[Frontiers in Bioscience E2, 1234-1240, June 1, 2010]

Species specificity of prorenin binding to the (pro)renin receptor *in vitro*

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1. ABSTRACT

In this study, binding properties of human prorenin to rat (pro)renin receptor [r(P)RR] and rat prorenin to human (pro)renin receptor [h(P)RR] were elucidated *in vitro* to investigate species specificity of prorenin bindings to (P)RR. Both (P)RRs were expressed *in vitro* based on wheat germ lysate and purified using His trap column. The association and dissociation rate constants of human and rat prorenin for the immobilized r(P)RR and h(P)RR were 6.64×10^6 and 1.01×10^6 $M^{-1}.s^{-1}$ and, 0.024 and 8.45×10^{-3} s^{-1}, respectively. Their K_D values were 3.7nM [3-fold higher than that of human prorenin vs h(P)RR (1.2nM)] and 8.3nM [1.2-fold lower than that of rat prorenin vs r(P)RR (10.2nM)], respectively. Additionally, human and rat prorenin bound to the pre-adsorbed rat and human (P)RR, respectively, performed enzymatic activities. Their molecular activities were $4.1h^{-1}$ [almost similar ($3.8h^{-1}$) to human prorenin vs h(P)RR] and $0.98h^{-1}$ [~2-fold lower than rat prorenin vs r(P)RR], respectively. Thus, these results suggest the species specificity for bindings of prorenin to (P)RR, which could be useful in elucidating the biochemical properties of prorenin binding to the receptor.

2. INTRODUCTION

The (pro)renin receptor [(P)RR] is a single transmembrane protein with 350 amino acid residues first reported by Nguyen *et al* (1). Since its finding, the renin-angiotensin system (RAS) has come across with new and unique perception regarding the physiological roles of not only renin/prorenin but also the receptor. This receptor binds both renin, the rate limiting enzyme of RAS as well as prorenin, the pre-active form of renin (2-4). Recently, evidence for the direct binding of renin and prorenin to the recombinant (pro)renin receptor have also been revealed (5-7). These bindings mediate dual effects by generating ang-II that regulates blood pressure/electrolyte balance and stimulating the second messenger pathway (7-10). Moreover, interaction of prorenin with the (P)RR leads to the non-proteolytic activation of prorenin by reversibly removing the prosegment from the active site through conformational change (2, 4, 10). The association of (P)RR in physiological and pathophysiological diseases (11-14) have been demonstrated in many animal models over expressing human (pro)renin receptor. For example, transgenic rat models targeting human (P)RR expression to

vascular smooth muscle cells suggest a pathological role of the (P)RR in raising blood pressure (15). Also, transgenic rats over expressing human (P)RR developed proteinuria and glomerulosclerosis by elevating glomerular phospho mitogen-activated protein kinase (MAPK) and profibrotic transforming growth factor (TGF)-β1 (10, 16).

The binding kinetics of rat and human renin/prorenin to their respective homologous receptors was reported in many *in vitro* studies (1, 2, 4-6). On the other hand, binding properties of the heterologous combination between prorenin and the receptor are unavailable, while based on sequence alignment of human and rat (P)RR, it could be assumed that in case of transgenic animals, over expressed human (P)RR and prorenin binds to endogenous prorenin and (P)RR, respectively. Transgenic animals permitted the specific interactions with the human transgene products *in vivo*. A unique species specificity of RAS was reported as human angiotensinogen expressed in the transgenic animal models could not cross react with the rat renin *in vivo* (17). So, the purpose of the present study was (i) to observe *in vitro* binding of human (P)RR to rat prorenin and rat (P)RR to human prorenin and (ii) to elucidate species specificity binding kinetics of their interactions *in vitro*.

3. MATERIALS AND METHODS

3.1. Preparation of human and rat prorenin

Chinese hamster ovary (CHO) cell lines harboring the cDNA's of human and rat prorenin were maintained in conditioned medium under humidified atmosphere of 5% CO_2 and 95% air in 25-cm^2 cell culture flasks. Prorenin preparations were harvested and purified as described previously (6).

3.2. Expression of human and rat (pro)renin receptor on the membrane of COS-7 cells

COS-7 cells were cultured in Dulbecco's Eagle medium at 37^{o}C in a humidified chamber under 5% CO_2 (2). The human and rat (pro)renin receptor containing FLAG tag at the N-terminus were expressed transiently on the membrane of the COS-7 cells and thus, identified according to the method described by Nurun *et al* (2).

3.3. Bindings of prorenin molecules to the (pro)renin receptor expressed on the membrane of COS-7 cells

Rat and human prorenin preparations at a concentration of 2.0 nM were allowed to incubate with the human and rat (P)RR, respectively, expressed on the membrane of the COS-7 cells at 37^{o}C for different time intervals (0, 6, 12 and 18 h). Before incubating with the receptor expressing COS-7 cells, prorenin preparations were incubated for 1 hour at 37^{o}C to minimize their cryo-activation and the level of activation was harmonized to less than 2%. The levels of unbound human and rat prorenins in the medium were measured according to the method described by Nurun *et al* (2). Briefly, after incubation of prorenin medium with the receptor expressing COS-7 cells, levels of prorenin in the medium was measured from its activity using standard Ang-I ELISA (18). In case of measuring the activity of human and rat renin, the substrate concentration was set according to their K_m values against sheep angiotensinogen as reported by Nagase *et al* (19). Thus, using specific activity of respective mature renin against sheep angiotensinogen, concentrations of prorenins were determined. This was considered as the levels of unbound prorenin. Then, bound amount was calculated by subtracting the unbound amount from initial amount.

3.4. *In vitro* expression of recombinant human and rat (pro)renin receptor

In recent time, method for *in vitro* expression of human (pro)renin receptor with 292 amino acids (32.5 kDa, N17-N308) with six histidine residues at the C-terminus lacking the transmembrane sequence has been described (6). Same protocol was followed for *in vitro* the expression of rat (pro)renin receptor containing 291 amino acids (~32.5 kDa, N18-N308) in this study. Briefly, the cDNA of the desired (pro)renin receptor with His-tag sequence was amplified by PCR using the restriction sites *Not* I and *Xho* I. The forward and the reverse primers were designed as 5'-tttgcggccgcAATGAATTTAGCATATTAAGATCGC-3' and 5'-tttctcgagGTTGAAAACCACCGAATACT-3', respectively. Followed by PCR purification, the product was cut with *Not* I and *Xho* I restriction enzymes and then inserted into pIVEX 1.3WG vector which was previously cut with the same restriction enzymes. After ligation and DNA sequencing analysis, the vector containing the insert was expressed in a continuous exchange cell free *in vitro* translation system based on wheat germ lysate using RTS 500 Wheat Germ CECF kit according to the instruction of manufacturer (Roche Diagnostics GmbH, Germany). This expressed receptor was then purified by affinity chromatography using His Trap column with the elution buffer consisting of 20 mM Phosphate, 500 mM NaCl and 500 mM Imidazol (pH 7.4). The purified protein was eluted, fractionated and then, checked by SDS-PAGE and Western blot analysis as reported previously (6).

3.5. Binding assay of prorenin molecules to the (pro)renin receptor using BIAcore assay system

Real-time binding assays of rat and human prorenin to human and rat (P)RR, respectively were performed using surface plasmon resonance technique by employing the BIAcore 2000 analyzer (BIAcore AB, Uppsala, Sweden). The binding quantities of the analytes were observed in terms of resonance units (RU). Highly purified anti-(P)RR-antibody against the epitope sequence E^{221}IGKRYGEDSEQFRD^{235} of the receptor was immobilized onto the CM5 surface through amine coupling. Production, purification and application of this anti-(P)RR-antibody for amine coupling on the CM5 sensor chip has recently been portrayed by Nabi *et al* (6). The sequence homology of this epitope between human and rat (P)RR is 100%. This amine coupled antibody was used to immobilize purified both human and rat (P)RR preparations according to the method described previously (5, 6).

Prorenin preparations at 0.5 nM, diluted in HBS-EP buffer (0.15 M NaCl, 3.0 mM EDTA, 0.005% polysorbate 20 and 0.01 M HEPES, pH 7.4), were injected into the sensor chip (flow rate: 10 μl/min) for real time

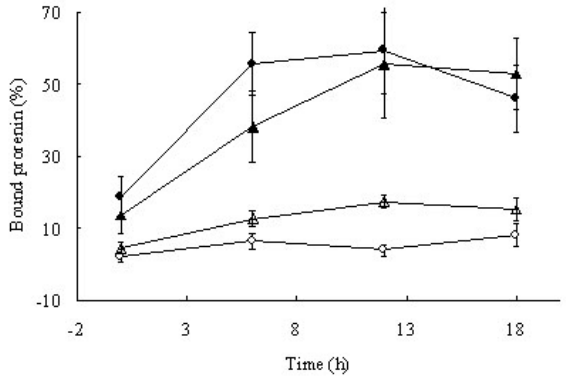

Figure 1. Time dependent bindings of rat and human prorenin to human and rat (pro)renin receptors, respectively expressed on the membrane of COS-7 cells. Recombinant rat and human prorenin preparations were incubated with the heterologous (pro)renin receptors expressing COS-7 cells. Prorenins incubated with the cells transfected only with the expression vector lacking the receptor cDNA were used as controls (open circle for rat prorenin and open triangle for human prorenin). The maximum percent binding was observed at 12 h for both rat prorenin bound to human (P)RR (closed circle) and human prorenin bound to rat (P)RR (closed triangle). Each data represents mean ± S.D. (n = 4).

kinetic studies to determine the receptor-protein association rate constants (k_a) and dissociation rate constants (k_d) by the BIA evaluation method, and the dissociation constant (K_D) was determined from Langmuir 1:1 kinetic binding model. In this case, sensor chip containing immobilized antibody lacking the (P)RR was used as control to determine the nonspecific binding of prorenin. BIAsimulation software program was used to draw the figures.

3.6. Determination of the molecular activities of receptor-bound prorenin molecules

To determine the activities of receptor-bound prorenin molecules, recombinant (P)RR preparations were immobilized onto the plastic surfaces of 96-well plates as described previously (4,6) with slight modification.

Three hundred microliter of (P)RR preparations (1.0 μg/ml) were allowed to immobilize on the wells of the 96-well plates overnight at 4^{o}C. Rat and human prorenin preparations (300 μl) at a concentration of 2.0 nM were incubated in each well containing the human and rat (P)RR, respectively, at 4^{o}C for 2 hours. After incubation, amounts of receptor-bound human and rat prorenin were estimated by subtracting the remaining amount of prorenin from their initial amount under standard assay conditions (4), and prorenin preparation lacking the receptor was used as control.

Also, renin activities of the receptor-bound human and rat prorenin molecules were measured by Ang-I ELISA (18) with the recombinant sheep angiotensinogen preparation (19) under standard assay condition as described previously (20). Molecular activities of prorenin were calculated as the ratio of the activity (fmole ang-I/well/h) of bound form of prorenin to the bound amount (fmole prorenin/well) of prorenin.

4. RESULTS

4.1. Binding of prorenin to (pro)renin receptor expressed on the membrane of COS-7 cells

Bindings of rat prorenin to human (P)RR and human prorenin to rat (P)RR were time-dependent. In both cases, under standard binding conditions, almost 60% of incubated prorenin molecules bound to the membrane-anchored receptors (Figure 1).

4.2. Real-time bindings of prorenin molecules to the immobilized (pro)renin receptors in BIAcore

In our previous study using surface plasmon resonance technique in BIAcore assay system it was found that human (P)RR associated with immobilized anti-221/235 antibody could bind to human prorenin with the dissociation constant (K_D) of 1.2±0.4 nM (5). In this study, real-time binding of rat prorenin to rat (P)RR was observed and the K_D was determined at 10.2±1.8 nM. The BIAcore analysis also showed the concentration dependent bindings of rat prorenin to human (P)RR and human prorenin to rat (P)RR (Figure 2A and B). From the real-time kinetic analyses for the interaction of prorenin to (P)RR, the association (k_a) and dissociation rate constants (k_d) were measured and, the dissociation constants (K_D) were determined at 8.3±1.5 and 3.7±0.9 nM, respectively by Langmuir 1:1 kinetic binding model using BIAevaluation software. All the data of the kinetic analyses were summarized in Table 1.

4.3. Molecular activities of the receptor-bound prorenin

Table 2 shows the bound amount of rat prorenin to immobilized human (P)RR and human prorenin to rat (P)RR as well as molecular activities of the receptor bound prorenin molecules. The amount of rat and human prorenin bound to human and rat (P)RR, respectively were determined as 50 ± 7 and 47 ± 6 fmole/well, respectively. The renin activities of the receptor-bound prorenin molecules were measured 49 ± 7 and 193 ± 18 fmole Ang-I/well/h for rat prorenin bound to human (P)RR and human prorenin bound to rat (P)RR, respectively. The values of molecular activities thus calculated were 0.98 ± 0.003 h^{-1} for rat prorenin and 4.1 ± 0.21 h^{-1} for human prorenin. Also, the molecular activities of human and rat prorenin for the bindings to their respective receptors were determined 3.8 ± 0.02 h^{-1} and 1.9 ± 0.05 h^{-1}.

5. DISCUSSION

The (pro)renin receptor plays a pivotal role in the renin-angiotensin system. The role of (P)RR in organ damage associated with hypertension and diabetes have been reported (11, 12, 14, 21, 22). For defining the pathogenic effects of the (pro)renin receptor, investigators have used transgenic rats that over expressed the human (P)RR (15, 23). Present study reports the binding properties of rat prorenin to human (P)RR and human prorenin to rat (P)RR

Table 1. Kinetic parameters for the bindings of prorenin molecules to the homologous and heterologous immobilized receptors using surface plasmon resonance technique

Combination of species		Association rate constant (k_a), $M^{-1}.s^{-1}$	Dissociation rate constant (k_d), s^{-1}	Dissociation constant (K_D), nM	Comparison of K_D (fold)[a]
Prorenin	(Pro)renin receptor				
human	human	$1.7x10^7 \pm 0.9x10^7$	0.0202 ± 0.01	1.2 ± 0.4[b]	1
human	rat	$6.64x10^6 \pm 1.93x10^6$	0.024 ± 0.011	3.7 ± 0.9	3.1
rat	rat	$0.95x10^6 \pm 0.21x10^6$	$9.69x10^{-3} \pm 2.14x10^{-3}$	10.2 ± 1.8	8.5
rat	human	$1.01x10^6 \pm 0.3x10^6$	$8.45x10^{-3} \pm 2.53x10^{-3}$	8.3 ± 1.5	6.9

[a]: all the values of K_D were compared with the K_D for the binding of human prorenin to human (pro)renin receptor; the data were expressed as Mean ± S.D. (n=7). [b]: Reference number (5).

Table 2. Molecular activities of human and rat prorenin molecules bound to homologous and heterologous receptors

Combination of species		Amount of bound prorenin (fmole/well)	Activity of the bound prorenin (fmole Ang-I/well/h)	Molecular activity (h^{-1})
Prorenin	(Pro)renin receptor			
human	human	74 ± 8	283 ± 32	3.8 ± 0.02
human	rat	47 ± 6	193 ± 18	4.1 ± 0.21
rat	rat	90 ± 11	170 ± 24	1.9 ± 0.05
rat	human	50 ± 7	49 ± 7	0.98 ± 0.003

Recombinant (P)RR preparations were immobilized onto the plastic surfaces of 96-well plates. Rat and human prorenin preparations were incubated in each well containing immobilized receptors and bound amount of prorenin was calculated. Molecular activities of prorenin were calculated as the ratio of the activity (fmole ang-I/well/h) of bound form of prorenin to the bound amount (fmole prorenin/well) of prorenin. The data were expressed as Mean ± S.D. (n=5).

Both human and rat (P)RR expressed on the membrane of COS-7 cells bound not only to homologous (2) but also to the heterologous prorenin molecules (Figure 1). Surface plasmon resonance technique using recombinant (pro)renin receptor also showed similar results (Figure 2A and B) and revealed the binding kinetics of prorenins to the immobilized receptors (Table 1). Further, receptor-bound heterologous prorenin molecules showed renin activities while sheep angiotensinogen was used as the substrate (Table 2).

Human (pro)renin receptor has been identified in the vascular smooth muscle cells (VSMCs) in human heart that binds prorenin (1). Also, rat VSMCs over expressing human (P)RR (3) and/or naturally expressing rat (P)RR (24) bound prorenin. These receptor-bindings mediate catalytic activation of prorenin that induces Ang-I generation (1, 3, 24). Our previous study reported the binding of rat and human prorenin to their respective (pro)renin receptors on the membrane of COS-7 cells with the K_D values of similar nanomolar range (0.89 and 1.8 nM, respectively) (2). Batenburg *et al* also reported the nanomolar order (K_D: 5.9 nM) of binding affinity of human prorenin for the (pro)renin receptor expressed on the membrane of the vascular smooth muscle cells (3). In addition, it was revealed that human (pro)renin receptor [human (P)RR-transgenic rats] bound to either human or rat prorenin molecule stimulated an intracellular signal that led to MAPK activation and expression of TGF-β1 was enhanced (25). Furthermore, bound prorenin activates cellular ERK phosphorylation in VSMCs which is independent of Ang-II generation or action (16, 26). Also, both human and rat recombinant (pro)renin receptor tagged with FLAG cDNA were confirmed to express on the membrane of the COS-7 cells and recognized by anti-FLAG antibody as it was shown previously (2). In the present study, time-dependent bindings of human prorenin to rat (P)RR and rat prorenin to human (P)RR have been shown in Figure 1.

In vitro binding kinetics of recombinant human prorenin to the immobilized recombinant human (pro)renin receptor has been reported recently by Nabi *et al* (5). Also in the present study, the real-time bindings of recombinant prorenins to recombinant receptors were directly observed by surface plasmon resonance technique using BIAcore assay system. The K_D values for the interaction of rat prorenin to human (P)RR and human prorenin to rat (P)RR were 8.3 ± 1.5 and 3.7 ± 0.9 nM, respectively, determined from their association and dissociation rate constants by BIAevaluation software using Langmuir 1:1 kinetic binding model. The K_D of rat prorenin for binding to its respective rat (P)RR was also determined at 10.2 ± 1.8 nM. The kinetic parameters i.e., the association and dissociation rate constants have been outlined in Table 1. The binding affinity of human prorenin for human (pro)renin receptor is 6.9-fold higher than that of rat prorenin to human (pro)renin receptor. Even binding affinity of human prorenin to rat (P)RR (K_D = 3.7 nM) is ~2.75-fold higher compared to that of the rat prorenin to rat (P)RR (K_D = 10.2 nM). Considering the values of dissociation constants between the homologous and heterologous combinations of rat and human prorenin as well as (P)RR, it is obvious from Table 1 that the K_D for the bindings of human prorenin to either homologous [h(P)RR] or heterologous [r(P)RR] receptor is lower than that of the K_D for the binding of rat prorenin to either of the receptor molecules. Thus, the data represented in Table 1 reflects that human prorenin molecule has higher flexibility for its binding to either of the receptor molecule.

We also determined the amount of prorenin molecules bound to the (pro)renin receptors immobilized on the synthetic surfaces and their enzymic activities to measure molecular activities. Human prorenin showed similar molecular activity of 3.8 ± 0.02 and 4.1 ± 0.21 h^{-1} when bound to the homologous and heterologous receptors, respectively, whereas rat prorenin showed almost two times higher molecular activity when bound to rat (P)RR than to human (P)RR (Table 2). Thus, this may explain why tissue

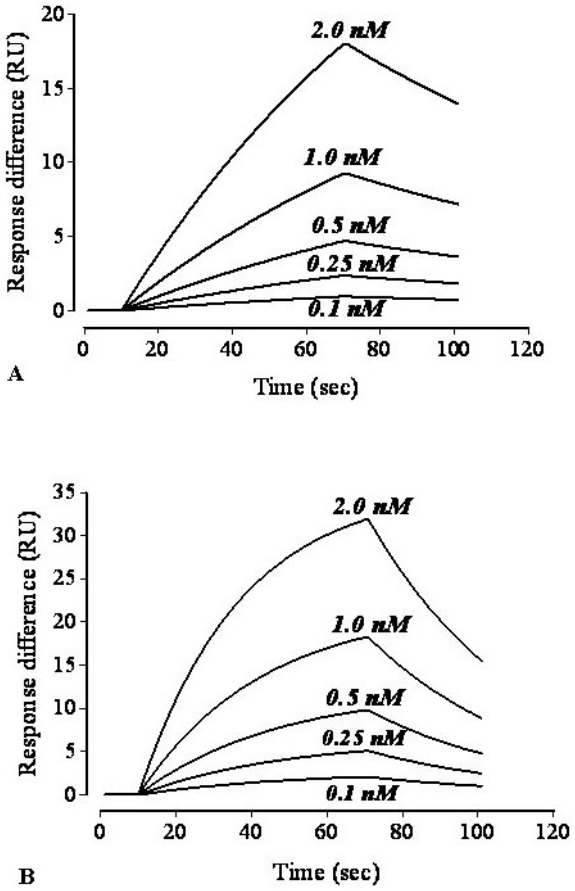

Figure 2. BIAsimulated representation of real-time bindings of rat prorenin to human (pro)renin receptor (A) and human prorenin to rat (pro)renin receptor (B) obtained by surface plasmon resonance technique in BIAcore assay system. Different concentrations of rat and human prorenin preparations were injected into the flow cell at a rate of 10 µl/min to bind to the immobilized human and rat (pro)renin receptors, respectively. The dissociation constants (K_D) were determined as 8.3 and 3.7 nM for binding of rat prorenin to human (pro)renin receptor and human prorenin to rat (pro)renin receptor, respectively. The results are representative of 7 experiments (mean ± S.D.). All the values shown in the figures are plotted after subtracting the specific binding from non-specific binding.

angiotensin II levels were not elevated in human (P)RR-transgenic rats as reported by Kaneshiro *et al* (23). On the other hand, the molecular activity exerted by rat prorenin bound to rat (pro)renin receptor was almost similar with the molecular activity (1.25 h^{-1}) of free form of rat mature renin as reported by Nabi *et al* (4). Furthermore, either bound to human (P)RR or rat (P)RR, molecular activity of human prorenin was, on an average, 2-4 fold higher than that of rat prorenin. This could be due to the slow activation rate of rat prorenin compared to that of human prorenin after protein-protein interaction through conformational change. A similar phenomenon of slow activation of rat prorenin was also reported *in vitro* by Suzuki *et al* (27). Using a novel chimeric approach for human and rat prorenins they demonstrated that chimera of human renin and rat prosegment showed very slow activation like native rat prorenin compared to the chimera of rat renin and human prosegment. Thus, they concluded that the prosegment sequence of prorenin played a pivotal role for the activation of prorenin molecules. They also showed that species specific regions within the prorenin prosegment like decoy region peptides (2, 5, 6) containing "handle" (2, 28) sequence might actually be crucial for not only the interaction of prorenin molecules to the receptor but also their non-proteolytic activation. Thus, the data of this study reveals that human prorenin is more flexible for its binding to either of the receptor molecules [h(P)RR or r(P)RR]. This could be due to the possibility of the spatial arrangement of the "handle" region sequence within the human prorenin molecule. This sequence might be protruded outward from the molecule in such a way which might have facilitated the easy binding and it has been reflected by the binding affinities (reciprocal of dissociation constant). The data presented so far only predicted the species specificity of prorenin molecule regarding its biochemical properties e.g., (P)RR binding kinetics and molecular activities *in vitro* rather the effects of species specificity of (P)RR itself. However, determination of the X-ray crystallographic structure of (P)RR would make possible to comprehensively remark on effects of species specificity for (P)RR. In conclusion, our *in vitro* study would help researchers to make strategy for producing transgenic animal needed for (pro)renin receptor study.

6. ACKNOWLEDGEMENTS

This study was supported in part by a Grant-in-aid for Scientific Research (1907165) from the Ministry of Education, Science and Culture of Japan. AHM Nurun Nabi is the recipient of a Postdoctoral Fellowship for Foreign Researchers (2007/2009: P07165) from Japan Society for the Promotion of Science.

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Key Words: Prorenin, (pro)renin receptor, Binding Affinity, Species Specificity, Surface Plasmon Resonance Technique

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