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The functional recombinant first extracellular (EC1) domain of PACAP receptor PAC1 normal form (PAC1-EC1(N)) recognizes selective ligands and stimulates the proliferation of PAC1-CHO cells

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ABSTRACT

PAC1 is a pituitary adenylate cyclase-activating polypeptide (PACAP) preferring receptor, which is abundant in the central and peripheral nervous systems. PAC1 belongs to the class B family of G protein-coupled receptors (GPCRs). The N-terminal first extracellular (EC1) domain of PAC1 is responsible for ligand recognition and binding. In this study, the recombinant EC1 domain of the PAC1 normal (N) form (amino acids 21–155) with 6His tag at the C-terminus (named PAC1-EC1(N)) was first expressed in an *Escherichia coli* strain and purified by an Ni-NTA affinity column. About 6–8 mg of recombinant PAC1-EC1(N) protein with purity above 95% was produced from 1 L of bacterial culture. Mass spectrum and western blot were used to identify the recombinant PAC1-EC1(N). Intrinsic tryptophan fluorescence (ITF) assays showed that the purified PAC1-EC1(N) protein was able to recognize and bind to the PAC1 selective agonist maxadilan, the antagonist M65 and vasoactive intestinal polypeptide (VIP). Maxadilan and M65 had higher affinities for PAC1-EC1(N) than VIP. The results of MTT assays showed that PAC1-EC1(N) stimulated the viability of PAC-CHO cells but blocked the effects of maxadilan on the proliferation of CHO cells expressing PAC1 (PAC1-CHO), indicating that the functional soluble PAC1-EC1(N) may act as a regulator for the activation of PAC1.

Pituitary adenylate cyclase-activating polypeptide (PACAP) belongs to the superfamily of metabolic, neuroendocrine and neurotransmitter peptide hormones that include vasoactive intestinal polypeptide (VIP), glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) [19]. PACAP binds to three different types of receptors: PAC1, VPAC1 and VPAC2, all of which belong to the class B G protein-coupled receptor (GPCR) family. PAC1 has a much higher affinity for PACAP than for VIP, whereas VPAC receptors recognize all peptides with a similar high affinity [12,19].

The PAC1 gene contains more than 18 exons, and alternative splicing of two regions (the first extracellular (EC1) domain and/or the third intracellular cytoplasmic (IC3) loop) of the PAC1 gene results in a relatively large number of PAC1 isoforms [1]. There are at least three isoforms in the EC1 domain of PAC1, including the normal (N) form (does not have a deletion), the short (S) form and very short form (VS) lacking 21 and 57 amino acids, respectively [8,9]. It has been demonstrated that the EC1 domains of PAC1 play an important role in determining ligand selectivity and binding [5,18].

PAC1 and its isoforms are abundantly expressed in the central and peripheral nervous systems [13,19]. Mice deficient in PAC1 displayed reduced expression of brain derived neurotrophic factors [23]. PAC1 mediates protective effects of PACAP in nerve cells [6,15]. PAC1 is considered as a potential target for the treatment of ischemic neuronal damage, Parkinson's disease and Alzheimer's disease [16,17,10].

In this article, we report the first production and characterization of functional soluble N-terminal EC1 domain of the mouse PAC1-normal form (amino acids 21–155) (Fig. 1), named PAC1-EC1(N).

The cDNA (Funeng Gene Company, Guangzhou, China), encoding PAC1-EC1(N) (Fig. 1) with 6× His purification tag at the C-terminal, was amplified by overlap PCR and cloned into the *Nde*I and *Xho*I sites of the expression vector pKYB (New England Biolab, USA). The positive clone named PKY-PAC1-EC1(N) was characterized by PCR and sequencing.

The recombinant plasmid PKY-PAC1-EC1(N) was introduced into the competent cells of *Escherichia coli* strain ER2566 (New

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England Biolab, USA). The positive clones were grown at 37 °C under agitation until the bacterial culture reached an absorbance (A_{600}) of about 0.7. Protein expression was induced by the addition of 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) to the bacterial culture. The expression continued for 4 h at 30 °C. The expression of the target protein PAC1-EC1(N) was identified by 10% SDS-PAGE. Bacteria were collected by centrifugation, weighed and resuspended in 10 mL/g binding buffer (20 mM phosphate, 500 mM sodium chloride, 20 mM imidazole, and pH 7.4). The cells were disrupted by pulse sonication for 20 min at 4 °C. After centrifugation at 12,000 × *g* for 30 min, soluble material was passed through a HisTrap FF crude prepacked column (GE Healthcare, USA) loaded with Ni-NTA agarose. The column was then washed sequentially by washing buffer (20 mM phosphate, 500 mM sodium chloride, 80 mM imidazole, and pH 7.4). Recombinant proteins were eluted from Ni-NTA agarose with elution buffer (20 mM phosphate, 500 mM sodium chloride, 150 mM imidazole, and pH 7.4). Dialysis was used to remove imidazole. Western blot analysis using a rabbit polyclonal antibody raised against amino acids 61–115 mapping within an N-terminal extracellular domain of mouse PAC1 (Santa Cruz Biotechnology, USA) was used to identify the recombinant PAC1-EC1(N) protein. Mass spectrometry was performed at the National Biology Test Center (Beijing, China).

Fluorescence measurements were performed with a Tean-safire spectrophotometer (TZCAN, Austria). Excitation and emission wavelengths were 290 and 337 nm, respectively, using spectral bandwidths of 4 nm. Briefly, intrinsic fluorescence of tryptophan residues (W58, W64, W90, and W123) from the purified N-ted was measured in 2 mL of hydroxyethylpiperazine ethane sulfonic buffer (pH 7.5) containing 1 μM purified PAC1-EC1(N) in the absence or presence of increasing concentrations (up to 8 μM) of either PAC1 agonist (Maxadilan), antagonist (M65), VIP or calcitonin gene related peptide (CGRP). Dissociation constants (K_d) were determined from titration curves using the analytical procedure developed by Bechet et al. [2]. The recombinant agonist maxadilan and PAC1 antagonist M65 were expressed and purified following the method described previously [22]. CGRP and VIP were purchased from Novagen (USA).

Chinese hamster ovary (CHO) cells were stably transfected with the mouse PAC1 receptor as previously described [21]. The clone used was referred to as PAC1-CHO (receptor density of 3.3 ± 0.3 pmol/mg protein). Cells were maintained in DMEM medium supplemented with 10% FBS and 0.8 mg/mL G418 with an atmosphere of 95% air and 5% CO_2 at 37 °C. To investigate the effect of PAC1-EC1(N) on the growth of PAC1-CHO cells, cells (2×10^5 cells/well) were plated in 96-well plates overnight at 37 °C in DMEM with 0.5% FBS. The next day, the cells were incubated with or without gradient concentrations of peptides in the absence of FBS for 24 h. The viability of the cells was determined by colorimetric MTT assay (Methylthiazoletetrazolium bromide, Sigma, USA). The assay is based on the reduction of MTT into a blue formazan dye by viable mitochondria. All experiments were run in at least four parallels and repeated six times. Results were expressed as percentage of control (treatment without any peptides) values. The data were compared by ANOVA followed by the Student–Newman–Keuls post hoc test. Groups of data from both were considered to be significantly different for $P < 0.05$.

The cDNA (459 bp) encoding PAC1-EC1(N) with 6× His at the C-terminal was cloned into the *Nde*I and *Xho*I sites of the vector pKYB to construct the recombinant expression vector pKY-PAC1-EC1(N). The positive clone was identified by PCR, enzyme cleavage and DNA sequencing.

As shown in Fig. 2A (lands 3 and 4), the recombinant PAC1-EC1(N) with a molecular size of 16 kDa was mainly expressed in bacterial cytoplasm. The recombinant PAC1-EC1(N) was purified above 95% by affinity chromatography of an Ni-NTA column dependent on the 6× His purification tag (Fig. 2A, land 7) and identified by the western blot (Fig. 2B). The result of mass spectrometry showed that the precise molecular weight of PAC1-EC1(N) was 16192u (Fig. 3), consistent with the theoretical molecular weight. The quantity of purified PAC1-EC1(N) obtained was 6–8 mg of target protein from a liter of bacterial culture.

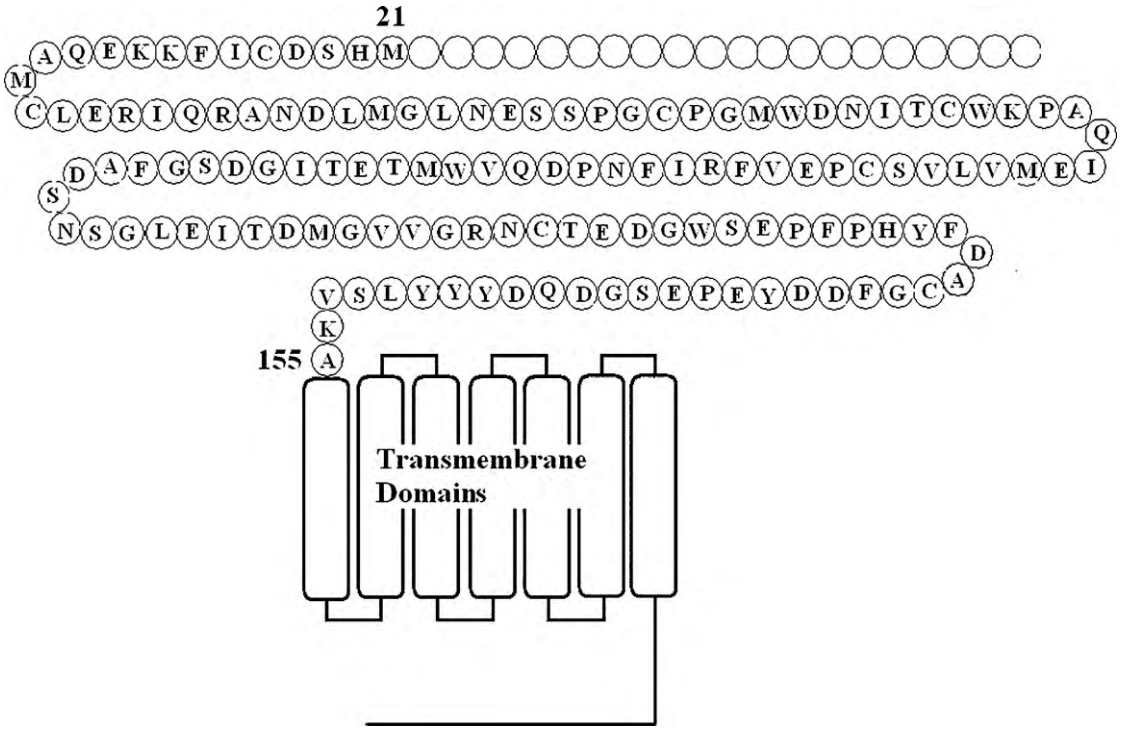

Fig. 1. Schematic representation of mouse PAC1 receptor. The solid circles represent amino acid residues included in the N-terminal EC1 domain sequence (amino acids 21–155) that was produced in bacteria. The blank circles represent amino acid residues of the signal sequence.