'Decoy peptide' region (RIFLKRMPSI) of prorenin prosegment plays a crucial role in prorenin binding to the (pro)renin receptor

A.H.M. NURUN NABI[1], KAZAL BORON BISWAS[2], TSUTOMU NAKAGAWA[1], ATSUHIRO ICHIHARA[3], TADASHI INAGAMI[4] and FUMIAKI SUZUKI[1,2]

[1]Faculty of Applied Biological Sciences, [2]United Graduate School of Agricultural Science, Gifu University, Gifu; [3]Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan; [4]Department of Biochemistry, Vanderbilt University School of Medicine, Nashville, TN, USA

Received February 6, 2009; Accepted April 10, 2009

DOI: 10.3892/ijmm_00000210

Abstract. This study investigated a role of decoy peptide region (R^{10P}IFLKRMPSI^{19P}) in prorenin prosegment for prorenin binding to the (pro)renin receptor using the surface plasmon resonance technique. Three kinds of anti-receptor antibodies labeled as anti-107/121, anti-221/235 and anti-His tag antibody were prepared. The respective antigens D^{107}SVANSIHSLFSEET^{121} (close to the N-terminal side of receptor), E^{221}IGKRYGEDSEQFRD^{235} (N-terminal side of the transmembrane part of receptor) and 10xHis sequence (C-terminus) were designed based on the sequence of the receptor. These antibodies were immobilized on the CM5 sensor chip by amine coupling and allowed to bind to the receptor. Human prorenin, renin and the decoy bound to the receptor associated with antibodies. Their association (k_a) and dissociation (k_d) rate constants were measured and the dissociation constants (K_D) were determined using Langmuir 1:1 kinetic binding model. The K_D for interaction of prorenin and receptor associated to anti-107/121, anti-221/235 and anti-His tag antibodies were 2.9, 1.2 and 7.8 nM, respectively and for renin they were 9.3, 4.4 and 7.1 nM. The decoy bound to the respective immobilized receptor-antibody complexes at K_D's of 6.2, 3.5 and 15.2 nM. Prorenin, renin and decoy had lower K_D at the nanomolar ranges compared to those of L^{1P}PTD^{4P} in the prorenin prosegment and A^{248}KKRLFDYVV^{257} in the C-domain of mature renin. The decoy reduced the binding of not only prorenin but also renin to (P)RR. These data are direct evidence that prorenin, renin and the peptides bind to (P)RR and the decoy reduces prorenin binding, supporting our hypothesis that decoy peptide region has a crucial role in prorenin binding.

Correspondence to: Professor Fumiaki Suzuki, Gifu University, 1-1 Yanagido, 501-1193, Gifu Shi, Gifu, Japan
E-mail: aob3073@gifu-u.ac.jp

Key words: (pro)renin receptor, renin, prorenin, decoy peptide, 'handle' region peptide, (P)RR blocker

Introduction

The human (pro)renin receptor, h(P)RR, discovered and cloned by Nguyen *et al* (1), binds renin then enhances its enzyme activity (1-3). Moreover, prorenin bound to (pro)renin receptor [(P)RR] undergoes substantial increase in its renin activity without proteolytic cleavage of the amino terminal prosegment. Prorenin was found to bind to h(P)RR with a higher affinity than renin (1,4). (P)RR is expressed ubiquitously, particularly in renal glomeruli (1), tubular systems (1), cardiac myocytes (3), including those in the cornea (5) and brain (6). Thus, locally anchored activated prorenin on (P)RR and renin not only supply angiotensins to local tissues, they also stimulate the receptor to transmit its own signals which include phospho-extracellular signal-regulated kinase (ERK 1/2) (1,7), phospho-p38 mitogen-activated protein kinase (MAPK) (8), profibrotic transforming growth factor (TGF)-ß1 (9), and inflamatogenic cyclooxygenase-2 (COX-2) (10) which are involved in end-stage organ damage of kidney, heart, vasculature, retina, etc. These signal responses were observed not only in cultured cells stimulated by renin and prorenin, but also from end-stage damaged tissues such as diabetic kidneys and pressure-overloaded heart (9,11,12). The degenerative effects of these signals seem to exert devastating effects, perhaps more severe than locally generated Ang II from immobilized renin and prorenin as attested by studies using mice lacking the Ang II type 1a receptor by Ichihara *et al* (13).

Suzuki and associates discovered a short pentameric sequence near N-terminal region of the prosegment sequence of prorenin and named it 'handle region peptide' (HRP), which protrudes out of the surface of the prorenin molecule and readily binds to specific antibodies targeting this segment resulting in activation of prorenin (14). This finding led Ichihara and his colleagues (15) to design a decameric decoy peptide by extending the C-terminus of HRP by 5 amino acid residues. This peptide has been found to inhibit the binding of renin/prorenin to the receptor as well as to block their receptor-mediated signal transduction in *in vivo* studies (2,4,6,12,13,16-18).

In diabetes, prorenin to renin ratio is markedly elevated in plasma. The elevated prorenin level has been considered as a

cause of microvascular damage and microalbuminuria (19-21). We found that chronic infusion of the decoy peptide effectively prevented the development of glomerulosclerosis and proteinuria for over a 28-week period (15,16).

A plausible mechanism of the therapeutic effects of the decoy peptide infusion may likely be due to the inhibition of prorenin-renin binding to (P)RR. For *in vivo* studies a specific inhibitor is a powerful tool for identifying a pathogenic mechanism of a disease as well as for developing a therapeutic strategy. However, direct evidence for the inhibition of prorenin-renin binding to the receptor by the decoy peptide has not been available. Without the solid mechanistic information, it is impossible to define the therapeutic mechanism of the decoy peptide. To shed light on the action of decoy peptide on prorenin-renin binding to the (P)RR we employed a novel approach for evaluating ligand binding to (P)RR by the surface plasmon resonance technique in BIAcore system which sensitively detects the binding of a ligand to a receptor immobilized onto antibodies coupled to the sensor chip. Here we report clear evidence for the high affinity binding of the decoy peptide with a dissociation constant (K_D) in a nanomolar range which makes it competitive enough against prorenin and renin binding whose K_D's are also at the level of nanomolar. Thus, we hypothesized that decoy peptide region in the N-terminal side of prosegment sequence of prorenin plays a crucial role in the binding of prorenin to (P)RR.

Materials and methods

Preparation of human renin, prorenin and (pro)renin receptor. Chinese hamster ovary (CHO) cell lines harboring human prorenin cDNA were maintained (22) under humidified atmosphere of 5% CO_2 and 95% air in 25-cm^2 cell culture flasks (CELLSTAR, Greiner, Bio-One, Germany) until attaining 100% confluent monolayer in the DMEM medium with 10% fetal bovine serum by the method described earlier by Nabi *et al* (2). A cDNA coding for human mature renin was inserted into the expression vector pcDNA3-sAgSP-10xHis (2). The resulting plasmid was transfected into CHO cells to establish a permanent cell line by the method described previously (2). The cell line secreted mature renin with a 10xHis tag into the culture medium. Recombinant prorenin with a 10xHis tag was prepared similarly. The media containing these preparations were collected and stored at -80˚C until further study.

A cDNA coding for the extracellular part of h(P)RR (N17-S304) with N-terminal FLAG tag (23) was inserted into the expression vector pcDNA3-sAgSP-10xHis. The extracellular part of h(P)RR with N-terminal FLAG tag and C-terminal deca histidine tag was transiently expressed in COS-7 cells and h(P)RR was purified by Ni Sepharose column chromatography.

Preparation of anti-receptor antibodies. Three antibodies against three epitopes of the 10xHis-tagged human (pro)renin receptor were used. The antigens D^{107}SVANSIHSLFSEET^{121} (close to the N-terminal side of the receptor), E^{221}IGKRYGE DSEQFRD^{235} (N-terminal side of the transmembrane part of the receptor) and 10xHis sequence (C-terminus) were designed based on the sequence of the receptor. The antibodies were named as anti-107/121, anti-221/235 and anti-His tag antibody, respectively. These were polyclonal antibodies, produced in rabbits and purified using affinity columns.

Immobilization of h(P)RR on the CM5 sensor chip using anti-receptor antibodies for real-time binding assay using BIAcore. BIAcore model 2000 (BIAcore AB, Uppsala, Sweden) was employed to study real-time binding of human renin and prorenin to h(P)RR which was immobilized to the CM5 sensor chip of the instrument at 25˚C. In the assay system the binding quantities of analytes can be observed in term of resonance units (RU). The response differences were calculated from the subtracted values of resonance units (RU) obtained from the specific and non-specific bindings of the analytes. The sensor chip in the flow cell of the instrument consisted of dextran matrix which contained carboxymethyl groups. This group was activated using *N*-ethyl-*N*^{1}-dimethyl-aminopropyl carbodiimide (EDC) and N-hydroxysuccinimide (NHS) and was allowed to couple with amino groups of proteins of interest. However, directly coupled purified (P)RR did not show appreciable binding capacity to renin. This problem was solved by direct coupling of anti-(P)RR-antibodies. The anti-107/121, anti-221/235 and anti-His tag antibodies were immobilized on the surface of the activated CM5 sensor chip using amine coupling method. Amount of immobilized antibodies varied from 0.35-0.40 ng/mm^2 (1,000 RU equivalent to 1 ng/mm^2 of flow cell). The h(P)RR preparations (6, 12, 24, 48, 60 nM) were injected (flow rate: 10 μl/min) to observe the real-time binding of h(P)RR to the immobilized antibody. Receptor at a concentration of 12 nM was sufficient for the binding assay of renin, prorenin and the peptides. Unreacted carboxymethyl groups of sensor chip lacking immobilized proteins were blocked with ethanolamine as a control for non-specific binding to compare the receptor binding with or without antibody. A buffer solution (pH 7.4) containing 0.01 M HEPES, 0.15 M NaCl and 3 mM EDTA was used as running buffer.

Binding assay of renin, prorenin and decoy peptide to the h(P)RR associated with anti-receptor antibodies using BIAcore. As described in the previous section a CM5 sensor chip affixed with immobilized h(P)RR via antibodies to the receptor epitopes were used in a BIAcore 2000. Renin and prorenin at concentrations of 0.1-2.0 nM as well as the decoy peptide (R^{10P}IFLKRMPSI^{19P}, including the 'handle region peptide') at concentrations of 20, 40, 80 and 100 nM were injected (flow rate: 10 μl/min) into the sensor chip for real-time kinetic study i.e., to determine their association (k_a) and dissociation rate constants (k_d) for the receptor from BIA evaluation method and their values of dissociation constant (K_D) were calculated from respective k_a and k_d values. In this case, flow cells within sensor chip containing immobilized anti-receptor antibodies lacking the h(P)RR were used as controls to determine the non-specific binding of renin, prorenin and the peptides. Also, different region peptides of prorenin and mature renin (L^{1P}PTD^{4P}, in the N-terminal side of prosegment and A^{248}KKRLFDYVV^{257} a sequence present in the C-domain, respectively) were tested in this study as reference peptides.

Table I. Kinetic parameters of decoy peptide binding to the receptor associated with anti-receptor antibodies immobilized to CM5 sensor chip.

	Decoy peptide[a] binding to h(P)RR associated with antibodies		
	Anti-107/121	Anti-221/235	Anti-His tag
k_a	$4.7x10^6$±$1.4x10^6$	$1.6x10^6$±$0.3x10^6$	$3.4x10^6$±$1.2x10^6$
k_d	0.0293±0.01	$5.6x10^{-3}$±$2.1x10^{-3}$	0.052±0.02
K_D	$6.20x10^{-9}$±$1.6x10^{-9}$	$3.50x10^{-9}$±$0.3x10^{-9}$	$1.52x10^{-8}$±$0.7x10^{-8}$

k_a, association rate constant [$M^{-1}.s^{-1}$]; k_d, dissociation rate constant (s^{-1}); K_D, dissociation constant (M); the data are indicated as mean ± SD (n=7). [a]Flown at 80 nM.

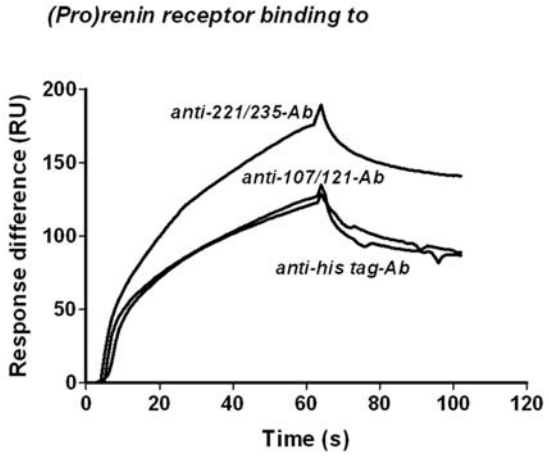

Figure 1. Bindings of h(P)RR to the antibodies (anti-107/121, anti-221/235 and anti-His tag antibodies) on CM5 sensor chip immobilized by amine coupling. Amount of immobilized antibodies were 0.35-0.40 ng/mm^2. The dissociation constants (K_D) for the interaction of h(P)RR to the antibodies were 1.05, 2.6 and 6.6 nM, respectively obtained from Langmuir 1:1 kinetic binding model. The tracings are representative of 7 identical experiments.

Interference of the binding of human renin and prorenin to the receptor by decoy peptide using BIAcore. Human prorenin or renin (0.5 nM) was co-incubated with decoy peptide (80 nM) to observe the inhibition of prorenin and renin binding to the immobilized receptor associated with different antibodies on CM5 sensor chip. The K_D for receptor binding as well as association (k_a) and dissociation (k_d) rate constants were determined by BIA evaluation program. The resonance units (RU) are not proportional to the concentration of analytes rather than represent binding mass of renin, prorenin and the decoy peptide. Decrease of resonance units in the presence of the decoy peptide was caused by that lower mass of the peptide which excluded the binding of high molecular mass, renin and prorenin, to the receptor.

Results

Binding of h(P)RR to anti-receptor antibodies immobilized on CM5 sensor chip. Concentration-dependent (using 6, 12, 24, 48 and 60 nM) binding of (P)RR to different antibodies immobilized to surfaces of the CM5 sensor chip were observed (data not shown). Fig. 1 shows typical binding of h(P)RR to the immobilized antibodies at a concentration of 12 nM. The binding affinities of (P)RR for different antibodies immobilized on different flow cells of CM5 sensor chip show almost similar resonance signal for their bindings. The values of dissociation constant (K_D) determined by Langmuir 1:1 binding model were 1.1, 2.6 and 6.6 nM for h(P)RR binding to immobilized anti-107/121, anti-221/235 and anti-His tag antibodies, respectively estimated from the association (k_a) and dissociation (k_d) rate constants.

Binding assay of decoy peptide to h(P)RR associated with different antibodies. Fig. 2A and C show the sensogram developed from the interaction of soluble phase decoy peptide with the receptor associated with different anti-receptor antibodies. Fig. 2C shows the binding of decoy peptide at a concentration of 80 nM to h(P)RR associated with anti-107/121, anti-221/235 and anti-His tag antibodies. We determined the association (k_a) and dissociation (k_d) rate constants as well as its dissociation constant (K_D) for the interaction of decoy peptide with the receptor associated with these three antibodies using BIAevaluation Software. Data are summarized in Table I.

Kinetic analysis of the decoy peptide binding to h(P)RR associated with anti-His tag antibody using BIAevaluation software. Using the surfaces with immobilized anti-receptor antibodies, decoy peptide showed dose-dependent binding to the h(P)RR. Fig. 2A shows the typical dose-dependent binding of the peptide (20, 40, 80 and 100 nM) to h(P)RR associated with anti-His tag antibody. The sensograms for different concentrations of decoy peptide were analyzed using Langmuir 1:1 kinetic binding model by fitting both the association and dissociation phases simultaneously. Curves derived from these concentrations of decoy peptide exhibited good individual fits in the binding model and could be subjected to K_D determination individually. The K_D values determined were 9.3, 3.8, 15.2 and 6.1 nM, respectively. Reference peptides $L^{1P}PTD^{4P}$ and $A^{248}KKRLFDYVV^{257}$ showed very low RU even at a concentration of 100 nM (Fig. 2D). The K_D values determined were $4.1x10^{-5}$ and $3.2x10^{-5}$ M.

Steady state analysis for the determination of dissociation constant (K_D) of decoy peptide to (P)RR using BIAevaluation software. In order to determine the dissociation constant (K_D)

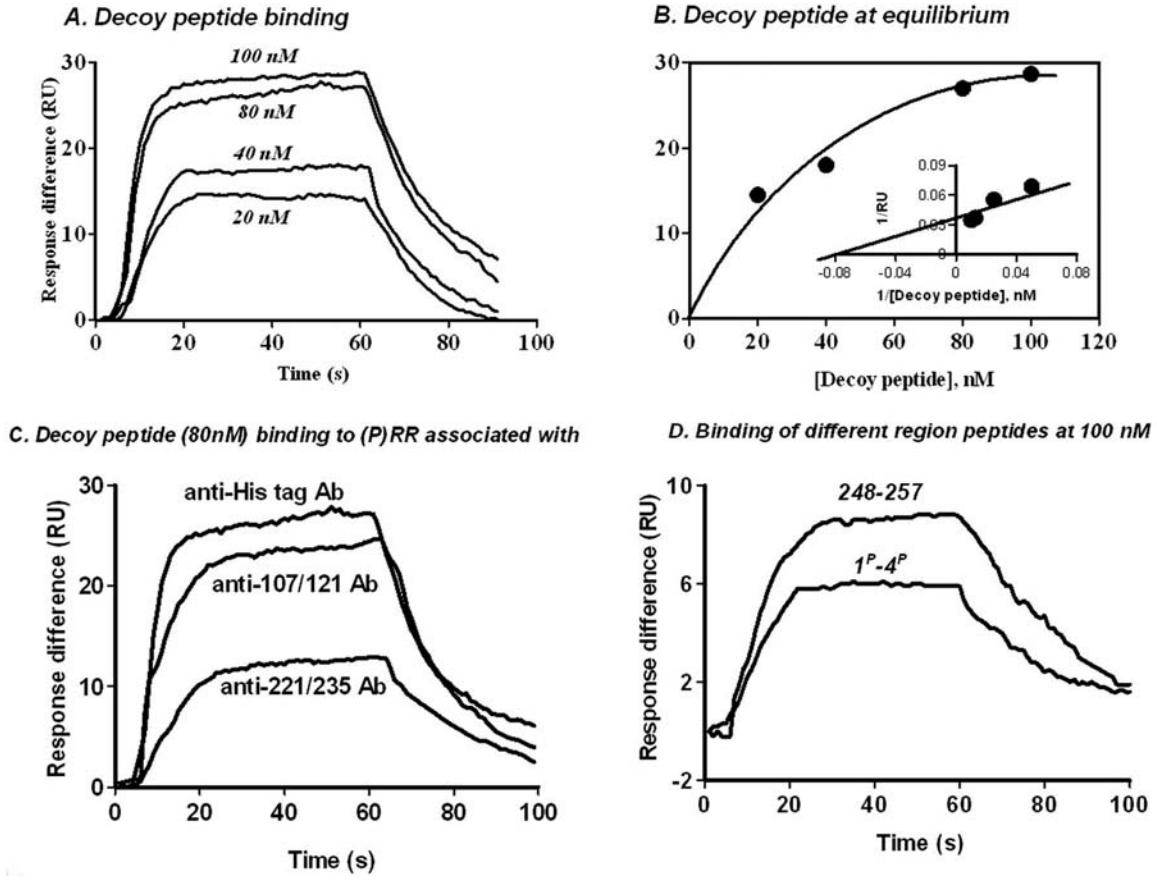

Figure 2. Surface plasmon resonance analysis of the interaction of peptides to h(P)RR. (A) This typical figure shows concentration-dependent binding of decoy peptide to h(P)RR associated with immobilized anti-His tag antibody. Decoy peptide at concentrations of 20, 40, 80 and 100 nM were injected at a flow rate of 10 μl/min. It shows that the binding sites of immobilized antibodies on CM5 sensor chip were practically saturated by the peptide at a concentration of 80 nM. (B) The steady state values of each different concentration being considered the R_{eq} (response at equilibrium) were plotted against the concentration of decoy peptide and thus, double reciprocal plot was replotted to determine the dissociation constant (K_D). (C) Decoy peptide binds to h(P)RR associated with anti-107/121, anti-221/235 and anti-His tag antibodies with different dissociation constant (K_D). (D) Binding of reference peptides from prorenin ($L^{1P}TDP^{4P}$) and renin ($A^{248}KKRLFDYVV^{257}$) at a concentration of 100 nM. These peptides bound to h(P)RR associated with anti-his tag-antibody with very high K_D. All the values shown in the figures are plotted after subtracting the specific binding from non-specific binding. The figure represents 7 identical experiments.

for a steady state binding between soluble phase of decoy peptide and immobilized receptor associated with antibody, equilibrium binding level was plotted vs. the concentration of the peptide (Fig. 2B), and thus, replotted in double reciprocal plot. The steady state values at every different concentration were considered as the response at equilibrium (R_{eq}). These plots were developed from the response plateau values of the sensograms (Fig. 2A) resultant of the interaction of peptide and immobilized receptor associated with antibody. The calculated K_D was 12.5 nM.

Assay of renin and prorenin bindings to h(P)RR associated with anti-receptor antibodies. Concentration-dependent bindings of prorenin and renin to (P)RR were observed in BIAcore assay system. The typical sensograms demonstrated in Fig. 3A and C were derived from the interaction of prorenin and renin with (P)RR while the receptor was associated with the immobilized anti-His tag antibody. The dissociation constants (K_D) for their steady state binding were 1.0 and 2.5 nM as described in the previous section and shown in Fig. 3B and D, respectively. Also, data summarized in Table IIA and B represents the association (k_a) and dissociation (k_d) rate constants as well as the dissociation constant (K_D) of only one concentration of prorenin and renin (0.5 nM) binding to (P)RR associated with anti-107/121, anti-221/235 and anti-His tag antibodies, respectively obtained by Langmuir 1:1 kinetic binding model.

Assay of interference of the bindings of renin and prorenin to h(P)RR associated with anti-receptor antibodies by decoy peptide. When decoy peptide was co-incubated with renin or prorenin, the response signal for their binding to (P)RR associated with different anti-receptor antibodies on the sensor chip decreased to a lower level than that without peptide. Considering the K_D values of the reference peptides, their effects on renin and prorenin binding to (P)RR was not performed. Fig. 4A and B show sensograms for the interference of the binding of prorenin and renin to (P)RR associated to anti-His tag antibody by the peptide, respectively.

Discussion

Although many studies have shown the effective blockade of prorenin activation by 'handle region peptide' (2,13,15,18), some incompatible results regarding the inhibitory effects of this peptide still persist (24-26). These studies have used

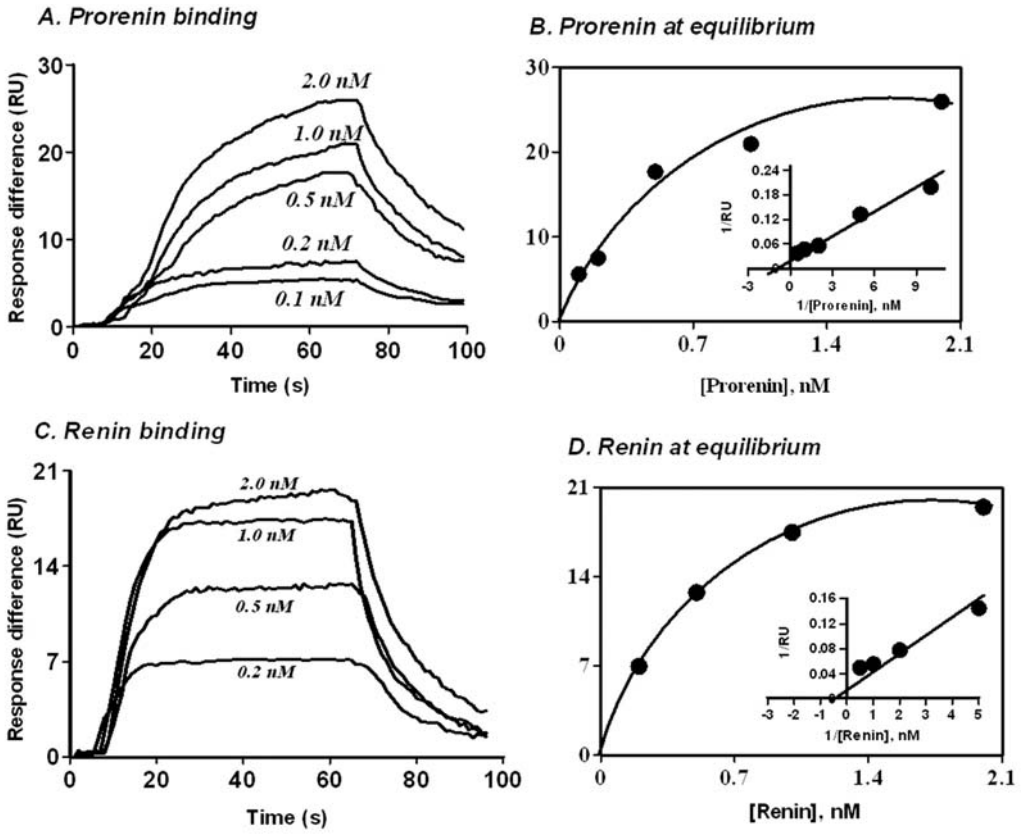

Figure 3. Surface plasmon resonance analysis of the interaction of prorenin and renin to (P)RR. Prorenin and renin (0.1, 0.2, 0.5, 1 and 2 nM) were injected into the flow cell over the CM5 sensor chip at a rate of 10 μl/min. Concentration-dependent bindings of prorenin (A) and renin (C) to h(P)RR associated with anti-His tag antibody were observed. B and D show the equilibrium state of the prorenin and renin binding to h(P)RR, respectively. The steady state values of each different concentration being considered the R_{eq} (response at equilibrium) were plotted against the concentration of prorenin and renin. The insets in the figures represent the double reciprocal plots constructed to get the K_D. All the values shown in the figures are plotted after subtracting the specific binding from non-specific binding. The figure represents 7 identical experiments.

Table II. Kinetic parameters of the binding of human prorenin (A) and renin (B) to the receptor associated with anti-receptor antibodies immobilized to CM5 sensor chip.

A, Prorenin

	Binding of prorenin[a] to h(P)RR associated with antibodies		
Parameters	Anti-107/121	Anti-221/235	Anti-His tag
k_a	$1.5x10^6$±$0.3x10^6$	$1.7x10^7$±$0.9x10^7$	$3.6x10^6$±$2.1x10^6$
k_d	$4.3x10^{-3}$±$1.3x10^{-3}$	0.0202±0.01	0.028±0.02
K_D	$2.9x10^{-9}$±$0.9x10^{-9}$	$1.2x10^{-9}$±$0.4x10^{-9}$	$7.8x10^{-9}$±$2.3x10^{-9}$

B, Renin

	Binding of renin[a] to h(P)RR associated with antibodies		
Parameters	Anti-107/121	Anti-221/235	Anti-His tag
k_a	$1.0x10^6$±$0.3x10^6$	$2.1x10^6$±$1.3x10^6$	$9.4x10^6$±$2.1x10^6$
k_d	$9.3x10^{-3}$±$3.6x10^{-3}$	$9.2x10^{-3}$±$2.2x10^{-3}$	0.067±0.06
K_D	$9.3x10^{-9}$±$2.5x10^{-9}$	$4.4x10^{-9}$±$1.7x10^{-9}$	$7.1x10^{-9}$±$2.9x10^{-9}$

k_a, association rate constant [$M^{-1}.s^{-1}$]; k_d, dissociation rate constant (s^{-1}); K_D, dissociation constant (M); the data are indicated as mean ± SD (n=7). [a]Flow at 0.5 nM.

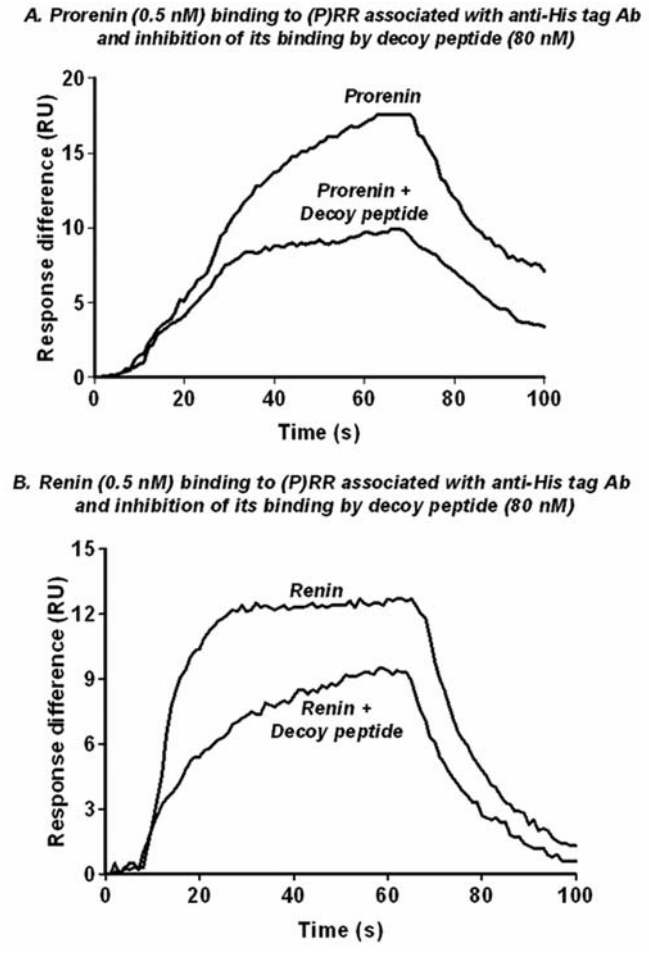

Figure 4. Binding of recombinant human prorenin (A) and renin (B) at 0.5 nM to h(P)RR associated with anti-His tag antibody on the surface of CM5 sensor chip using the surface plasmon resonance technique. Sensor chip containing immobilized anti-h(P)RR-antibody lacking the h(P)RR was used as control to determine non-specific bindings. Differences of resonance units were calculated by subtracting the resonance signal of non-specific binding from that of total binding. Decreased resonance unit in the presence of decoy peptide (80 nM) indicated interference of the binding of human renin and prorenin by the peptide, because the resonance unit represents the total binding mass of the analytes. The figure represents 7 identical experiments.

different experimental approaches with different cell lines or animal models. Such *in vivo* studies could be influenced by some membrane associated proteins that might participate positively or negatively while observing the renin and prorenin activation, their (P)RR-mediated signalling pathways as well as blockade of their activation by HRP. To avoid such possibilities, intact form of highly purified renin, prorenin, peptides and (P)RR were used *in vitro* in surface plasmon resonance technique. Using this approach, we have tried to answer the following questions: a) does decoy peptide bind to (pro)renin receptor directly? b) if so then does it inhibit prorenin binding to the receptor? This study thus, demonstrated the direct binding of decoy peptide to the receptor, for the first time, using the surface plasmon resonance technique (BIAcore assay system) *in vitro* (Fig. 2A and C and Table I). Both renin and prorenin bound to the immobilized receptor and the decoy peptide inhibited renin and prorenin binding to the receptor (Fig. 3A and C, Fig. 4A and B and Table II).

Kato *et al* (27) using BIAcore assay system reported binding of h(P)RR to the immobilized human renin on the surface of CM5 sensor chip. In these studies very high concentrations (in the range of μM) of h(P)RR was needed to observe binding to renin. At the same concentration, the h(P)RR did not bind immobilized prorenin. This might be due to the effects of amine coupling. To avoid such hazard in the present study on (P)RR binding we preferred to use biochemically intact renin, prorenin and h(P)RR preparations. Indeed, we observed very poor bindings of renin, prorenin and the peptide to the receptor when h(P)RR was directly coupled on the CM5 sensor chip by amine coupling as described in methods. Amine coupling required for their immobilization on the sensor chip might cause a change in conformation in those parts of molecules that are involved in the binding mechanism. To overcome this problem, anti-107/121, anti-221/235 and anti-His tag antibodies were used as connectors to intact h(P)RR to enable us to study interaction of intact renin, prorenin and receptor. Fortunately, antibodies were not affected by the amine coupling (Fig. 1).

Human renin, prorenin and decoy peptide had different K_D values for the receptor when associated with different antibodies (Tables I, IIA and B). Furthermore, this study revealed that decoy peptide reduced the binding of renin and prorenin to the h(P)RR (Fig. 4A and B). Highest amount of binding was observed within 60-70 sec when ligands were allowed to flow through the flow cells at a rate of 10 μl/min. Using independent t-test it was revealed that resonance signals of renin and prorenin binding to h(P)RR were reduced significantly when co-incubated with decoy peptide ($p<0.001$ in both cases, n=7). Here, data of the 60 sec were chosen arbitrarily for the comparison. On the other hand, peptides from other region of prorenin and mature renin bound to (P)RR (Fig. 2D), but had >1000 times higher K_D compare to that of the decoy peptide. Due to high values of K_D, we did not perform the competitive binding study of prorenin and renin with these peptides. Also, we reported in our previous study that peptides from the prorenin prosegment region 7^P-10^P (termed as 'gate' region in ref. 14) and 30^P-36^P (close to the C-terminal end of prosegment) were unable to inhibit binding of prorenin to (P)RR expressed on the membrane of COS-7 cells (2). It was also found that prorenin had lower K_D than renin for the (P)RR while associated with antibodies against the amino acid sequences 107-121 (K_D values: 2.9 and 9.3 nM, respectively) and 221-235 (K_D values: 1.2 and 4.4 nM, respectively). However, anti-His tag antibody associated receptor had K_D values for renin and prorenin 7.1 and 7.8 nM, respectively obtained by Langmuir 1:1 kinetic binding model. The peptides of 10xHis tag was attached to the C-terminus of the receptor lacking the C-terminal transmembrane region in the intact (P)RR sequence. Therefore, association of h(P)RR with this antibody by the C-terminal 10xHis probably kept plenty of space for binding of renin and prorenin with their binding site(s) remaining unaffected.

The reason why prorenin has a lower K_D for the receptor than renin (Table IIA and B) can be explained by the fact that the sequence of decoy peptide is present only in the prorenin molecule. On the other hand, we observed that the decoy peptide also inhibited the binding of renin (Fig. 4B). In the

present study, we determined the K_D of the decoy peptide in the range of nanomolar by BIAcore assay system (Table I). Moreover, the K_D values of prorenin and renin to (P)RR were also in the range of nanomolar (Table IIA and B). These data suggest that the decoy peptide competitively inhibits prorenin and renin binding to (P)RR. Such competitive inhibition has been estimated by other binding assay systems on the COS-7 cell membrane (2). Therefore, the most probable explanation for the inhibition of renin binding could be the change in the conformation of h(P)RR due to binding of the decoy peptide. This might ultimately slowed down the access of renin to its binding site within the receptor. This study clearly demonstrates not only the inhibitory effects of decoy peptide but also its binding properties to the h(P)RR *in vitro*. Therefore, it supports our hypothesis that decoy peptide plays a crucial role in the binding of prorenin to human (pro)renin receptor. Nevertheless, though our *in vitro* study showed evidence of direct binding of the peptide to (P)RR and blockade of prorenin binding, it still remains to be clarified why *in vivo* studies are not compatible with each other.

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, and NIH grant (H58205). A.H.M. Nurun Nabi is the recipient of a Postdoctoral Fellowship for Foreign Researchers (2007/2009: P07165) from Japan Society for the Promotion of Science.

References

1. Nguyen G, Delarue F, Burckle C, Bouzhir L, Giller T and Sraer JD: Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin. J Clin Invest 109: 1417-1427, 2002.
2. Nabi AHMN, Uddin NM, Nakagawa T, Iwata H, Ichihara A, Inagami T and Suzuki F: Role of 'handle' region of prorenin prosegment in the non-proteolytic activation of prorenin by binding to membrane anchored (pro)renin receptor. Front Biosci 12: 4810-4817, 2007.
3. Batenburg WW, Krop M, Garrelds IM, de Vries R, de Bruin RJ, Burcklé CA, Müller DN, Bader M, Nguyen G and Danser AH: Prorenin is the endogenous agonist of the (pro)renin receptor. Binding kinetics of renin and prorenin in rat vascular smooth muscle cells overexpressing the human (pro)renin receptor. J Hypertens 25: 2441-2453, 2007.
4. Nabi AHMN, Kageshima A, Uddin MN, Nakagawa T, Park EY and Suzuki F: Binding properties of rat prorenin and renin to the recombinant rat renin/prorenin receptor prepared by a baculovirus expression system. Int J Mol Med 18: 483-488, 2006.
5. Satofuka S, Ichihara A, Nagai N, Tsubota K, Itoh H and Ishida S: Pathologic roles of prorenin and (pro)renin receptor in the eye. Front Biosci 13: 3884-3895, 2008.
6. Shan Z, Cuadra AE, Sumners C and Raizada MK: Characterization of a functional (pro)renin receptor in rat brain neurons. Exp Physiol 93: 701-708, 2008.
7. Sakoda M, Ichihara A, Kaneshiro Y, Takemitsu T, Nakazato Y, Nabi AHMN, Nakagawa T, Suzuki F, Inagami T and Itoh H: (Pro)renin receptor-mediated activation of mitogen-activated protein kinases in human vascular smooth muscle cells. Hypertens Res 30: 1139-1146, 2007.
8. Saris J, 't Hoen P, Garrelds I, Dekkers D, denDunnen J, Lamers J and Danser AHJ: Prorenin induces intracellular signaling in cardiomyocytes of angiotensin II. Hypertension 48: 564-571, 2006.
9. Nguyen G and Danser AHJ: Prorenin and (pro)renin receptor: a review of available data from *in vitro* studies and experimental models in rodents. Exp Physiol 93: 557-563, 2008.
10. Kaneshiro Y, Ichihara A, Takemitsu T, Sakoda M, Suzuki F, Nakagawa T, Hayashi M and Inagami T: Increased expression of cyclooxygenase-2 in the renal cortex of human prorenin receptor gene-transgenic rats. Kidney Int 70: 641-646, 2006.
11. Campbell DJ: Critical review of prorenin and (pro)renin receptor research. Hypertension 51: 1259-1264, 2008.
12. Ichihara A, Sakoda M, Kurauchi-Mito A, Kaneshiro Y and Itoh H: Involvement of (pro)renin receptor in the glomerular filtration barrier. J Mol Med 86: 629-635, 2008.
13. Ichihara A, Suzuki F, Nakagawa T, Kaneshiro Y, Takemitsu T, Sakoda M, Nabi AHMN, Nishiyama A, Sugaya T, Hayashi M and Inagami T: Prorenin receptor blockade inhibits development of glomerulosclerosis in diabetic angiotensin II type 1a receptor-deficient mice. J Am Soc Nephrol 17: 1950-1961, 2006.
14. Suzuki F, Hayakawa M, Nakagawa T, Nasir UM, Ebihara A, Iwasawa A, Ishida Y, Nakamura Y and Murakami K: Human prorenin has 'gate and handle' regions for its non-proteolytic activation. J Biol Chem 278: 22217-22222, 2003.
15. Ichihara A, Hayashi M, Kaneshiro Y, Suzuki F, Nakagawa T, Tada Y, Koura Y, Nishiyama A, Okada H, Uddin MN, Nabi AHMN, Ishida Y, Inagami T and Saruta T: Inhibition of diabetic nephropathy by a decoy peptide corresponding to the 'handle' region for nonproteolytic activation of prorenin. J Clin Invest 114: 1128-1135, 2004.
16. Ichihara A, Kaneshiro Y, Takemitsu T, Sakoda M, Nakagawa T, Nishiyama A, Kawachi H, Shimizu F and Inagami T: Contribution of nonproteolytically activated prorenin in glomeruli to hypertensive renal damage. J Am Soc Nephrol 17: 2495-2503, 2006.
17. Ichihara A, Kaneshiro Y, Takemitsu T, Sakoda M, Suzuki F, Nakagawa T, Nishiyama A, Inagami T and Hayashi M: Nonproteolytic activation of prorenin contributes to development of cardiac fibrosis in genetic hypertension. Hypertension 47: 894-900, 2006.
18. Susic D, Zhou X, Frohlich ED, Lippton H and Knight M: Cardiovascular effects of prorenin blockade in genetically hypertensive rats (SHR) on normal and high salt diet. Am J Physiol Heart Circ Physiol 295: H1117-H1121, 2008.
19. Veniant M, Ménard J, Bruneval P, Morley S, Gonzalez MF and Mullins J: Vascular damage without hypertension in transgenic rats expressing prorenin exclusively in the liver. J Clin Invest 98: 1966-1970, 1996.
20. Deinum J, Ronn B, Mathieson E, Derky FHM, Hop WCJ and Schalenkamp MADH: Increase in serum prorenin precedes onset of microbalbuminuria in patients with insulin dependent diabetes mellitus. Diabetologia 42: 1006-1010, 1999.
21. Deinum J and Danser AHJ: Renin, prorenin and putative (pro)renin receptor. Hypertension 46: 1069-1076, 2005.
22. Poorman RA, Palermo DP, Post LE, Murakami K, Kinner JH, Smith CW, Reardon I and Heinrikson RL: Isolation and characterization of native human renin derived from Chinese hamster ovary cells. Proteins 1: 139-145, 1986.
23. Nakagawa T, Nishiuchi K, Akaki J, Iwata H, Satou R, Suzuki F and Nakamura Y: Efficient production of recombinant human (pro)renin utilizing a decahistidine tag attached at the C-terminus. Biosci Biotechnol Biochem 71: 256-260, 2007.
24. Feldt S, Batenburg WW, Mazak I, Maschke U, Wellner M, Kvakan H, Dechend R, Fiebeler A, Burckle C, Contrepas A, Jan Danser AH, Bader M, Nguyen G, Luft FC and Muller DN: Prorenin and renin-induced extracellular signal-regulated kinase 1/2 activation in monocytes is not blocked by aliskiren or the handle-region peptide. Hypertension 51: 682-688, 2008.
25. Feldt S, Maschke U, Dechend R, Luft FC and Muller DN: The putative (pro)renin receptor blocker HRP fails to prevent (pro)renin signaling. J Am Soc Nephrol 19: 743-748, 2008.
26. Muller DN, Klanke B, Feldt S, Cordasic N, Hartner A, Schmieder RE, Luft FC and Hilgers KF: (Pro)renin receptor peptide inhibitor 'handle-region' peptide does not affect hypertensive nephrosclerosis in Goldblatt rats. Hypertension 51: 676-681, 2008.
27. Kato T, Kageshima A, Suzuki F and Park EY: Expression and purification of human (pro)renin receptor in insect cells using baculovirus expression system. Protein Expr Purif 58: 242-248, 2008.