-HCl and 1 mM EDTA, pH 7.0, and sonicated (three strokes of 30 s each) to perform cell lysis. Cell lysate was then incubated for 30 min at room temperature in the presence of 10 μg/mL DNase I and 3 mM $MgCl_2$. The solution was then mixed with 0.5 volume of 100 mM Tris-HCl, 150 mM NaCl, and 1.5% Triton X-100 at pH 7.0, and the inclusion bodies were pelleted by centrifugation at 30000*g* for 30 min. The pellet was resuspended in 100 mM Tris-HCl and 150 mM NaCl, pH 7.0, containing 0.5% Triton X-100 and washed once with the same buffer followed by two washes without Triton X-100. The inclusion bodies were suspended in 6 M guanidine hydrochloride (GdnHCl), 100 mM DTT, and 1 mM EDTA, pH 8.0, at room temperature and centrifuged at 30000*g* for 30 min. DTT was removed by dialyzing the supernatant against 150 volumes of 4 M guanidine hydrochloride and 100 mM sodium phosphate, pH 6.0, for 48 h with buffer changes at 12 and 24 h. The protein was then loaded onto a Ni-NTA column after the pH was adjusted to 8.0, washed with 0.1 M sodium phosphate and 4 M guanidine hydrochloride, pH 6.3, and eluted by lowering the pH to 4.5. Renaturation was achieved by dialyzing the protein against 15 volumes of 1 M L-arginine, 1 mM EDTA, 0.05 M sodium phosphate, 5 mM reduced GSH, and 1 mM oxidized GSSG, pH 8.0, at a protein concentration of 0.3 mg/mL at 4 °C. Refolded protein was dialyzed against a buffer containing 10 mM Tris-HCl, 1 mM EDTA, 10% glycerol, and 50 mM NaCl, pH 7.3, and centrifuged at 30000*g* for 30 min. The supernatant containing protein was further purified on an anion-exchange Resource Q column from Amersham Biosciences Corp. (Piscataway, NJ) by using a NaCl gradient (50−600 mM). The peak fractions were size fractionated on SDS−PAGE, and the molecular mass was assessed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) spectrometry. Protein concentrations were determined using bicinchoninic acid reagent (BCA) (Pierce Biotechnology, Rockford, IL).

Analytical Ultracentrifugation. Sedimentation equilibrium experiments were performed using a Beckman Model XL-A ultracentrifuge at 20 °C at rotor speeds of 27000 and 32000 rpm. Absorbance was measured at 280 nm, and the data were collected as an average of five successive radial scans using a 0.003 cm step size. The data were fitted to a single species or to a monomer−dimer model using the equations:

$$C_r = C_0 \exp(MHd) + E$$

or

$$C_r = C_0 \exp(MHd) + C_0^2 K_a \exp(2MHd) + E$$

where d is $r^2 - r_0^2$, $H = (1 - \upsilon r)(\omega^2/2RT)$, C_r and C_0 are the concentrations at radius r and r_0, respectively, M is the molecular weight of the monomer, υ is the partial specific

volume, r is the solvent density, ω is the angular velocity of the rotor, K_a is the association constant of the monomer−dimer equilibrium, and E is the baseline offset. Partial specific volumes were calculated from the weight average of the partial specific volumes for individual amino acids. Data were fitted to the equation by nonlinear least squares using the Microcal Origin 4.1 software provided by Beckman for the XL-A. The quality of the fit was characterized by χ^2, the sum of the squares of the residuals, and examination of the residuals for systematic deviation. The data obtained for Nt-CRLR could be fitted to a monomer. Protein concentrations used were 0.5 mg/mL in 20 mM sodium phosphate with 100 mM NaCl at pH 7.3.

Sedimentation velocity experiments were performed at 20 °C at a rotor speed of 50000 rpm with standard double sector Epon charcoal-filled centerpieces. The UV absorption of the cells was scanned using continuous mode. The data were analyzed with the SedFit 8.7 program, and the solvent density and viscosity were obtained from the Sednterp program. The calculated sedimentation coefficient ($s_{20,w}$) of 1.8 corresponds to a MW of 18.1 kDa, indicating that the CRLR N-domain is a monomer.

Circular Dichroism. CD spectra were collected at 25 °C on an AVIV 62DS spectropolarimeter. Spectra are the average of three to five scans and were corrected for buffer contribution. The sample concentration was 0.16 mg/mL in phosphate buffer containing 20 mM sodium phosphate and 100 mM NaCl, pH 7.3, and the sample was filtered through a 0.22 μm syringe filter.

Fluorescence Spectroscopy. Fluorescence spectroscopic studies were performed in buffer containing 20 mM sodium phosphate and 100 mM sodium chloride, pH 7.3, with or without 6 M GdnHCl. The final concentration of the protein used was 4 μM. Spectra were collected with an excitation of 295 nm and emission from 310 to 400 nm. Appropriate buffer blank spectra were subtracted.

Preparation of Membrane Protein from Rat Uterus. Membranes were prepared from day 20 pregnant rat uterine tissue for ligand-binding assay as previously described (*7*). Briefly, rat uterus was homogenized in ice-cold Tris-HCl buffer (50 mM Tris-HCl, pH 7.4) containing 0.32 mM sucrose, 1 mM dithiothreitol, 5 mM EDTA, and 200 KIU/L aprotinin. After centrifugation at 1000*g* for 10 min at 4 °C, the supernatant was further centrifuged at 10000*g* for 20 min at 4 °C. The crude pellet was then resuspended in Tris-HCl buffer and centrifuged again. The final pellet was resuspended in Tris-HCl buffer, and the protein concentration was determined and adjusted to 1 mg of protein/mL with assay buffer (50 mM Tris-HCl, 10 mM KCl, 100 KIU/L aprotinin, 3 mM sodium azide).

^{125}I-CGRP and ^{125}I-AM Binding Assay in Pregnant Rat Uterine Membranes. Rat uterus is reported to have CGRP and AM binding sites, and these sites are reported to be elevated during pregnancy (*33*, *35*). Inhibition by Nt-CRLR of ^{125}I-CGRP and ^{125}I-AM binding to uterine membranes was measured using CGRP and AM binding as previously described (*35*). Since $RAMP_1$ is a common receptor activity modifying protein for CGRP as well as amylin receptor, we also tested cross-reactivity of amylin for CGRP receptor by performing ^{125}I-CGRP binding assay in the presence of varying concentrations of unlabeled amylin. Briefly, ^{125}I-CGRP or ^{125}I-AM (10^{-11} M) was incubated with membrane proteins (50 μg per tube) in the absence or presence of increasing amounts of unlabeled CGRP or AM (10^{-11} to 10^{-8} M), Nt-CRLR (3×10^{-9} to 5×10^{-6} M), or amylin (2.3×10^{-6} to 3×10^{-3} M). The binding was performed in a total reaction volume of 300 μL in assay buffer (50 mM Tris-HCl, 10 mM KCl, 100 KIU/L aprotinin, 3 mM sodium azide) containing 0.5% heat-inactivated bovine serum albumin (BSA) for 150 min at 4 °C. After incubation, 600 μL of cold assay buffer was added to each tube, and the mixture was centrifuged at 12000*g* for 5 min at 4 °C. The bound radioactivity remaining in the pellets was counted in a Packard Auto-gamma counter. ^{125}I-CGRP or ^{125}I-AM binding to rat uterine membrane was analyzed using the method of Scatchard (*28*). The percent of inhibition of the ^{125}I-CGRP and ^{125}I-AM binding to uterine membranes in the presence of varying amounts of unlabeled CGRP, AM, Nt-CRLR, or amylin was determined. The concentrations of unlabeled CGRP, AM, Nt-CRLR, or amylin at which 50% (IC_{50}) of the ^{125}I-CGRP or ^{125}I -AM binding was inhibited were calculated, and binding constants were determined by Scatchard analysis. All reactions were done in triplicate and values determined were the mean of three separate assays.

Direct Binding of ^{125}I-CGRP to the Nt-Domain of CRLR. Nt-CRLR (200 nM) was incubated with 0.5 nM ^{125}I-CGRP for 150 min at 4 °C. These conditions were found to be optimal from our previous CGRP binding studies (*35*). The binding buffer consisted of 50 mM sodium phosphate, pH 7.4, 10 mM KCl, and 1 mM phenylmethanesulfonyl fluoride (PMSF). ^{125}I-CGRP binding was performed in the presence (nonspecific binding) or absence (total binding) of 1 μM unlabeled CGRP. As a control for nonspecific binding of ^{125}I-CGRP, binding was performed with an unrelated protein, insulin-like growth factor binding protein 3 (IGFBP-3). The radioligand binding at equilibrium was determined by Ni-NTA pull-down assay (*36*). Briefly, 40 μL of the Ni-NTA resin preequilibrated in binding assay buffer was added after the completion of the binding reaction. The reaction was further incubated for 5 min at 4 °C and centrifuged at 1000*g*. The pellet was washed with ice-cold assay buffer. The bound fraction was counted for radioactivity using a γ counter (Beckman). Counts were corrected for any nonspecific binding of ^{125}I-CGRP to Ni-NTA alone in the presence or absence of cold CGRP. All reactions were done in triplicate and values determined were the mean of three assays.

Specific binding of ^{125}I-CGRP with Nt-CRLR was also assayed by cross-linking studies using EDC, which is a zero-length cross-linker. Purified Nt-CRLR (200 nM) was incubated with 0.5 nM ^{125}I-CGRP for 150 min at 4 °C in the presence or absence of 1 μM unlabeled CGRP or amylin. Nonspecific binding was assessed by cross-linking 1 μM IGFBP3, a nonspecific protein, with ^{125}I-CGRP. The buffer consisted of 50 mM sodium phosphate, pH 7.4, 10 mM KCl, and 1 mM PMSF. Freshly prepared EDC (25 μM) was added, and the reaction was incubated at room temperature for 30 min. The reaction was then quenched by adding 4× SDS−PAGE loading buffer and electrophoresed on 12% SDS−PAGE along with prestained markers. The gel was dried and exposed to Kodak X-Omat film with an enhancing screen at −70 °C.

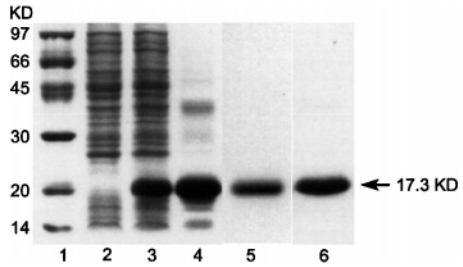

FIGURE 1: SDS−PAGE of bacterial extract or N-terminal domain protein of CRLR. Lanes: 1, marker; 2, bacterial protein obtained before induction; 3, bacterial protein obtained after induction with IPTG; 4, inclusion body; 5, protein after Ni-NTA column; 6, purified protein after Resource Q column.

RESULTS

Cloning and Expression of the Nt-CRLR. We have cloned the Nt-domain of CRLR without the signal sequence (the first 22 amino acid sequence of CRLR) in pCRT7/NT-TOPO, an expression vector from Invitrogen. The sequence of the Nt-domain was calculated using the PHDhtm Predict Protein algorithm (*27*). A cDNA encoding Nt-domain of CRLR (Glu 23−Glu 133) was amplified using polymerase chain reaction (PCR) and inserted into a pCRT7/NT-TOPO expression vector (Invitrogen). The Nt-domain, Nt-CRLR, was expressed in *E. coli* with the yield of 20−25% (∼100 mg/L) of the total *E. coli* extract (Figure 1). Total *E. coli* protein fractions when run on 12% SDS−PAGE showed that Nt-CRLR was present exclusively as inclusion bodies (lane 4, Figure 1). We observed that addition of L-arginine facilitated refolding and considerably minimized protein loss due to aggregation (*3*, *31*). In addition, the redox system employed for renaturation was also necessary, indicating the importance of the disulfide bond for proper folding. Refolded protein was further purified on an anion-exchange Resource Q column (Figure 1, lane 6), and the final yield of the refolded protein was 5−10 mg/L of induced *E. coli* culture. With this clone and the purification system, we now have a well-established recombinant production system for the CRLR Nt-domain for structural and functional studies.

Biophysical Characterization of the Nt-Domain of CRLR. Purified and refolded Nt-CRLR obtained was verified for its mass by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectroscopy, confirming the monomeric mass of Nt-CRLR as 17.3 kDa. All cysteines of the protein participate in disulfide bond formation as confirmed by an assay using Ellman's reagent (*26*). Data from ultracentrifugation studies as shown in sedimentation equilibrium (Figure 2A) and sedimentation velocity (Figure 2B) measurements indicate that Nt-CRLR exists as single species corresponding to its monomeric form. Unlike CD spectra of α-helical proteins that are similar to those of model α-helices, CD spectra of β-proteins exhibit profiles that are similar to either those of model β-sheets or unordered polypeptides and are classified as either β_I- or β_{II}-proteins, respectively. It has recently been shown that the source of β_{II}-protein is due to contribution of the poly(Pro)II-type (P2) structure, and the relative ratio of P2 to β-sheet determines the CD profile to be either β_I or β_{II} (*29*). The CD profile of the CRLR N-domain suggests that it belongs to the β_{II} class of β-sheet proteins (Figure 3A). Other class B GPCR receptors such as the parathyroid hormone receptor N-domain

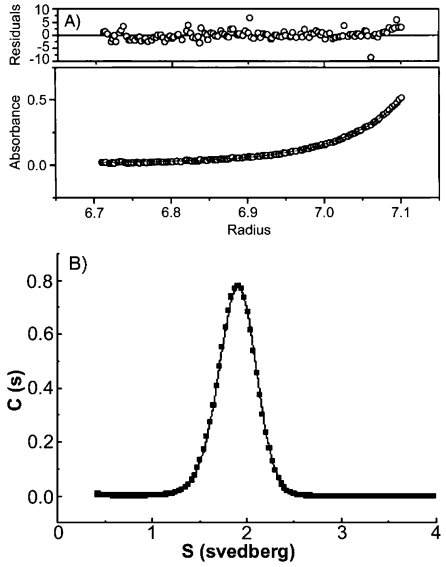

FIGURE 2: Ultracentrifugation studies. (A) Sedimentation equilibrium profile of the purified N-terminal domain of CRLR. The molecular mass of Nt-CRLR was determined by analytical ultracentrifugation. The data for the protein could be fitted to a single species of molecular weight corresponding to that of monomers. Randomness of the residuals (χ^2) indicates the quality of the fit. (B) Sedimentation velocity profile of purified Nt-CRLR. The calculated molecular weights from measured sedimentation coefficients indicated that Nt-CRLR is a monomeric protein.

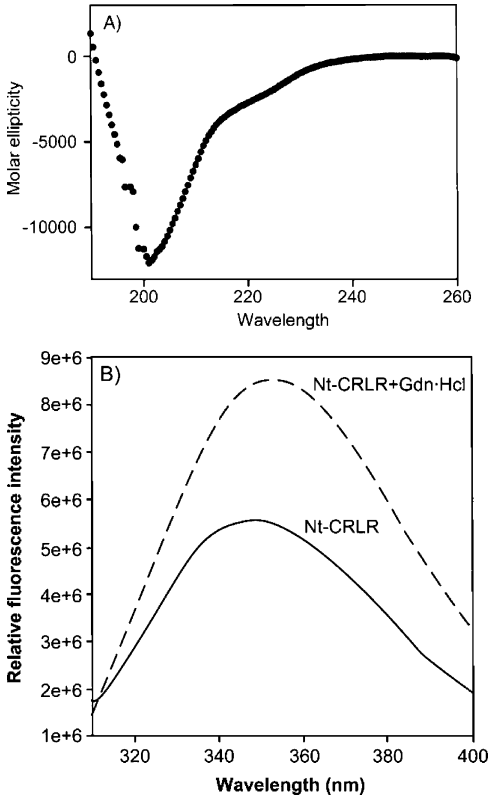

FIGURE 3: Biophysical characterization of Nt-CRLR. (A) Far-UV circular dichroism spectra of a 0.16 mg/mL solution of the CRLR N-terminal domain in 20 mM sodium phosphate and 50 mM NaCl, pH 7.3. (B) Fluorescence spectroscopy of Nt-CRLR in the presence and absence of 6 M GdnHCl.

also show a CD spectrum characteristic of the β_{II} class of β-sheet proteins (*28*).

On addition of guanidine hydrochloride, the fluorescence emission maximum is red shifted, indicating that the tryp-

tophans are in a hydrophobic environment in the folded protein, and interestingly, it is observed that the fluorescence intensity actually increases on unfolding, suggesting that the fluorescence is quenched in the folded state due to the proximity of other aromatic amino acids (Figure 3B). CD data also show loss of structure in the presence of GdnHCl (data not shown). Taken together, our results show that the refolded Nt-CRLR protein is homogeneous, monomeric, and stable and possesses a well-defined secondary structure.

Inhibition of ^{125}I-CGRP and ^{125}I-AM Binding to Pregnant Rat Uterine Membrane-Bound Full-Length CRLR by Nt-CRLR. The ability of Nt-CRLR to compete with native CGRP and AM receptors in rat uterus for binding to ^{125}I-CGRP and ^{125}I-AM was assessed. We measured ^{125}I-CGRP and ^{125}I-AM binding to membrane receptors from pregnant rat uterus in the presence of varying concentrations of unlabeled CGRP, AM, Nt-CRLR, or amylin, and the IC_{50} for CGRP, AM, Nt-CRLR, and amylin were calculated. Scatchard analysis of ^{125}I-CGRP binding in the presence of increasing amounts of unlabeled CGRP and AM confirmed that rat uterus contains high-affinity CGRP (CRLR + $RAMP_1$) and AM (CRLR + $RAMP_2$/$RAMP_3$) with an apparent K_d of 1.8×10^{-9} and 8×10^{-9} M, respectively. The calculated concentrations of unlabeled CGRP, Nt-CRLR, and amylin causing half-maximal inhibitory effects (IC_{50}) of ^{125}I-CGRP binding to rat uterine membrane were 2×10^{-9}, 2.15×10^{-7}, and 6.5×10^{-5} M, respectively. Cross-reactivity of amylin with CGRP receptor in rat uterus is evident as both CGRP and amylin share a common receptor activity modifying protein $RAMP_1$ (Figure 4A). However, IC_{50} for amylin is 100-fold higher than Nt-CRLR for ^{125}I-CGRP binding, indicating an important role of Nt-CRLR in ligand binding and selectivity. The concentrations of AM and Nt-CRLR causing half-maximal inhibitory effect (IC_{50}) of ^{125}I-AM binding to rat uterine membranes were 9×10^{-9} and 2.9×10^{-7} M, respectively (Figure 4B). Amylin up to 3 mM was unable to displace ^{125}I-AM binding in rat uterine membrane protein. The specificity of Nt-CRLR binding to ^{125}I-CGRP and ^{125}I-AM is further evidenced by the observation of reduced ^{125}I-CGRP and ^{125}I-AM binding to rat uterine membrane protein in the presence of Nt-CRLR protein ($r^2 = 0.98$) (Figure 4). ^{125}I-CGRP binding to Nt-CRLR is further confirmed by a direct ligand-binding assay with the Nt-CRLR (Figure 5). The specificity of this interaction was further assessed by ligand binding followed by cross-linking in the presence of unlabeled amylin. As shown in Figure 6, amylin is unable to displace CGRP binding to Nt-CRLR, which is consistent with amylin receptor phenotype (*25*). In addition, ^{125}I-CGRP did not bind to a nonspecific protein, IGFBP3, further supporting the specificity of ^{125}I-CGRP binding to the Nt-domain of CRLR. These studies support our hypothesis that the Nt-domain of CRLR possesses ligand-binding activity and is capable of binding to CGRP and AM in the absence of RAMPS.

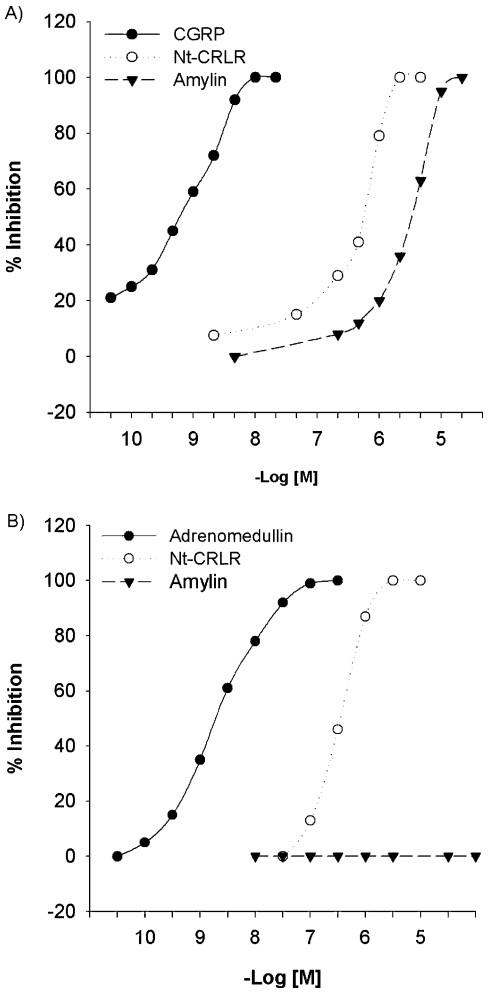

FIGURE 4: Competition binding assay. (A) ^{125}I-CGRP binding to rat uterine membrane receptors and its inhibition by unlabeled CGRP, Nt-CRLR, and amylin. ^{125}I-CGRP binding was dose-dependently inhibited by unlabeled CGRP, Nt-CRLR, and amylin. (B) ^{125}I-AM binding to rat uterine membrane receptors and its inhibition by unlabeled AM, Nt-CRLR, and amylin.^{125}I-AM binding was dose-dependently inhibited by both unlabeled AM and Nt-CRLR whereas amylin did not displace ^{125}I-AM. Binding assays were performed in duplicate, and each point represents the mean of two assays, and the inhibition was expressed as percentage of total ^{125}I-CGRP binding to rat uterine membranes.

DISCUSSION

CRLR is a 7-TM class B GPCR, which forms a functional CGRP receptor when coexpressed with $RAMP_1$ and a functional AM receptor when coexpressed with $RAMP_2$ or $RAMP_3$ (*1*, *20*). However, a recent study suggests that RAMPs also associate with other class B GPCRs, suggesting their role in modulating cell signaling, but unlike CRLR and CTR, this association does not cause a change in receptor phenotype (*5*). Although it has been recently established that signaling by CGRP, AM, and amylin is unique among peptide hormones and requires the formation of a receptor/RAMP complex (*20*), the exact role of these peptides and their cognate receptors in different physiologies remains to be investigated. It is evident that the structure and functional knowledge of these receptor components is of paramount importance for understanding ligand selectivity and affinity.

In this study, we have focused on structural and functional properties of the Nt-domain of CRLR. We have expressed Nt-CRLR in *E. coli* as an inclusion body. Formation of an inclusion body is a common phenomenon of heterogeneously expressed protein in *E. coli.* As Nt-CRLR has three disulfide bridges, more than 80% of the expressed protein was deposited as aggregates (Figure 1). Reduced and oxidized glutathione and L-arginine were used to refold the protein. L-Arginine, a low molecular weight folding enhancer has been shown to be very effective in other refolding protocols

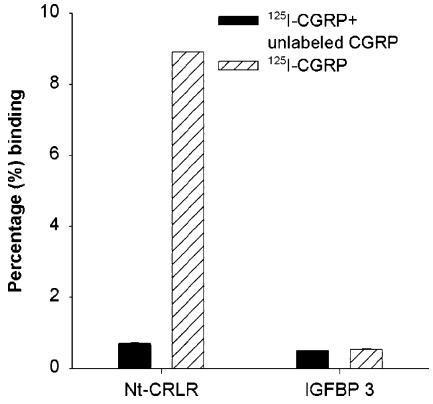

FIGURE 5: Specificity of ^{125}I-CGRP binding to the purified Nt-CRLR. This figure shows the percentage of ^{125}I-CGRP bound to Nt-CRLR in the absence (total binding) or presence of 1 μM unlabeled CGRP (nonspecific binding). The binding complex was pulled down by Ni-NTA. IGFBP3 protein was utilized to determine if ^{125}I-CGRP specifically binds to an unrelated protein. Each bar represents the mean ± SEM of three separate assays.

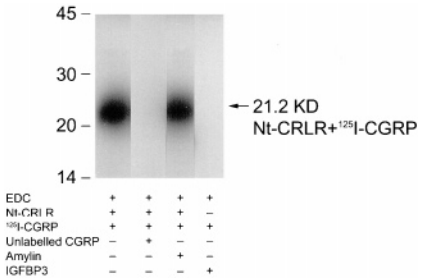

FIGURE 6: Autoradiogram illustrating cross-linking of ^{125}I-CGRP to the Nt-CRLR protein. ^{125}I-CGRP binds specifically to the Nt-CRLR (21.2 kDa). Note that the bands obtained with ^{125}I-CGRP are completely displaced with unlabeled CGRP and amylin did not alter ^{125}I-CGRP binding to Nt-CRLR. ^{125}I-CGRP does not show any cross-linked product with the nonspecific protein IGFBP-3.

including refolding of the N-terminal domain of parathyroid hormone receptor (*15*) and antibody FAB fragments (*4*). L-Arginine is reported to stabilize the native state of the protein by preferential hydration (*32*). As an oxido-shuffling system a 5:1 ratio of reduced GSH to oxidized GSSG was used, which allows rapid reshuffling of the incorrect disulfide bonds (*15*). This refolding method yielded 30−40% renaturation of Nt-CRLR from the inclusion body material. As shown in Figure 2, the refolded Nt-CRLR was monomeric and structurally well-defined as demonstrated in Figure 3.

The binding property of Nt-CRLR is determined by two separate methods: (1) by the ability of Nt-CRLR to compete with CGRP and AM receptors in rat uterus for binding ^{125}I-CGRP and ^{125}I-AM and (2) by specific direct binding of ^{125}I-CGRP to Nt-CRLR. The binding of ^{125}I-CGRP and ^{125}I-AM to rat uterine receptors is reduced in the presence of Nt-CRLR, with an IC_{50} of 2.15×10^{-7} and 2.9×10^{-7} M, respectively. Scatchard analysis of ^{125}I-CGRP binding to rat uterine tissue indicated a K_d of 1.8×10^{-9} M and an IC_{50} of 1.9×10^{-9} M for CGRP, which is similar to our previous reports (*35*). The K_d for ^{125}I-AM binding to rat uterine membranes was 8×10^{-9} M, and the IC_{50} was 9×10^{-9} M. The IC_{50} value of Nt-CRLR to compete for both of the ligands shows binding interaction of Nt-CRLR with CGRP and AM. Additionally, in direct ligand-binding studies using an Ni-NTA pull-down assay (Figure 5) and cross-linking studies (Figure 6), we show that Nt-CRLR is bound and cross-linked with the radiolabeled CGRP, suggesting a direct protein−protein interaction between the Nt-domain of CRLR with the ligand CGRP. Further, the binding of ^{125}I-CGRP to the Nt-domain of CRLR is specific, since unlabeled CGRP, but not amylin, displaced ^{125}I-CGRP binding (Figure 6), thus supporting the known pharmacology of amylin receptor (*25*, *34*). However, amylin competes dose dependently for ^{125}I-CGRP but not ^{125}I-AM binding in rat uterine membrane protein with half-maximal displacement, $IC_{50} = 6.5 \times 10^{-5}$ M. Cross-reactivity of amylin with CGRP receptor is evident as the receptor for both of these ligands shares a common receptor activity modifying protein $RAMP_1$. However, IC_{50} for amylin is 100-fold higher than that for the N-terminal domain of CRLR (Nt-CRLR, $IC_{50} = 2.15 \times 10^{-7}$ M). This further demonstrates the importance of Nt-CRLR in ligand specificity and selectivity. The difference in the affinities of the intact CGRP and AM receptor in rat uterus in the presence and absence of Nt-CRLR clearly indicates that the N-terminal domain alone is not sufficient for ligand binding and that RAMPS and/or other domains of the CRLR protein are also involved in the formation of high-affinity CGRP and AM receptor.

CRLR has been shown to be an active CGRP and AM receptor only when coexpressed with RAMPS (*11*, *20*). Distinct pharmacology acquired by CRLR in the presence of RAMPS reflects either a direct participation of RAMPS in the selective binding pocket of the ligand or an indirect conformational effect on CRLR (*10*). Hilairet et al. have demonstrated in their cross-linking studies that CGRP forms a cross-linked product with the CRLR/$RAMP_1$ complex (*17*), suggesting that either CGRP binds to both CRLR and $RAMP_1$ or that CGRP binds to CRLR and cross-linking to $RAMP_1$ is simply because of proximity effects. In that case interaction of $RAMP_1$ with CRLR could induce in a conformational change in CRLR resulting in a high-affinity receptor by providing an important ligand-binding site. Current evidence suggests that the transmembrane helices are responsible for the homodimer and heterodimer formation (*30*). According to the known pharmacology of class B 7-TM GPCRs, $RAMP_1$ is not involved in direct binding to the ligand, and CGRP binds only to CRLR. However, with the recent reports, there remains a possibility that $RAMP_1$ could provide an important site for ligand binding (*8*, *19*). In a recent study, $RAMP_1$ chimera were used to dissect the role of the three different structural domains of $RAMP_1$ (Nt-domain, TM domain, and cytoplasmic domain). It was shown that the extracellular domain of $RAMP_1$ is absolutely critical for CGRP binding and affinity (4000-fold decrease in CGRP binding and affinity in the case of the $RAMP_1$ Nt-domain mutant) and thereby stimulation of receptor-mediated signal transduction (*19*). Nevertheless, this does not preclude the possibility that both $RAMP_1$ and CRLR can bind CGRP independent of each other but likely not be able to induce signaling.

With respect to the contribution of factors such as exoloops of 7-TM CRLR and RAMP accessory protein in ligand binding, the energetics of ligand binding and receptor activation is extremely complicated, and the contribution of binding affinity from CRLR and RAMPS is not trivial. In the case of GPCR receptors, ligand-binding affinity (free energy, *G*) comes from binding to both the Nt domain and the extracellular loops (exoloops). If the binding events are

coupled, there is an additional coupling energy involved (*18*). Our data deal with binding to the Nt domains and do not address the role of ligand binding to the exoloops. Therefore, our study does not address all of the binding events (and binding affinities) in vivo. Our data capture the earliest of the binding events and so do not address ligand-induced conformational changes and a possible role of RAMPS that could be important for binding and signal transduction. Thus, with the Nt-CRLR protein we can now begin to address some of these issues.

These studies are performed with the recombinant Nt-CRLR protein synthesized in a prokaryotic system. Therefore, ligand binding observed is devoid of factors which are known to influence ligand binding, such as glycosylation, TM domains, exoloops of the CRLR receptor, and effect of RAMP protein. In addition, for the first time, the Nt-domain of CRLR has been expressed independent of RAMPs, and thus, we were able to address the contribution of Nt-CRLR in CGRP and AM actions independent of the reported role of RAMPS as a chaperone and the glycosylation state of CRLR. Although analysis of the receptor−ligand interaction and defining the precise nature of the interaction between CRLR and RAMPS with CGRP and AM will require detailed structural and functional studies, the present investigation has provided considerable insight into their mode of interaction and the feasibility of overexpression of the Nt-domain of CRLR and its subsequent refolding and purification to obtain homogeneous proteins for future structural studies.

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