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Towards three-dimensional structural analysis of (pro)renin receptor

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1. ABSTRACT

The (pro)renin receptor is a single-spanning membrane protein that binds both renin and its inactive precursor prorenin. The receptor binding enhances the catalytic activity of renin and induces non-proteolytic activation of prorenin as well as triggers intracellular signaling with either renin or prorenin as a ligand. Three-dimensional structural information of (pro)renin receptor is important to understand the receptor binding. This information is not available due to the lack of its three-dimensional structure. In this review, we summarize the binding properties of (pro)renin receptor, provide the results of structure prediction and point out the issues to be tackled towards three-dimensional structural analysis of this receptor.

2. INTRODUCTION

The renin-angiotensin system plays an important role in the regulation of blood pressure and electrolyte balance (1). A key enzyme of this system, renin (EC 3.4.23.15), specifically cleaves its macromolecular substrate, angiotensinogen, to release the N-terminal decapeptide, angiotensin I. This enzymatic reaction is the first step to produce the potent vasoconstricting octapeptide, angiotensin II. Prorenin is the inactive precursor of renin (2, 3). It has a prosegment of 43 amino acid residues attached to the N-terminus of mature renin consisting of 340 amino acid residues. The prosegment masks the active site of renin, thereby preventing the access of angiotensinogen. Prorenin does not self-activate. The blood circulating level of prorenin is 10 times higher than

that of renin, but the physiological role of prorenin remains unclear. In 2002, (pro)renin receptor [(P)RR] was identified as a new molecule of the renin-angiotensin system (4). Since its discovery, many investigators have undertaken molecular, cellular and physiological studies to clarify how this receptor participates in the system (5-9).

(P)RR is a 350-amino acid single-spanning membrane protein that binds both renin and prorenin (4). The receptor binding increases the catalytic activity of renin and induces non-proteolytic activation of prorenin, which leads to angiotensin-dependent pathway (2, 4). Such binding also results in angiotensin-independent intracellular signaling pathway that involves mitogen activated protein kinase activation (4, 7). The receptor binding to either renin or prorenin is the initial step to trigger these two pathways and probably requires a specific protein-protein interaction between (P)RR and renin/prorenin. In this review, we summarize the binding properties of (P)RR, provide the results of structure prediction and point out the issues to be tackled towards three-dimensional structural analysis of this receptor.

3. AMINO ACID SEQUENCE

The amino acid sequences of (P)RR are highly homologous between vertebrates such as human, rat, mouse, chicken and fish (10) (Figure 1). The homologous sequence is also found in an invertebrate fruit fly (Figure 1), nematode (GenBank: CAB07401) and Arabidopsis (GenBank: AAD11797). (P)RR has four domains (4, 10) (Figure 1): a signal peptide (residues 1-16; human (P)RR numbering), an extracellular domain (residues 17-304), a transmembrane domain (residues 305-324) and a cytoplasmic domain (residues 325-350). (P)RR has no potential *N*-linked glycosylation site and no Cys residue. The invariant residues shown in Figure 1 would be important in the structure and function of (P)RR, since evolutionally-conserved residues often cluster in a defined region to form a functional site on protein surface (14, 15). Putative protein processing site RxxR is conserved among the homologous sequences (10) (Figure 1). Near this processing site, there is the start residue of M8-9 fragment (10, 16) that is associated with vacuolar H^+-ATPase. A C-terminal region containing the transmembrane and cytoplasmic domains is highly conserved among vertebrate and invertebrate (10) (Figure 1). The C-terminal region is suggested to have a common function as a subunit of the ATPase (7, 10), which is supported by the recent finding on (P)RR-knockout mice (9). The cytoplasmic domain has candidate phosphorylation sites (4) (Figure 1), which is possibly involved in the intracellular signaling pathway.

4. RECEPTOR BINDING

(P)RR was found to bind to renin and prorenin (4). Such binding has been confirmed by using (P)RR expressed on the cell membrane of COS-7 cells (17) and vascular smooth muscle cells (18) as well as using recombinant (P)RR immobilized on a plastic well (19) and on a sensor chip for surface plasmon resonance analysis (20). Prorenin binds to (P)RR with a higher affinity than renin (19), which suggests that the prosegment facilitates the binding. Therefore, (P)RR should bind not only to the prosegment of prorenin, but also to the mature renin moiety.

Recently, Nabi *et al*. (21) provided a new insight into the receptor binding to renin and prorenin. This study investigated whether an exogenous peptide fragment related to prorenin can inhibit the binding of renin and prorenin to the extracellular domain of (P)RR. The peptides examined include the decoy peptide and the "hinge" peptide. The decoy peptide (R^{10P}IFLKRMPSI^{19P}) contains the "handle" region (I^{11P}FLKR^{15P}) that corresponds to amino acids 11-15 of the prorenin prosegment. The "handle" region is shown to play a crucial role for non-proteolytic activation of prorenin by protein-protein interaction (22). Based on this finding (22), the decoy peptide was designated to inhibit prorenin binding to (P)RR (23) and its efficacy has been confirmed *in vivo* studies (23-27). The "hinge" peptide (S^{149}QGVLKEDVF^{158}) corresponds to a flexible region that connects two domains of renin and is located near the bottom part of the active site (21). This region is common to both renin and prorenin. Using surface plasmon resonance and equilibrium state analyses, Nabi *et al*. (21) showed that the decoy peptide and the "hinge" peptide have a higher binding affinity to (P)RR than reference peptides used to compare low bindings. The dissociation constant K_d values of (P)RR for the decoy peptide, the hinge peptide, and a reference peptide (residues 248-257 of prorenin) were estimated 3.5, 17, and 4 x 10^4 nM, respectively (21). Moreover, the "hinge" peptide competitively inhibits the receptor binding to renin and prorenin (21). This finding indicates that renin and prorenin can interact with (P)RR through a common site involving the "hinge" region. The decoy peptide inhibits the receptor binding not only to prorenin, but also to renin (21). This is possibly because the decoy peptide competes for the "handle" region-binding site of (P)RR, thereby inducing a conformational change that hinders the receptor binding to the renin moiety. Collectively, prorenin binds to the receptor through at least two binding sites including the "hinge" and "handle" regions (21). Since the "hinge" region is located distant from the "handle" region, there could be an extensive contact between prorenin and (P)RR.

Another recent paper (28) reported an intriguing result of the receptor binding. The K_d values of (P)RR for renin alone and renin in complex with a direct renin inhibitor aliskiren were estimated 4 nM (21) and 5 x 10^4 nM (28), respectively. This result indicates that renin-aliskiren complex has a much lower affinity to (P)RR than renin alone. As described above, renin binds to (P)RR through at least the "hinge" region site. Binding with aliskiren into the active site of renin may lead a local conformational change of the "hinge" region site, which interferes with the receptor binding (28).

(P)RR has been reported to possibly form a dimer (4, 29). The functional site formed by two extracellular domains of (P)RR possibly contributes to make the extensive contact that recognizes the "hinge" and

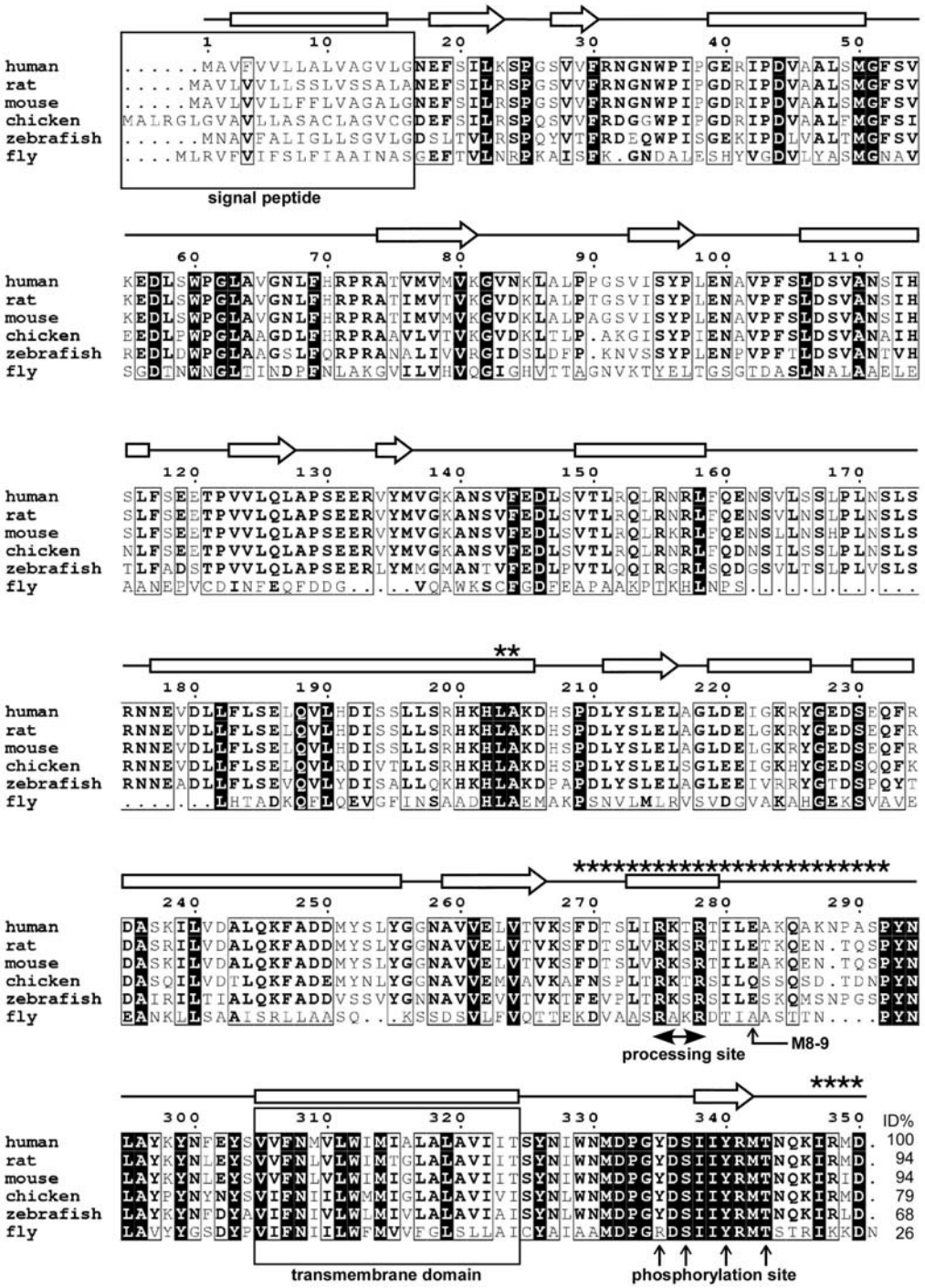

Figure 1. Sequence comparison of (P)RR homologs. The sequence homologs were obtained using BLAST search (11) and the sequences were aligned using CLUSTAL W (12). The figure was generated with ESPript (13). Species names and the GenBank sequence entry numbers are as follows: human, AAM47531; rat, BAD67178; mouse, AAH14706; chicken, BAE92902; zebrafish, AAH59542; fly, AAF54350. "ID%" represents percentage identity to human (P)RR. Invariant and conserved residues between these sequences are highlighted with a black background and a boxed area, respectively. The signal peptide and transmembrane domain are boxed and labeled. The putative protein processing site, the start residue of M8-9 fragment and candidate phosphorylation sites are indicated *below* the sequences. Predicted secondary structure elements and intrinsically-disordered regions of human (P)RR are shown *above* the sequence as rectangles (alpha-helices), arrows (beta-strands) and asterisks (intrinsically-disordered region).

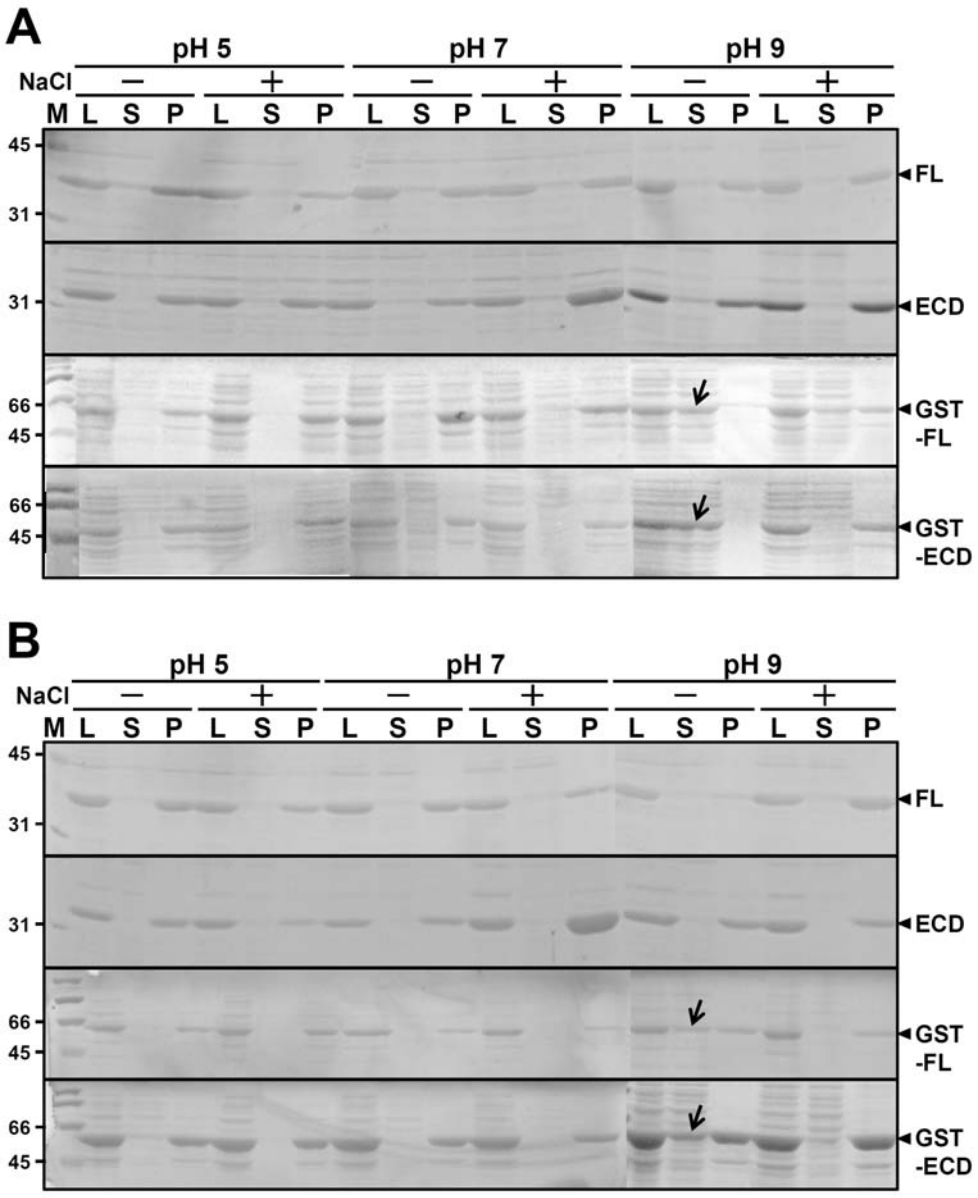

Figure 2. Expression and solubility test of (P)RR. The following four forms of human (P)RR were expressed in *E. coli* at 25°C (A) and 37°C (B) upon induction with isopropyl-beta-D-thiogalactopyranoside: full-length protein (FL); extracellular domain (ECD); GST-fused full-length protein (GST-FL); GST-fused extracellular domain (GST-ECD). These four proteins are designated to have no signal peptide. The expression plasmids, pET-11a (Novagen) and pGEX-4T-2 (GE Healthcare), were used to express (P)RR alone and GST-fused (P)RR, respectively. The *E. coli* cells were lysed by sonication in different kinds of buffer (pH 5, 7 and 9) in the presence (+) or absence (−) of 0.2 M NaCl. The cell lysate (L), supernatant (S) and pellet (P) as well as molecular weight markers (M) were subjected to SDS-polyacrylamide gel electrophoresis, followed by Coomassie brilliant blue staining. Each arrow indicates the position of the protein with enhanced solubility.

"handle" regions. A conformational change may occur in (P)RR upon binding with the "handle" region. Even after the receptor-binding, renin and prorenin retain the similar affinity (the Michaelis constant) to angiotensinogen (28), which indicates that the receptor binding does not affect the access of angiotensinogen. To gain a deeper insight into the molecular mechanism of receptor binding, three-dimensional structure of (P)RR alone and in complex with the relevant binding partner is essential.

5. STRUCTURE PREDICTION

To date, there is no three-dimensional structural information available for (P)RR. This is because three-dimensional structure of (P)RR has not been determined. Instead, secondary structure and disordered region of (P)RR can be predicted from its amino acid sequence alone by using recent bioinformatics programs. The PSIPRED program is a secondary structure prediction method (30),

which utilizes a neural network to predict secondary structure using multiple sequence alignments produced by PSI-BLAST search (11). The DISOPRED program can predict intrinsically (natively)-disordered region of protein based on a probability estimate of the amino acid residue being disordered (31). These programs are available through the web servers (32). The amino acid sequence of (P)RR was submitted to the servers. The PSIPRED analysis gives values of 39% of alpha-helix and 14% of beta-strand for (P)RR (Figure 1). The transmembrane domain is predicted to adopt an alpha-helix. The DISOPRED analysis suggests that there is a long disordered region (residues 269-292) in (P)RR (Figure 1). This region contains the putative processing site RxxR and the first residue of M8-9 fragment of vacuolar H^+-ATPase. To obtain the detailed structural information of (P)RR, the three-dimensional structure should be determined by the experiments on the bench work.

6. TOWARDS THREE-DIMENSIONAL STRUCTURAL ANALYSIS

The three-dimensional structure of protein is mainly determined by the two experimental methods: X-ray crystallography and nucleic magnetic resonance spectroscopy (33). Both methods produce the information on the relative positions of the atoms of the molecule. The choice of method depends on several factors, such as the molecular weight, solubility and ease of crystallization of the protein of interest (33). Structure determination methods by nucleic magnetic resonance spectroscopy are limited to a protein with molecular weight less than 40 k (34), whereas X-ray crystallography will be applied to larger proteins and protein complexes (33, 34). Since the apparent molecular weight of dimeric (P)RR is about 75 k, X-ray crystallography is the method of choice for the three-dimensional structure analysis of (P)RR.

The prerequisite for determining a protein structure by X-ray crystallography is to prepare protein crystals suitable for X-ray analysis. When attempting to obtain such crystals by screening, we need to produce a close-to-milligram quantity of pure protein. Because of efficient and cost-effective means for expressing recombinant proteins, *E. coli* is a suitable host for the first attempt to produce any recombinant protein (35). But the recombinant proteins sometimes have unfavorable properties that hinder attempts to determine the three-dimensional structure. Such properties include low expression level, low solubility and difficulty of purification. Various types of tag protein have been elaborated to overcome some of these problems (36, 37) and glutathione *S*-transferase (GST) is one of the tag proteins. Lowering the growth temperature to express recombinant proteins often makes the expressed proteins more soluble, presumably because slower rates of protein production can allow the proteins to fold properly (35). Optimization of cell lysis buffer to extract recombinant proteins is important to enhance protein solubility and stability (35).

As a first step to prepare (P)RR protein in a large amount, we expressed it in *E. coli* in a small-scale experiment, examining the effects of length of protein, GST-tag, growth temperature and cell lysis buffer on protein expression and solubility. The tag-free (P)RR proteins (FL and ECD) were almost insoluble in all the conditions examined (upper two panels in Figures 2A and 2B). On the other hand, the GST-tagged (P)RR proteins (GST-FL and GST-ECD) became soluble when they were extracted with the cell lysis buffer at pH 9 in the absence of NaCl (see arrows in Figures 2A and 2B). Moreover, the GST-tagged proteins were more soluble when expressed at lower temperature (25°C). These results show that expression as a GST-tagged protein at 25°C is very effective to obtain soluble (P)RR preparations.

7. CONCLUSION

Binding of (P)RR to either renin or prorenin is the initial step to trigger the angiotensin-dependent and angiotensin-independent pathways. The three-dimensional structural information of (P)RR is valuable to understand the molecular mechanism of receptor binding. The large-scale production of (P)RR in *E. coli* is the key towards the three-dimensional structural analysis of this receptor.

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