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Participation of the extracellular domain in (pro)renin receptor dimerization

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ABSTRACT

The (pro)renin receptor [(P)RR] induces the catalytic activation of prorenin, as well as the activation of the mitogen-activated protein kinase (MAPK) signaling pathway; as such, it plays an important regulatory role in the renin–angiotensin system. (P)RR is known to form a homodimer, but the region participating in its dimerization is unknown. Using glutathione S-transferase (GST) as a carrier protein and a GST pull-down assay, we investigated the interaction of several (P)RR constructs with full-length (FL) (P)RR in mammalian cells. GST fusion proteins with FL (P)RR (GST-FL), the C-terminal M8-9 fragment (GST-M8-9), the extracellular domain (ECD) of (P)RR (GST-ECD), and the (P)RR ECD with a deletion of 32 amino acids encoded by exon 4 (GST-ECDd4) were retained intracellularly, whereas GST alone was efficiently secreted into the culture medium when transiently expressed in COS-7 cells. Immunofluorescence microscopy showed prominent localization of GST-ECD to the endoplasmic reticulum. The GST pull-down analysis revealed that GST-FL, GST-ECD, and GST-ECDd4 bound FLAG-tagged FL (P)RR, whereas GST-M8-9 showed little or no binding when transiently co-expressed in HEK293T cells. Furthermore, pull-down analysis using His-tag affinity resin showed co-precipitation of soluble (P)RR with FL (P)RR from a stable CHO cell line expressing FL h(P)RR with a C-terminal decahistidine tag. These results indicate that the (P)RR ECD participates in dimerization.

1. Introduction

The (pro)renin receptor [(P)RR] is a newly identified regulator of the renin–angiotensin system that is essential for blood pressure control and electrolyte balance [1]. Human (P)RR [h(P)RR] is a 350-amino acid receptor comprised of a signal peptide, a large extracellular domain (ECD), a single transmembrane domain, and a short cytoplasmic domain from the N-terminus to the C-terminus. (P)RR binds renin (EC 3.4.23.15) and its inactive proenzyme prorenin, which triggers the phosphorylation of extracellular signal-related protein kinase (ERK) 1/2 [1]. On the other hand, (P)RR increases the catalytic activity of renin and activates prorenin non-proteolytically to generate angiotensin I [1,2]. (P)RR is related to the organ damage associated with hypertension and diabetes (reviewed in [3,4]).

In addition to its roles as the cell surface receptor of renin and prorenin, other roles in Wnt/β-catenin signaling [5] and intracellular vacuolar H^+-ATPase function [6,7] have recently been reported for (P)RR. Accordingly, many reports have shown that endogenous and exogenously expressed (P)RR is predominantly localized intracellularly [8–13].

To date, three molecular forms of (P)RR have been identified: the full-length (FL) form [1], a truncated transmembrane form with the C-terminal region (M8-9 fragment) [14], and the most recently identified form, a truncated soluble form with the N-terminal region [soluble (P)RR; s(P)RR] [11]. s(P)RR is generated intracellularly through proteolytic cleavage of FL (P)RR by furin [11] or ADAM19 [12] at the Golgi apparatus. s(P)RR has been detected in the plasma [11,15] and urine [16]. It binds renin [11] and also binds and activates prorenin *in vitro* [12,16,17], indicating its potential as a biomarker for certain diseases [4,15].

Many biochemical studies have shown that FL (P)RR forms a homodimer [1,9]. A mutant (P)RR with a deletion of 32 amino acids encoded by exon 4 of the (P)RR gene that is observed in patients with X-linked mental retardation and epilepsy [18] has been demonstrated to bind to FL (P)RR and to exert a dominant negative

Abbreviations: ECD, extracellular domain; ER, endoplasmic reticulum; FL, full-length; (P)RR, (pro)renin receptor; s(P)RR, soluble (pro)renin receptor.

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effect [19]. However, the specific region responsible for its dimerization remains unknown. In this study, the region important for dimerization was elucidated by expressing several (P)RR constructs in mammalian cells. Moreover, we investigated the intracellular localization of (P)RR.

2. Materials and methods

2.1. Antibodies

Anti-(P)RR antibody (GTX114169) was purchased from GeneTex (Irvine, CA, USA), and its binding epitope has been mapped to amino acid (aa) residues 146–281 of h(P)RR as described in the Supplementary data section. Other antibodies used were anti-GST (Santa Cruz Biotechnology; Dallas, TX, USA), anti-6His 9F2 (Wako Pure Chemical Industries; Osaka, Japan), anti-actin and anti-FLAG M2 (Sigma–Aldrich; St. Louis, MO, USA), anti-KDEL and anti-GM130 (MBL; Nagoya, Japan), and Cy2-conjugated anti-rabbit immunoglobulin G and Rhodamine Red X-conjugated anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories; West Grove, PA, USA).

2.2. Plasmid constructions

The h(P)RR cDNA was obtained by polymerase chain reaction (PCR) from a human kidney cDNA library (Clontech Laboratories; Mountain View, CA, USA). pcDNA3-h(P)RR-10His, which encodes h(P)RR with a foreign signal peptide at the N-terminus and a decahistidine tag at the C-terminus, was constructed by inserting the DNA fragment corresponding to aa 17–350 of h(P)RR into pcDNA3-10His [20], which contains a substitution of an ILVPRGS sequence for the IVPRGS sequence (thrombin cleavage site) encoded by the original plasmid. To construct pEBMulti-FLAG-h(P)RR, a DNA fragment encoding FL h(P)RR with a FLAG epitope (DYKDDDDK) between the signal peptide (aa 1–16) and the ECD was amplified by PCR and subcloned into the mammalian expression plasmid pEBMulti-Hyg (Wako Pure Chemical Industries). The h(P)RR constructs [aa 17–350 (FL), aa 17–304 (ECD), the ECD with deletion of aa 101–132 (ECDd4), and aa 282–350 (M8-9)] and the endoplasmic reticulum (ER) retention sequence of human calreticulin (QAKDEL) fused to *Schistosoma japonicum* glutathione S-transferase (GST) that was preceded by a 24-amino acid signal peptide from the sheep angiotensinogen sequence at the N-terminus, as shown in Fig. 1, were generated in the mammalian expression plasmid pcDNA3 (Invitrogen; Carlsbad, CA, USA). The sequence integrity of all inserts was confirmed by DNA sequencing.

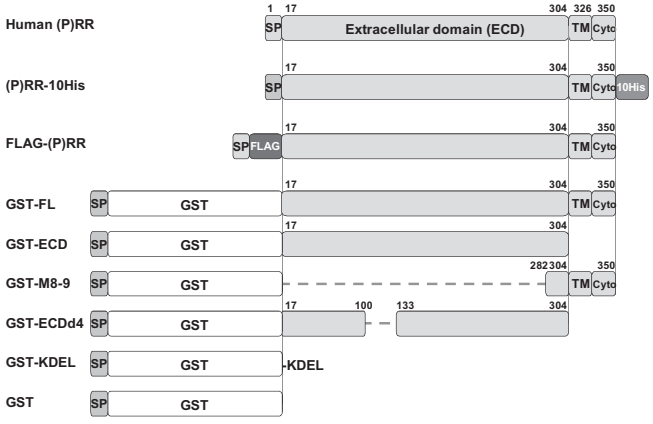

Fig. 1. Schematic representation of human (P)RR and constructs used in this study. (P)RR-10His, full-length (FL) h(P)RR with a C-terminal decahistidine tag (10His); FLAG-(P)RR, FL h(P)RR with an N-terminal FLAG tag (FLAG); GST-FL, FL h(P)RR fused to GST; GST-ECD, the extracellular domain (ECD) of h(P)RR fused to GST; GST-M8-9, M8-9 fragment of h(P)RR fused to GST; GST-ECDd4, the h(P)RR ECD carrying a deletion fused to GST; GST-KDEL, GST fused with the KDEL ER-retention sequence; GST, GST alone. Numbers indicate amino acid positions in the h(P)RR sequence. Dashed lines indicate the deleted regions. SP, signal peptide; TM, transmembrane domain; Cyto, cytoplasmic domain.

2.3. Cell culture, transfection, and establishment of stable cell lines

COS-7 (RCB0539) and HEK293T (RCB2202) cells were provided by the RIKEN BRC through the National Bio-Resource Project of MEXT, Japan, and maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO_2.

Cells cultured for 24 h were transiently transfected with the expression plasmid(s) based on the polyethyleneimine-mediated method [21]. Briefly, for COS-7 cells grown in a 6-well plate, plasmid DNA (2 μg) was mixed with 6 μg of polyethyleneimine (Polysciences; Warrington, PA, USA) in 0.2 ml of 150 mM NaCl and incubated for 20 min at room temperature. The culture medium was replaced by fresh medium (1.5 ml/well), and the DNA–polyethyleneimine mixture was plated to the cells. The cells were cultured for a further 48 h.

A Chinese hamster ovary (CHO) cell line expressing h(P)RR-10His was established by employing our previously described method [20]. Briefly, pcDNA3-h(P)RR-10His and pmDHFR, encoding mouse dihydrofolate reductase, were transfected into dihydrofolate reductase-deficient CHO cells using the polyethyleneimine-mediated method. A clonal cell line with high production of the recombinant protein, CHO/h(P)RR-10His, was used in this study.

2.4. Cell lysate and culture supernatant

COS-7 cells in serum-free medium were transfected for 48 h with plasmids as described above. Culture supernatants were collected and concentrated using Amicon Ultra-0.5 10K (Millipore; Billerica, MA, USA). The cells were washed with phosphate-buffered saline (PBS), pH 7.4, and lysed in ice-cold PBS containing 1% Triton X-100 and centrifuged to remove debris. CHO cells at 90–100% confluence were cultured for 48 h in a 60-mm diameter dish. Culture supernatants were collected. Cells were washed with PBS, lysed in ice-cold PBS containing 0.5% Triton X-100, and centrifuged to remove debris.

2.5. Immunofluorescence microscopy

COS-7 and CHO cells were grown on a gelatin-coated cover glass placed in a 6-well plate. COS-7 cells were transiently transfected with the desired plasmids as described above and cultured for 48 h. The cells were fixed in 3.7% formaldehyde in PBS on ice for 30 min, permeabilized in PBS containing 0.1% Triton X-100 and 0.1% Tween 20 at room temperature for 30 min, and then incubated for 30 min in blocking solution (PBS containing 2% fetal bovine serum and 0.1% Tween 20). The cells were then incubated at 4 °C overnight with either anti-(P)RR or anti-GST antibodies together with either anti-KDEL or anti-GM130 antibodies diluted in the blocking solution. After washing with 0.1% Tween 20 in PBS, cells were incubated at room temperature for 1 h with Cy2-conjugated anti-rabbit immunoglobulin G antibody to detect either (P)RR or GST and Rhodamine Red X-conjugated anti-mouse immunoglobulin G antibody to detect either KDEL or GM130 in blocking solution. The nuclei were stained with H33342 (Nacalai Tesque; Kyoto, Japan). Fluorescence images were obtained using an LSM 710 confocal microscope (Carl Zeiss; Germany).