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P7, a novel antagonist of corticotropin releasing factor receptor type 1 (CRFR1) screened from phage display library

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ABSTRACT

The corticotropin releasing factor (CRF) plays a central role in regulating the activities of hypothalamic-pituitary-adrenal (HPA) axis in the presence of a variety of stressful stimuli via binding to its type 1 receptors (CRFR1). Despite that many peptidic or non-peptidic antagonists of CRFR1 have been developed to serve as therapeutic tools to CRF-related pathologies, none of them have been utilized clinically. Targeting the extracellular domain 1 (EC1) of CRFR1, the CRF-binding site, represents a new strategy to inhibit the function of the receptor. However, no such agents have been identified up to now. Herein, by using an 87-amino acid fragment corresponding to the EC1 region as the bait, we screened the binding polypeptides from a phage display (Ph.D.-12) peptide library. After 3-round biopanning, positive clones were selected and the polypeptides carried by them were identified. 5 polypeptides were found to bind with the target specifically. Among them, the P7 exhibited the highest affinity. By evaluating the cAMP accumulation in the CRFR1 or CRFR2-expressing HEK293 cells, we demonstrated that P7 blocking the function of CRFR1, but not CRFR2. In addition, we also found that P7 and CRF act on CRFR1 competitively. Taken together, we reveal that P7, a novel polypeptide identified from phage display library, inhibits the function of CRFR1 effectively and specifically by binding at its EC1 domain. The new polypeptide might provide a promising agent for diagnostic or therapeutic utilities in CRF-related disorders.

1. Introduction

The corticotropin releasing factor or hormone (CRF or CRH), a 41amino-acid peptide, was found in hypothalamus 30 years ago [1]. It is synthesized and secreted by the paraventricular nucleus (PVN) of the hypothalamus in response to stress. The peptide plays crucial roles in modulating the hypothalamic-pituitary-adrenal (HPA) axis activated by stressful stimuli, as well as behavioral, autonomic, endocrine, reproductive, cardiovascular, gastro-intestinal, metabolic and immune systemic functions, or homeostasis and viability of peripheral organs [2–5]. Moreover, CRF effects have also been found in tumorgenesis [6,7], mood disorders [8], neuroplasticity [9,10], skin stress response system [11], inflammation [12,13], addiction [14–16] and Alzheimer's disease [17]. CRF exerts its biological function via binding to its receptors (CRFR). Mammalian tissues express two types of specific receptors, termed R1 and R2 [18]. Both of them belong to class B1 of G-protein coupled receptors (receptors for 'brain-gut' neuropeptides) that signal primarily through the stimulation of cAMP. CRF binds to CRFR1 with a higher affinity, whereas with a much lower affinity to CRFR2 [19]. Mechanistically, CRF binds to the N-terminal extracellular domain 1 (EC1), which triggers the tethering of the peptide to the juxtamembrane (J-domain). J-domain is the region where CRF activates the receptor with very high affinity resulting in intracellular signaling (two-domain model) [20].

Given that the CRF-CRFRs system are involved in diverse pathologies, CRFR antagonists are being actively studied and developed as possible tools or treatments. Since 1991, a large number of peptide and non-peptide such antagonists have been developed [21,22]. However, these antagonists have not revealed clinical utility despite over 30 years of research. The affinity and the blocking effect of most non-peptide antagonists are provided

Abbreviations: CRF, corticotropin releasing factor; CRFR, CRF receptor; EC1, extracellular domain 1; Ph.D., phage display; GST, glultathione S-transferase; HPA, hypothalamic-pituitary-adrenal; HA, hemagglutinin.

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mainly by the J-domain, and thus may be inadequate to interfere the binding of CRF to EC1 domain. For the peptide antagonists, higher immunogenicity might be a problem during utility given that most of them are usually the analogs of CRF [21]. Compared with small molecule and peptide, polypeptides gain more and more attention due to their advantages of low immunogenicity, a high degree of safety and efficiency, easy to synthesis and purification, less toxicity mild side effects, and have the potential to penetrate further into tissues owing to their small size [23]. However, no CRFR1-specific polypeptides have been identified up to now.

In the present study, aiming to develop polypeptidic antagonists that blocking the binding of CRF to CRFR1, we screened a commercial phage display (Ph.D.) - 12 peptide library (NEB, Beverly, MA, USA) by using the extracellular domain 1 (EC1) of the receptor, the CRF-binding site, as our bait. Several clones were selected and specific binding was confirmed. One of binding peptides, P7, disturbs the function of CRFR1 in a specific and competitive manner, implicating that the polypeptidic antagonists targeting the EC1 domain, rather than the J domain, attenuate the function of CRFR1 effectively.

2. Materials and methods

2.1. Reagents

Ph.D.-12 Phage Display Peptide Libraries [1.5×10^{13} plaque forming units (pfu)/ml] and the host bacterial strain *Ecoli* ER2738 were purchased from New England Biolabs (Beverly, MA, USA). (HRP)-anti-M13 mAb was a product of Amersham Pharmacia Biotech (Uppsala, Sweden). Human hemagglutinin (HA)-tagged CRFR1 and CRFR2-expressing vectors (pcDNA3.1-CRFR1 and pcDNA3.1-CRFR2) were purchased from UMR cDNA Resource Center. pcDNA3.1 was obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). LANCE™ cAMP 384 Kit was purchased from PerkinElmer Life and Analytical Sciences, Inc (Shelton, CT, USA). Other chemicals used in this study were of analytical grade and commercially available.

2.2. In-vitro phage biopanning

The selection procedure was mainly referenced from the Ph.D.-12 library kit manual. Briefly, the glultathione S-transferase fused, 87-amino acid of EC1 domain of CRFR1 (GST-$EC1_{87}$) and GST were coated on a 96-well plate with 100 μl (5 g/l) carbonate buffer (0.1 M $NaHCO_3$, pH 8.6) and incubated at 4 °C overnight. Approximately 2×10^{11} pfu phages were preincubated with the GST-coated plate for 1 h and then were transferred to the GST-$EC1_{87}$-coated plate and incubated for 1 h at room temperature with gentle agitation. Unbound phages were removed by washing with 0.01% TBST. GST-$EC1_{87}$-bound phages were eluted and neutralized. The eluted phages were amplified and purified to be used for subsequent rounds of selection. To increase the stringency, the concentration of target and incubation time were decreased, the concentration of tween20 in washing solution and washing times were decreased in the second and third round selection.

2.3. Phage ELISA for positive clones

GST-$EC1_{87}$ were coated into 96-well plate at 4 °C overnight. The phage clones (4.8×10^{10} pfu) were added to wells and incubated for 1 h at room temperature. After six times washing with 0.05% TBST, the bound phages were incubated with the anti-M13 phage antibody (1:5000). The development was performed by the addition of 3, 3′, 5, 5′-tetramethyl-benzidine (TMB) and incubation in the dark for 20 min. After terminated by adding 50 μl/well 2 M H_2SO_4, the absorbance was read at 450 nm. Blank well was used as the blank control and GST as the negative control.

2.4. Cell culture, transfection and western blotting

HEK293 cells were cultured in Dulbecco's Modified Eagle Medium (GIBCO, Invitrogen Corporation) supplemented with 10% fetal bovine serum. pcDNA3.1-CRFR1 (human) was transfected into HEK293 cells using the Lipofectamine™ 2000 (Invitrogen Corporation). Geneticin (800 mg/ml) was used to select CRFR1-expressing clones. pcDNA3.1 and pcDNA3.1-CRFR2 (human) were transiently transfected into HEK293 cells. After harvested, all the transfected cells were washed with ice-cold PBS, treated with Buffer X (50 mM Tris pH7.4, 270 mM NaCL, 1% Triton X-100) for 30 min in ice with gentle agitation, and centrifuged at 14,000 rpm for 30 min at 4 °C. The lysates were subjected to SDS-PAGE on 12% gel and transferred onto a PVDF membrane. The anti-HA primary antibody (1:1000, GIBICO) was used to detect the target proteins in Western blotting.

2.5. cAMP assays

HEK293-CRFR1 cells were washed with PBS, detached with enzyme-free versene (0.48 mM EDTA-4Na). 3×10^4 cells/well were seeded in a 384-well white plate and incubated for 30 min with CRF and CRFR1-binding peptides. Then the cells were incubated with Detection Mix for 4 h at room temperature in dark. Followed the manuals of LANCE™ cAMP 384 Kit for the determination of cAMP production, fluorescence resonance energy transfer was measured.

2.6. Data analysis

For all the phage ELISA and cAMP assays, all the experiments were repeated for three times independently. Data were analyzed with origin 8.0 (OriginLab) and GraphPad prism version 5 (GraphPad Software) and were shown as mean ± SEM. Concentration-response curves were fitted to the Hill equation separately. The overall statistical significance was tested for individual groups by using an unpaired Student's t test if necessary. Differences were considered statistically significant if $p < 0.05$.

3. Results

3.1. Identification of P7 as a high-affinity binding polypeptide to CRFR1 extracellular domain 1(EC1)

Considering that the EC1 of CRFR1 is the key binding site of CRF and related peptides [24] and also the low homologous region compared with that of CRFR2 (48.7% identity, whereas the overall's is 70%), we decided to use this domain as our bait to screen specific binding peptides from a (Ph.D.)-12 peptide library. The GST-fused $EC1_{87}$ (from Pro20 to Leu106) fragment was purified as described previously [25]. To increase the specificity, we decreased the amount of target and incubation time, and increased the concentration of Tween20 and wash times every round simultaneously. After three rounds of bio-panning, the efficiency (recovery ratio) was increased about 84 folds (from 5.0×10^{-7} to 4.2×10^{-5}, Table 1), suggesting the specific binding phages to EC1 domain were enriched effectively. 8 clones were randomly picked out from the third-round product and their binding ability was measured by using phage ELISA. Among them, clone C2, C4, C5, C7 and C8 exhibited specific binding to target proteins (their absorbance at 450 nm at 4.8×10^{10} pfu phage was 0.77 ± 0.06, 1.38 ± 0.11, 0.77 ± 0.04, 1.55 ± 0.004 and 0.11 ± 0.10, respectively) when compared with the blank and GST groups (Fig. 1A). Such bindings

Table 1
Enrichment of EC1-binding phages for each panning round.

Round	Target (μg)	[Tw20] (V/V)[a]	Wash times	Incubation time (min)	Input phages (pfu)	Output phages (pfu)	Recovery ratio (output/input)
1	5.0	0.1%	10	60	5×10^{11}	2.5×10^{5}	5.0×10^{-7}
2	2.5	0.2%	12	45	7×10^{11}	1.5×10^{6}	2.1×10^{-6}
3	1.0	0.5%	15	30	6×10^{11}	2.5×10^{7}	4.2×10^{-5}

[a] Concentration of tween20.

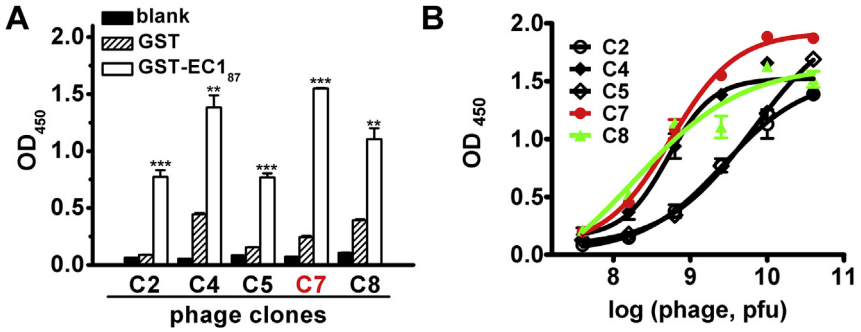

Fig. 1. Binding profiles of the polypeptides identified from phage display-12 peptide library against the 87-amino acid extracellular 1(EC1) domain segment ($EC1_{87}$) of CRFR1. (A) Binding ability of positive phage clones to $EC1_{87}$. 4.8×10^{10} pfu phages were used for each clone. $^{**}p < 0.01$, $^{***}p < 0.001$, versus GST sample. (B) Concentration-dependent response of the positive clones.

were also concentration-dependent, as shown in Fig. 1B. The sequence of the displayed peptide in these clones was detected and summarized in Table 2. Obviously, C7 displayed the highest binding affinity, with a K_d of $(5.58 \pm 1.20) \times 10^8$ pfu. The sequence for P7, the peptide carried by C7, is 'YAGDFWDLDALA'. We next investigated the possible function of peptide P7 in antagonizing the role of CRFR1.

3.2. P7 inhibits the cAMP releasing induced by the activation of CRFR1 in HEK293 cells

Both CRFR1 and CRFR2 are mainly coupled to Gs-protein, and thus activation of these receptors will trigger the release of cAMP and the according accumulation of cAMP [26]. To measure the possible antagonistic function of P7 to CRFR1, the peptide was commercially synthesized and its effects on cAMP release induced by activation of CRFR1 were assayed. To gain higher expression level, a CRFR1-stably expressed HEK293 cell line was established and the expression of the receptor was confirmed by Western blotting (HEK293-hCRFR1, Fig. 2A). In the presence of different concentrations of CRF ([CRF]), the cAMP accumulation (donated as Fluorescent Intensity, FI) in this cell line was concentration-dependent, with an EC_{50} of 8.44 ± 0.68 nM (Fig. 2B). When the [CRF] was set at EC_{80} (32 nM), P7 inhibited cAMP release also in a concentration-dependent manner, with an IC_{50} of only 0.14 ± 0.06 nM (Fig. 2C). Importantly, P7 did not affect the level of cAMP in the absence of CRF, suggesting P7 does not influence the basal release of cAMP in the cell line. Taken together, our results demonstrate that P7 inhibits the cAMP production induced by activation of CRFR1.

Table 2
The sequence of the polypeptides displayed by the corresponding positive phage clones.

Clone/peptide	Sequence (N to C)
C2	YLDAWENEVINF
C4	YGDRWFMPVMRS
C5	DGYNGEPWIWQQ
C7 (P7)	YAGDFWDLDALA
C8	WGYDWWENRSLT

3.3. P7 competitively antagonizes the function of CRFR1

The EC1 domain of CRFR1, which was selected as the bait for screening, serves as CRF binding site. Furthermore, we have revealed that P7 antagonize the function of CRFR1 in a concentration-dependent manner. Hence, we speculate that P7 might be a competitor of CRF in the stimulatory process of CRFR1. To test this, we investigated the cAMP accumulation in the absence or presence of 1 nM and 10 nM P7 in HEK293-CRFR1 cells. As the dose–responsive curves shown in Fig. 3A, compared with the one in the absence of P7 (P7-free), introduction of P7 made the curves right-shifted significantly. The EC_{50} was 72.5 ± 10.6, 105.1 ± 10.4 and 142.9 ± 10.6 nM for P7-free, 1 nM P7 and 10 nM P7, respectively (Fig. 3B). 10 nM P7 increased the EC_{50} by about 2 folds. These data suggest that P7 is a competitive ligand of CRFR1 and is able to inhibit the function of the receptor effectively.

3.4. P7 does not affect the function of CRFR2

Since CRFR2 shares a higher homology with CRFR1, we also investigated the possible role of P7 on the function of CRFR2. Similar with the situation of CRFR1, cAMP formation was also induced in HEK293-hCRFR2 cells in the presence of human UCNIII, the selective ligand of the receptor. The EC_{50} the activation curve was 36.5 ± 2.5 nM (Fig. 4A). The effects of P7 were assessed at the presence of 144 nM UCNIII, approximately EC_{80} of the curve. As illustrated in Fig. 4B, P7 did not influent the cAMP formation evoked by UCNIII-CRFR2 signal pathway in HEK293 cells, implicating the peptide does not disturb the function of CRFR2.

4. Discussion

Considering the central role of CRF in the acute and chronic stresses, and CRFR1 receptors are present in CNS and many peripheral tissues, it is reasonable to predict that blocking CRF-CRFR1 pathway might alleviate a diverse of stress-related pathologies.

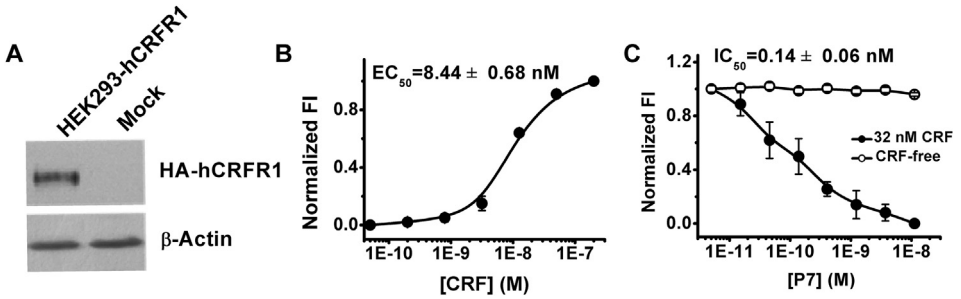

Fig. 2. P7 specifically inhibits the cAMP release induced by the activation of CRFR1 in HEK293 cells. (A) Human CRFR1 (hCRFR1) was highly expressed in HEK293 cells, as confirmed by Western blotting. The HA-tagged target protein (HA-hCRFR1) was detected in the stably transfected (HEK293-hCRFR1) and vector-transfected (mock) HEK293 cells by HA primary antibodies. (B) Concentration-dependent stimulation of CRF to CRFR1 in HEK293 cells. The concentration of CRF ([CRF]) was plotted as a function of normalized fluorescent intensity (normalized FI). (C) Concentration-dependent inhibition of P7 to the cAMP accumulation induced by the activation of CRFR1 in the absence (CRF-free) or presence of 32 nM CRF. Data were presented as mean ± SEM.

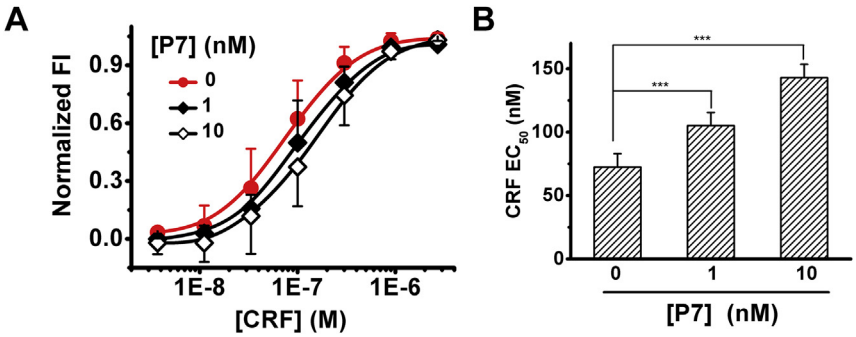

Fig. 3. P7 and CRF act on CRFR1 competitively. (A) Concentration-dependent curves for CRF stimulation to CRFR1 in the absence (P7-free) and presence of 1 nM, 10 nM P7. Data were presented as mean ± SEM. (B) Quantification of EC50 of CRF stimulation in the absence or presence of 1 nM, 10 nM P7. Data were presented as mean ± SEM. ***$p < 0.001$, versus the absence of P7 group.

Despite that many peptide or non-peptide antagonist of CRFR1 have been developed in the recent 30 years, it is important to note that some issues are still need to be considered: 1) Due to lower molecular weight and acting at J domain, small molecule antagonists are not sufficient to inhibit the function of CRFR1, implicating that targeting the EC1 domain might be more potent. 2) Peptide antagonists do bind to the EC1 domain of CRFR1 and demonstrate higher potency to block the CRF-CRFR1 system and also important potential as clinical tools. Nevertheless, due to higher molecular weight, they are not able to penetrate the brain and might have higher immunogenicity. Given the above issues, we used the EC1 domain of CRFR1 as the bait to screen a Ph.D.-12 displayed peptide library to obtain polypeptidic antagonist in the present study. After 3 rounds of biopanning, positive clones were enriched effectively. 8 clones were selected from the 3rd products to confirm their binding. 5 of them exhibited higher affinity to EC1 domain, with the C7 phage representing the highest one. The deduced amino acid sequence of P7, which was carried by C7 phage, is 'YAGDFWDLDALA'. In the regard of molecular weight, these polypeptides are higher than those of small molecules and lower than those of CRF analogs (peptides). Moreover, they are designed to bind with the EC1 domain.

We also demonstrated that the dose–response curves of CRF were right-shifted by the increment of [P7]. Together with the data that P7 inhibits the function of CRFR1 in a dose-dependent manner, our results are consistent with the notion that P7 and CRF bind to

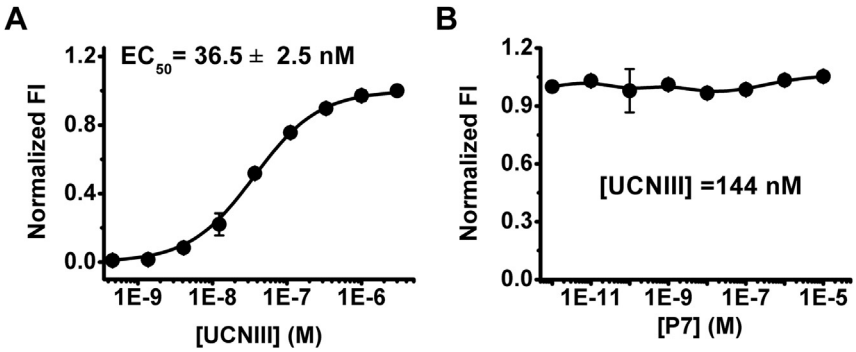

Fig. 4. P7 does not inhibit the cAMP formation triggered by the activation of UCNIII-CRFR2 system in HEK293 cells. (A) Concentration-dependent stimulation of UCNIII to CRFR2 in CRFR2-transfected HEK293 cells. (B) Concentration-dependent effect of P7 on the cAMP accumulation induced by the activation of UCNIII-CRFR2 pathway in the presence of 144 nM UCNIII.

CRFR1 competitively. In addition, it appears that the potency of P7 is higher than that of CRF when considering the degree of their opposite effects on CRFR1. When the [CRF] was set at 32 nM, the IC_{50} of P7 is approximately 0.14 nM, whereas the EC_{50} of CRF is about 105.1 nM at the presence of 1 nM P7. Note that there exist about 10 folds difference in the EC_{50}s of CRF calculated from Fig. 2C and Fig. 3, which might be originated from the different expression level of CRFR1 in the stable-expressed HEK293 cells.

Structurally, CRFR1 and CRFR2 are approximately 70% identical at the amino acid level, but exhibit considerable divergence at the N-terminal EC1 domain, which comprises the major ligand binding site, serves to dock peptide ligands or antagonists, and can also discriminate the ligands [27–29]. The $EC1_{87}$ fragment, which serves as our bait for screening, is corresponding to the region of amino acids 20–106, a domain overlapped with CRF binding site [28]. Furthermore, the residue Glu104, which might be responsive for the selectivity, is also located within the domain [27]. Consistent with this structural basis, P7 was found to inhibit the cAMP release triggered by the interaction of CRF-CRFR1, but not UCNIII-CRFR2, suggesting that the polypeptide is able to attenuate the function of CRFR1 specifically. It is assumed that the electrostatic interactions between the Arg35 and Glu39 of CRF and Glu104 of CRFR1 play important roles in receptor binding [27]. However, no such residue combination was found in the peptides carried by the positive clones in our experiments, suggesting their binding mechanism may be different.

Increasing evidence has demonstrated that activation of CRF receptors in the gastrointestinal tract affects intestinal permeability and motility [13]. Specially, CRFR1 promotes intestinal inflammation and endogenous angiogenesis, and antagonist of the receptor exhibits drastic therapeutic effect in animal models [30–32]. Based on these results, we speculate that P7 might acts potentially to alleviate the pathologies of intestinal inflammation. Indeed, the therapeutic roles of the polypeptide were found in inflammatory bowl diseases (unpublished data).

In summary, several polypeptides were screened out from the Ph.D. 12 library by using EC1 segment as the bait. Among them, P7 binds the target with the highest affinity and inhibits the function of CRFR1 in specific and concentration-dependent manner. In addition, P7 and CRF act on the receptor competitively. Our results illustrate that the polypeptides targeting the CRF binding domain, rather that the J domain, inhibit the function of CRFR1 effectively and specifically. After additional modifications, these polypeptides might be promising to use as diagnostics and therapeutic agents to CRF-related disorders.

Conflict of interest

None.

Acknowledgments

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Transparency document

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