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Bacterial expression and purification of a heterodimeric Adrenomedullin receptor extracellular domain complex using DsbC-assisted disulfide shuffling

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Abstract

Adrenomedullin (AM) is a peptide hormone that is a potent vasodilator and is essential for vascular development. The AM receptor is a heterodimeric cell surface receptor composed of the calcitonin receptor-like receptor (CLR), a class B G protein-coupled receptor, in association with either of two receptor activity modifying protein (RAMP) coreceptors, RAMP2 or -3. The extracellular domains (ECDs) of CLR and the RAMPs form the primary AM binding site. Here, we present novel methodology for expression and purification of a heterodimeric AM receptor ECD complex as an MBP-CLR ECD fusion protein in association with the RAMP2 ECD. Co-expression of the RAMP2 ECD with the disulfide bond isomerase DsbC in the oxidizing cytoplasm of *E. coli trxB gor* enabled proper disulfide formation *in vivo*. The isolated RAMP2 ECD was purified to homogeneity. Co-expression of a soluble MBP-CLR ECD fusion protein with DsbC in *E. coli trxB gor* yielded a heterogeneous mixture of species with misfolded ECD. Incubation of affinity-purified MBP-CLR ECD *in vitro* with purified RAMP2 ECD, DsbC, and glutathione redox buffer promoted proper folding of the CLR ECD and formation of a stable MBP-CLR ECD:RAMP2 ECD complex that was purified by size-exclusion chromatography and which exhibited specific AM binding. Approximately 40 mg of highly purified complex was obtained starting with 6 L bacterial cultures for each protein. The methodology reported here will facilitate structure/function studies of the AM receptor.

Keywords

G protein-coupled receptor; Co-receptor; Peptide hormone; Vasodilator

Introduction

Adrenomedullin (AM) is a 52-amino acid peptide hormone that was originally isolated from a human pheochromocytoma [1] and has since been shown to have multiple functions. AM is produced by a wide range of cells including smooth muscle and vascular endothelial cells where it functions as a potent vasodilator [2, 3]. AM also has important functions during development, being essential for vascularization [3, 4]. Altered AM levels are associated

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with several pathophysiological states including acute myocardial infarction, pulmonary hypertension, preeclampsia, and sepsis [2, 3, 5]. The protective effect of AM in these conditions suggests that AM receptor agonism may be a treatment strategy. In addition, the angiogenic activity of AM has led to the suggestion that AM receptor antagonism might block tumor angiogenesis [6]. The AM receptor is thus of considerable interest as a drug target. The AM receptor is a heterodimeric cell surface receptor that is comprised of the calcitonin receptor-like receptor (CLR), a class B G protein-coupled receptor (GPCR), in association with a receptor activity modifying protein (RAMP) coreceptor. Three RAMPs associate with CLR to determine hormone binding specificity for AM or the related vasodilatory peptide calcitonin gene-related peptide (CGRP) [7]. Heterodimerization of CLR with RAMP2 or -3 gives rise to AM receptors, whereas the CLR-RAMP1 heterodimer is a CGRP receptor. AM and CGRP are members of the calcitonin family of peptide hormones that also includes calcitonin (CT), which regulates calcium homeostasis, and amylin (AMY), which regulates blood glucose levels [8]. CT signals through a related class B GPCR, the calcitonin receptor (CTR), in the absence of RAMPs [9]. Association of any of the three RAMPs with CTR gives rise to AMY receptors [10–12].

CLR, like CTR and other class B GPCRs, is comprised of an N-terminal extracellular domain (ECD) and a membrane-embedded 7-transmembrane (TM) domain. The ECD contains six conserved cysteine residues that form three disulfide bonds that are essential structural elements of the short consensus repeat fold common to class B GPCR ECDs [13, 14]. Peptides bind to class B GPCRs in a bipartite manner; the C-terminal half of the peptide binds to the ECD to impart high affinity, and the N-terminal half of the peptide binds to the 7-TM domain to activate the receptor [15]. RAMPs consist of an N-terminal ECD linked to a single TM helix. RAMPs 1 and 3 each have three disulfide bonds in their ECD, whereas RAMP2 has only two. The RAMP ECDs form a 3-helix bundle in which the disulfides play a structural role by linking helices [16, 17]. Cell-based chimeric receptor and mutagenesis studies indicated that the CLR and RAMP ECDs interact with each other and that the RAMP ECD determines peptide hormone specificity [18–22]. Several groups have purified isolated CLR and RAMP1 and -2 ECDs as well as CLR-RAMP1 and CLR-RAMP2 ECD complexes for structure/function studies [16, 17, 23, 24]. In all cases, the CLR and RAMP ECDs were expressed in *E. coli* as inclusion bodies and the ECDs were purified using traditional refolding protocols with denaturants, glutathione redox buffer, and L-arginine. Co-refolding of the CLR and RAMP ECDs yielded the heterodimeric complexes. Crystal structures of isolated RAMP1 and RAMP2 ECDs and heterodimeric CLR-RAMP1 and CLR-RAMP2 ECD complexes have been reported [16, 17, 25], but no peptide-bound structures are available.

We previously developed methodology for the bacterial expression and purification of class B GPCR ECDs engineered in a form that facilitates proper disulfide bond formation and promotes their crystallization for structural studies [14]. Our methodology, which is distinct from traditional refolding protocols, utilizes *in vitro* disulfide bond isomerase catalyzed disulfide shuffling in the absence of denaturants. The technology incorporates the following features: *i)* The GPCR ECD is tagged at the N-terminus with maltose binding protein (MBP) to promote solubility and permit affinity purification on amylose resin; *ii)* The ECD is tagged at the C-terminus with a hexa-histidine (H_6) tag to permit purification by immobilized metal affinity chromatography (IMAC) and ensure isolation of full-length product; *iii)* The MBP-ECD- H_6 fusion protein is expressed in the oxidizing cytoplasm of *E. coli* *trxB* *gor* to permit disulfide bond formation; *iv)* The fusion protein is co-expressed with the bacterial chaperone/disulfide isomerase DsbC; *v)* If co-expression with DsbC is insufficient to generate the proper disulfide bond pattern *in vivo*, the affinity-purified fusion protein is incubated overnight *in vitro* with purified DsbC in a glutathione redox buffer to resolve incorrectly formed disulfide bonds; and *vi)* Properly folded MBP-ECD fusion

protein is purified away from misfolded multimers and aggregates by size-exclusion chromatography. MBP plays a crucial role by solubilizing misfolded ECD throughout the procedure. Moreover, MBP facilitates crystallization of the fusion protein by providing a large hydrophilic surface area for crystal contacts. This technology enabled the purification, crystallization, and structure determination of several class B GPCR ECDs in peptide-bound and ligand-free states [14, 26–30]. Here, we show that our expression and purification technology can be adapted to produce a heterodimeric AM receptor ECD complex comprised of an MBP-CLR ECD fusion protein and the RAMP2 ECD.

Materials and Methods

Plasmid construction

The cDNAs encoding human RAMP2 and CLR were obtained from the Missouri S&T cDNA resource center. Two expression plasmids were constructed by inserting PCR-amplified fragments encoding the RAMP2 or CLR ECDs into previously described plasmids [14, 29] using standard cloning techniques. The bacterial expression plasmids are based on the T7 promoter-driven, IPTG inducible pETDuet1 vector (Novagen) for co-expression of two proteins in *E. coli*. For both plasmids, the first multiple cloning site encodes the ECD of interest as an MBP fusion protein and the second multiple cloning site encodes the bacterial disulfide isomerase protein DsbC. The RAMP2 ECD expression plasmid encodes MBP followed by a flexible GSSSGGG linker, thrombin cleavage site LVPRGS, and a fragment encoding residues 36–140 of RAMP2 with a C-terminal (His)₆ tag and a stop codon. A BamHI site in the parental vector overlaps the GS of the thrombin cleavage site and permits insertion of the RAMP2 fragment as a BglII-NotI insert. The plasmid for expression of MBP-CLR ECD encodes MBP followed by an NAAAEF linker and then a fragment encoding residues 29–144 of CLR with a C-terminal (His)₆ tag and a stop codon. An EcoRI site in the parental plasmid overlaps the EF sequence in the linker and permits insertion of the CLR-encoding fragment as an EcoRI-NotI insert. The PCR primers were as follows: CLR sense: 5'-GCATTAGAATTTCGAGGACTCAATTCAG-3'; CLR anti: 5'-ATAGAAGCGGCCGCTTAATGATGGTGGTGATGATGCAGGTAAAACAAATTTAG-3'; RAMP2 sense: 5'-GCATTAAGATCTAATCCCCACGAGGCC-3'; RAMP2 anti: 5'-ATAGAAGCGGCCGCTTAATGATGGTGGTGATGATGGTCAGAGAAGGTGGG-3'. Automated DNA sequencing by the Microgen core facility at OUHSC verified the plasmids.

Protein expression

The MBP-Thrombin-RAMP2.36-140-H₆/DsbC and MBP-CLR.29-144-H₆/DsbC constructs were separately expressed in the *E. coli* strain Origami B (DE3) [Novagen], which contains the *trxB gor* mutations. Cells transformed with the expression plasmid were grown in baffled shake flasks at 37°C in LB Lennox broth supplemented with 50 µg/ml ampicillin. After the growth reached mid-log phase, the temperature was reduced to 16°C and the cultures were induced with 0.4 mM IPTG and incubated with shaking overnight. Cells from a 6L culture were harvested by centrifugation, resuspended in 100 ml buffer A (50 mM Tris-HCl, pH 7.5, 10% (vol/vol) glycerol, 150 mM NaCl, 25 mM Imidazole) and stored at –80°C.

Purification of RAMP2 ECD

All purification steps were performed at 4°C. The column chromatography steps utilized an AKTA purifier (GE Healthcare). The frozen, resuspended MBP-Thrombin-RAMP2.36-140-H₆ cell pellet was thawed and the cells were lysed by sonication (Branson). The lysate was clarified by centrifugation at 25,000×g for 20 min. The supernatant was loaded on a 50 ml bed volume Nickel-chelating Sepharose (GE Healthcare) column pre-equilibrated in buffer

A. The column was washed with buffer A and eluted with buffer B (50 mM Tris-HCl, pH 7.5, 10% (vol/vol) glycerol, 150 mM NaCl, 265 mM Imidazole).

The peak fractions were pooled and loaded on a 40 ml bed volume Amylose high flow (NEB) column pre-equilibrated in buffer C (50 mM Tris-HCl, pH 8.0, 5% (vol/vol) glycerol, 150 mM NaCl). The column was washed with buffer C and eluted with a linear gradient of buffer C to buffer D (50 mM Tris-HCl, pH 8.0, 5% (vol/vol) glycerol, 150 mM NaCl, 10 mM Maltose). The peak fractions were pooled and human α -thrombin (HTI) was added at a 1:750 (thrombin weight:protein weight) ratio and the digestion mixture was incubated overnight at 4°C. The digestion mixture was subjected to IMAC as above to remove the untagged MBP and thrombin. The peak fractions containing RAMP2 ECD-H₆ were spin concentrated (Pierce spin concentrator device; 9 kDa MWCO) and applied to a 320 ml bed volume Superdex S200HR gel-filtration column (GE Healthcare) equilibrated in buffer E (50 mM Tris-HCl, pH 7.5, 10% (vol/vol) glycerol, 150 mM NaCl, 0.5 mM EDTA). Peak fractions eluted from the gel-filtration column in buffer E were pooled, dialyzed to storage buffer (50 mM Tris-HCl, pH 7.5, 50% (vol/vol) glycerol, 150 mM NaCl), and stored at -80°C. Protein concentration was determined by Bradford assay with a BSA standard curve. Concentration is stated in terms of the monomer.

Purification of the MBP-CLR:RAMP2 ECD complex

The resuspended MBP-CLR.29-144-H₆ cell pellet was lysed by sonication and the fusion protein was purified by IMAC and Amylose affinity chromatography performed as above for the RAMP2 ECD construct. The affinity-purified MBP-CLR.29-144-H₆ was subjected to *in vitro* disulfide shuffling in the presence of purified RAMP2 ECD, a glutathione redox buffer, and purified DsbC. Purification of DsbC and non-denaturing (native) polyacrylamide gel electrophoresis for analyzing the protein folding states were performed as described previously [14]. The large-scale shuffling reaction contained MBP-CLR.29-144-H₆ (9 μ M), RAMP2.36-140-H₆ (13.5 μ M), DsbC (4.5 μ M dimer), 1 mM each reduced and oxidized glutathione in a buffer of 50 mM Tris-HCl, pH 8.0, 5% (vol/vol) glycerol, 150 mM NaCl, ~1 mM Maltose. The reaction was incubated overnight at 20°C and then subjected to IMAC (as above) to remove the untagged DsbC. The eluted AM receptor ECD complex was pooled and solid ammonium sulfate was added to 80% saturation to precipitate the complex. This step was used to concentrate the protein in preparation for gel-filtration chromatography. The precipitate was collected by centrifugation, resuspended in a minimal volume of 25 mM Tris-HCl, pH 7.5, 10% (vol/vol) glycerol, and applied to a Superdex S200HR gel filtration column as above for the RAMP2 ECD. Peak fractions containing the AM receptor ECD complex were pooled, dialyzed to storage buffer (50 mM Tris-HCl, pH 7.5, 50% (vol/vol) glycerol, 150 mM NaCl), and stored at -80°C. Protein concentration was determined by Bradford assay with a BSA standard curve. Concentration is stated in terms of the heterodimer.

Synthetic Peptides

Peptides were custom synthesized and HPLC-purified by RS Synthesis (Louisville, KY). All peptides were C-terminally amidated unless otherwise noted. Biotinylated peptides were labeled at the N-terminus. The lyophilized peptides were reconstituted in water and stored as aliquots at -80°C. Peptide concentrations were determined by UV absorbance at 280 nm using molar absorptivity calculated based on tyrosine residues. Peptide sequences were as follows:

B-sCT(1-32)NH₂[C1A/C7A]: Biotin-
ASNLSTAVLGKLSQELHKLQTYPRNTGSGTP-NH₂

B- α CGRP(8–37)NH₂: Biotin-GGYVTHRLAGLLSRSGGVVKNFVPTNVGSKAF-NH₂

B-AM(19–52)NH₂[C21A]: Biotin-GTATVQKLAHQIYQFTDKDKDNVAPRSKISPQGY-NH₂

B-rAMY(5–37)NH₂[C7A]: Biotin-ATAATQRLANFLVRSSNNLGPVLPPTNVGSNTY-NH₂

AM(22–52)NH₂: TVQKLAHQIYQFTDKDKDNVAPRSKISPQGY-NH₂

AM(22–52)OH: TVQKLAHQIYQFTDKDKDNVAPRSKISPQGY-OH

α CGRP(8–37)NH₂: YVTHRLAGLLSRSGGVVKNFVPTNVGSKAF-NH₂

AlphaScreen luminescent peptide binding assay

Peptide binding was assessed with an AlphaScreen luminescent proximity assay (Perkin-Elmer). The reaction mixtures contained 15 μ g/ml each (for saturation binding) or 10 μ g/ml each (for competition binding) streptavidin-coated donor beads and nickel-chelate-coated acceptor beads in a buffer of 50 mM MOPS pH 7.4, 150 mM NaCl, and 7 mg/ml fatty acid-free BSA (PAA Laboratories). The reactions were prepared in the dark under green light and incubated at room temperature. For saturation binding assays, the purified MBP-CLR ECD-H₆:RAMP2 ECD-H₆ complex (10 nM) was incubated with the indicated concentrations of biotinylated peptides and allowed to equilibrate for 5 h. For competition assays, biotinylated AM (40 nM) and the MBP-CLR ECD-H₆:RAMP2 ECD-H₆ complex (10 nM) were mixed with un-labeled competitor peptides as indicated, and the reactions were incubated 5 h to reach equilibrium. Photon counts were recorded in 384-well white optiplates using a PolarStar Omega plate reader equipped with filters for AlphaScreen (BMG Labtech). The competition data were fit to a variable-slope dose-response inhibition equation for determination of IC₅₀ values using Prism 5.0 (GraphPad Software, San Diego).

Results and Discussion

Plasmid construction and protein expression in *E. coli* *trxB gor*

A strategy for bacterial expression and purification of the heterodimeric AM receptor ECD complex was devised that involved purifying the isolated RAMP2 ECD and using it to promote *in vitro* folding of the CLR ECD and formation of a stable, heterodimeric complex. Two expression plasmids were constructed for the separate expression of the RAMP2 and CLR ECDs as soluble maltose binding protein (MBP) fusion proteins in *E. coli*. We reasoned that because the RAMP2 ECD contains only two disulfide bonds, co-expression of it with the disulfide bond isomerase DsbC in the oxidizing cytoplasm of *E. coli* *trxB gor* [31] would permit proper folding of the RAMP2 ECD *in vivo*, obviating the need for inclusion body solubilization and traditional refolding protocols as employed by other groups to purify RAMP ECDs [16, 17, 24]. To this end, a pETDuet1-based plasmid was constructed that encoded an MBP-Thrombin cleavage site-RAMP2 ECD-(His)₆ fusion protein in the 1st multiple cloning site and untagged DsbC in the 2nd multiple cloning site. RAMP2 residues 36–140 were used as the ECD. The thrombin cleavage site permits isolation of the ECD free of MBP. A similar plasmid was constructed that encoded an MBP-CLR ECD-(His)₆ fusion protein in the 1st multiple cloning site and untagged DsbC in the 2nd multiple cloning site. CLR residues 29–144 were used as the ECD. This plasmid differed from the previous plasmid in that instead of a thrombin cleavage site, an NAAAEF linker sequence was included between MBP and the ECD. We expected that the three CLR ECD disulfide bonds would not be properly formed *in vivo* because our previous experience with several other class B GPCR ECDs indicated that their co-expression with DsbC in *E. coli* *trxB gor* was insufficient for proper folding. However, we reasoned that our previously developed *in vitro*

DsbC-assisted disulfide shuffling methodology could be adapted to the MBP-CLR ECD:RAMP2 ECD complex. Each of the plasmids was separately introduced into the *E. coli* Origami B (DE3) expression strain, which contains the *trxB* *gor* mutations. Protein expression was performed in 6 L cultures with IPTG induction overnight at 16°C.

Purification of the RAMP2 ECD

Our strategy for purifying the RAMP2 ECD is outlined in Fig. 1A. We started with a 6 L culture co-expressing MBP-Thrombin-RAMP2.36-140-H₆ and DsbC. The fusion protein was purified by IMAC and amylose affinity chromatography via the H₆ and MBP tags, respectively (Fig. 1B, lanes 1 and 2). Approximately 340 mg of fusion protein was obtained. The affinity-purified sample exhibited predominantly a single band on non-reducing SDS-PAGE at the molecular weight of a monomer and a very small amount of high molecular weight disulfide-linked multimers (Fig. 1B, lane 2). These results supported the hypothesis that most of the fusion protein was properly folded *in vivo*. The affinity-purified sample was digested overnight with thrombin protease (Fig. 1B, lane 3), and the RAMP2 ECD-H₆ was purified away from MBP and the protease by IMAC (Fig. 1B, lane 4). The RAMP2 ECD was concentrated and applied to a Superdex200 size-exclusion column from which it eluted as a single peak (Fig. 1C and Fig. 1B, lanes 6–10). The purified RAMP2 ECD contained disulfide bonds as evidenced by its slower mobility on SDS-PAGE in the presence of the reducing agent DTT (Fig. 1C, inset). The RAMP2 ECD eluted from the gel filtration column at a volume corresponding to a molecular weight of ~ 38 kDa, which indicated a trimer (Fig. 1D). This result was consistent with the observation of trimers in two distinct crystal structures of the isolated RAMP2 ECD [17] [and PDBID 2XVT] and strongly suggested that the RAMP2 ECD was properly folded. A final yield of ~ 40 mg of purified RAMP2 ECD was obtained.

Purification of the MBP-CLR:RAMP2 ECD complex

Our strategy for purifying MBP-CLR ECD and forming the complex with purified RAMP2 ECD is outlined in Fig. 2A. We started with a 6 L culture co-expressing MBP-CLR.29-144-H₆ and DsbC. The MBP-CLR protein was purified by IMAC and amylose affinity chromatography, yielding ~120 mg protein (Fig. 2C, lanes 1 and 2). The amylose affinity step enabled quick exchange of the protein into a buffer suitable for *in vitro* disulfide shuffling. The affinity-purified MBP-CLR was a heterogeneous mixture of misfolded species, many of which were high molecular weight disulfide-linked multimers (species *i*) as evidenced by non-reducing SDS-PAGE (Fig. 2C, lane 2) and native gel electrophoresis (Fig. 2B, lane 1). The band of fastest mobility on lane 1 of the native gel (Fig. 2B) is likely misfolded, monomeric MBP-CLR (species *ii*). We tested several *in vitro* disulfide-shuffling conditions to assess the effects of glutathione redox buffer, RAMP2 ECD (13.5 μM), and DsbC (4.5 μM dimer) on the folding of MBP-CLR (9 μM). DsbC was purified as previously described [14]. Small-scale reactions were incubated overnight at 20°C and the MBP-CLR folding states were monitored by native gel electrophoresis (Fig. 2B). Redox buffer alone had little effect on the MBP-CLR population (Fig. 2B, lanes 2 and 3). Incubation of MBP-CLR with RAMP2 ECD in the presence of redox buffer resulted in the formation of a new MBP-CLR species (*iii*) of faster mobility (Fig. 2B, lanes 4 and 5), but did not eliminate species *ii*. Incubation of MBP-CLR with RAMP2 ECD and DsbC in redox buffer significantly decreased the misfolded multimer (*i*) and monomer (*ii*) species and generated a significant amount of species *iii* (Fig. 2B, lanes 6 and 7). We hypothesized that species *iii* was properly folded MBP-CLR. Notably, an MBP-CLR ECD:RAMP2 ECD complex was not observed on the native gel, suggesting that the complex was unstable under the electrophoretic conditions.

The *in vitro* disulfide shuffling condition used for Fig. 2B, lane 7 was chosen as the optimal condition for a large-scale disulfide shuffling reaction containing MBP-CLR ECD (~110 mg) and RAMP2 ECD (~39 mg) at a 1:1.5 molar ratio. After overnight incubation at 20°C, the presence of disulfide-linked MBP-CLR ECD multimers was significantly reduced (Fig. 2C, lane 3). The mixture was applied to an IMAC column to remove untagged DsbC (Fig. 2C, lanes 4 and 5), and the eluted sample containing MBP-CLR ECD-H₆ and RAMP2 ECD-H₆ was subjected to Superdex 200 gel-filtration chromatography. MBP-CLR ECD and RAMP2 ECD co-eluted from the gel filtration column indicating their interaction (Fig. 2D). The complex was well separated from remaining MBP-CLR ECD multimers and excess RAMP2 ECD. The elution volume of the complex indicated a molecular weight of ~139 kDa, which closely corresponds to a dimer of MBP-CLR ECD:RAMP2 ECD heterodimers (Fig. 2E). This result is consistent with the observation of a dimer of heterodimers in the crystal structure of the ligand-free CLR ECD:RAMP2 ECD complex [17]. We obtained a final yield of ~40 mg of MBP-CLR ECD:RAMP2 ECD complex. It is interesting that in our system the CLR ECD did not properly fold in the absence of the RAMP2 ECD. Other groups have reported the proper folding of bacterially expressed CLR ECD in the absence of RAMP ECDs using traditional refolding protocols [17, 23]. Our observation of a RAMP2 ECD requirement for CLR folding is consistent with CLR existing in cells as an obligate heterodimer with RAMPs [7].

The purified AM receptor ECD complex specifically binds AM

The peptide-binding ability of the recombinant AM receptor MBP-CLR ECD:RAMP2 ECD complex was assessed with an AlphaScreen luminescent proximity assay (Perkin-Elmer), which is based on the transfer of singlet oxygen molecules between “donor” and “acceptor” beads. Singlet oxygen molecules released by the donor beads activate light emission from the acceptor beads when the beads are brought into proximity by a molecular interaction. We previously used this assay to examine peptide binding to the ECDs of other class B GPCRs [14, 27, 29, 30]. The recombinant protein complex was attached to the surface of Nickel-chelate coated acceptor beads via the H₆ tags and N-terminally biotinylated peptides were attached to the surface of streptavidin coated donor beads. In a saturation binding assay, the MBP-CLR ECD:RAMP2 ECD complex bound AM, but did not bind other calcitonin family peptides (Fig. 3A). In a competition assay, non-biotinylated AM displaced the interaction of biotin-AM and the receptor with an IC₅₀ value of ~10 μM and binding was dependent on C-terminal amidation (Fig. 3B), which is consistent with the requirement of C-terminal amidation for AM bioactivity. CGRP(8–37)NH₂ did not exhibit significant binding to the AM receptor ECD complex in the competition assay. Multivalency resulting from the two-bead assay format complicates K_D determination, however, under our assay conditions the IC₅₀ is likely a reasonable approximation of the affinity [29]. Affinity in the low micromolar range has been observed for other class B GPCR ECD-peptide pairs [14, 27, 29, 30]. The binding results indicated that the recombinant AM receptor ECD complex was properly folded and that the CLR and RAMP2 ECDs were sufficient to determine AM specificity. These results are consistent with chimeric receptor studies that indicated that the RAMP ECD is sufficient to determine the hormone binding specificity of CLR [18].

Conclusions

How RAMP coreceptors determine the peptide hormone binding specificity of CLR remains unclear. Here, we developed novel methodology that is both time- and cost-efficient for producing mg quantities of highly purified AM receptor CLR:RAMP2 ECD complex. The peptide-binding assay indicated that the recombinant protein was properly folded and exhibited the expected AM binding specificity. Thus, the bacterially expressed protein recapitulated the desired characteristics of the native receptor. The methodology reported

here will facilitate future structure/function studies of the AM receptor that will define how RAMPs determine CLR hormone binding specificity and may inform the rational design of therapeutic agents for disorders including acute myocardial infarction, pulmonary hypertension, preeclampsia, sepsis, and cancer.

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Abbreviations

CLR	calcitonin receptor-like receptor
CTR	calcitonin receptor
RAMP	receptor activity modifying protein
MBP	maltose binding protein
ECD	extracellular domain
AM	adrenomedullin
CGRP	calcitonin gene-related peptide
sCT	salmon calcitonin
rAMY	rat amylin
DsbC	<i>E. coli</i> disulfide bond isomerase
BSA	bovine serum albumin
PCR	polymerase chain reaction
IMAC	immobilized metal affinity chromatography
GSH	reduced glutathione
GSSG	oxidized glutathione
DTT	dithiothreitol
IPTG	isopropyl β -D-1-thiogalactopyranoside

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Highlights

- A heterodimeric AM receptor ECD complex was purified using novel folding methodology
- The RAMP2 ECD was properly folded by co-expression with DsbC in *E. coli* *trxB gor*
- Proper folding of an MBP-CLR ECD fusion protein was dependent on RAMP2 ECD
- DsbC-assisted disulfide shuffling enhanced RAMP2-dependent CLR ECD folding *in vitro*
- The recombinant MBP-CLR:RAMP2 ECD complex selectively bound AM

Fig. 1. Purification of the RAMP2 ECD. (A) Expression and purification strategy. “Th” is a thrombin cleavage site. (B) Non-reducing SDS-PAGE analysis of the RAMP2 purification. Molecular weight markers are shown in kDa. The gel was stained with coomassie blue. Lanes are as follows: 1, IMAC elution; 2, amylose elution; 3, after overnight thrombin digest; 4, 2nd IMAC elution; 5, gel-filtration void fraction; 6–10, gel-filtration peak fractions. (C) Gel-filtration elution profile. The inset shows an SDS-PAGE analysis of the final RAMP2 sample in the absence or presence of 25 mM DTT. (D) Molecular weight determination of the RAMP2 ECD by gel-filtration chromatography. Std, Bio-Rad gel-filtration molecular weight standards.

Fig. 2. Purification of the MBP-CLR ECD:RAMP2 ECD complex. (A) Expression and purification strategy. Species *i* represents intermolecular disulfide-linked MBP-CLR multimers, species *ii* represents monomeric MBP-CLR with misfolded CLR ECD, and species *iii* represents MBP-CLR with properly folded CLR ECD. (B) Non-denaturing (native) PAGE analysis of affinity-purified MBP-CLR folding states after overnight incubation at 20°C under the following conditions: 1, MBP-CLR alone; 2, 5 mM GSH/1 mM GSSG; 3, 1 mM each GSH/GSSG; 4, 5 mM GSH/1 mM GSSG and RAMP2; 5, 1 mM each GSH/GSSG and RAMP2; 6, 5 mM GSH/1 mM GSSG and RAMP2 and DsbC; 7, 1 mM each GSH/GSSG and RAMP2 and DsbC; 8, RAMP2 and DsbC (no GSH/GSSG); 9, 1 mM each GSH/GSSG and RAMP2 and DsbC (no MBP-CLR); 10, 25 mM DTT and MBP-CLR and RAMP2. RAMP2 is not visible on the gel because the MBP-CLR ECD:RAMP2 ECD complex was unstable during electrophoresis and the isolated RAMP2 ECD ran off the gel. The gel was stained with coomassie blue. (C) Non-reducing SDS-PAGE analysis of the purification. Molecular weight markers are shown in kDa. The gel was stained with coomassie blue. Lanes are as follows: 1, IMAC elution; 2, amylose elution; 3, after large-scale overnight disulfide shuffling in the presence of DsbC, RAMP2, and glutathione redox buffer; 4, 2nd IMAC flow through; 5, 2nd IMAC elution. (D) Gel-filtration elution profile. The insets show lanes from a non-reducing SDS-PAGE analysis of the two main peaks. (E) Molecular weight determination of the MBP-CLR ECD:RAMP2 ECD complex by gel-filtration chromatography. Std, Bio-Rad gel-filtration molecular weight standards.

Fig. 3. AlphaScreen luminescent peptide binding assay. (A) Saturation binding. The recombinant AM receptor ECD complex (10 nM) was incubated with the indicated concentrations of biotinylated calcitonin family peptide hormones. (B) Competition binding. The recombinant AM receptor ECD complex (10 nM) and biotin-AM (40 nM) were incubated with the indicated concentrations of unlabeled competitor peptides. Data shown are the average of duplicate samples.

Table 1

Summary of protein yields.

Purification Steps	Protein Yield (mg)
MBP-Th-RAMP2 ECD (6 L culture)	
IMAC, Amylose	340
Thrombin digest, 2 nd IMAC, gel-filtration	40 (isolated RAMP2 ECD)
MBP-CLR ECD (6 L culture)	
IMAC, Amylose	120
MBP-CLR ECD:RAMP2 ECD complex	
Initial 1:1.5 mixture for disulfide shuffling	110 (MBP-CLR) and 39 (RAMP2)
2 nd IMAC to remove DsbC, gel-filtration	40 (complex)