[Frontiers in Bioscience E2, 1211-1217, June 1, 2010]

Functional characterization of the decoy peptide, $R^{10P}IFLKRMPSI^{19P}$

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1. ABSTRACT

Decoy peptide ($R^{10P}IFLKRMPSI^{19P}$) showed its beneficial role in ameliorating the end-stage organ damage related disorders. Subsequently, *in vivo* and *in vitro* studies have been carried out to verify its effectiveness in several models using different experimental approaches. These studies with decoy peptide including the "handle" sequence have focused on the association of the (pro)renin receptor and prorenin in the pathogenesis of diabetes and hypertension. However, the function of (pro)renin receptor might be more complex than it was anticipated as it is not only distributed intracellularly and appeared on the cell membrane but also found in plasma. The decoy resembling the N-terminal sequence of prorenin has been useful in determining the structure-function relationship of prorenin and (P)RR. Therefore, this review tries to shed light on the use of decoy peptide in elucidating the functional properties of both prorenin and (pro)renin receptor by pointing out recent studies.

2. INTRODUCTION

The decoy peptide ($R^{10P}IFLKRMPSI^{19P}$) mimics the N-terminal sequence of human prorenin molecule (1-4). Its efficacy for improving organ damage was first reported by Ichihara *et al.* (1) designed on the basis of the sequence of "handle" ($I^{11P}FLKR^{15P}$) region peptide proposed by Suzuki and his group (5). Prorenin, the pre-active form of renin with no enzymatic activity, has reportedly been performed renin activity when associated with the newly identified (pro)renin receptor, (P)RR (6-9) via local conformational change and generates Ang-I at tissue sites (4,6-8,10,11). Receptor-bound prorenin also stimulates signal transduction via second messenger pathways (6,12-15).

The (P)RR is a multifunctional protein with 350 amino acid residues having a single transmembrane domain (6). This receptor is ubiquitously available in different places- from cell membrane (6,8,10) to the intracellular

(A) NH_2-R^{10P}IFLKRMPSI^{19P}-COOH

(B) 5(6)-Carboxyfluorescein- NH_2- R^{10P}IFLKRMPSI^{19P}-COOH

(C) NH_2- R^{10P}IFLKRMPSI^{19P}-COOH -K-5(6)-Carboxyfluorescein

(D) 5(6)-Carboxyfluorescein-Ahx- NH_2- R^{10P}IFLKRMPSI^{19P}-COOH

Figure 1. Primary structures of the native decoy peptide (A), FITC-decoy with 5(6)-Carboxyfluorescein tagged at the NH_2-terminal end (B), FITC-decoy with 5(6)-Carboxyfluorescein tagged at the CO_2-terminal end through a Lys (K) residue (C) and FITC-Ahx-decoy, 5(6)-Carboxyfluorescein was tagged with decoy by the help of a spacer denoted as Ahx (6-aminohexnoyl) (D). These peptides were tested in BIAcore assay system for their binding properties to the immobilized recombinant (pro)renin receptor.

organelles e.g., endoplasmic reticulum (16), golgi apparatus (17), in the cytosol (17,18) and plasma (18). The hydrophobic part of this receptor (after furin cleavage), similar to the ATPase associated protein 2, binds vacuolar H^+-ATPase (18-20). Further, expression of (P)RR and activation of local renin-angiotensin system through renin binding in the brain have been confirmed (17,21). Patients having mutation in (P)RR gene develop epilepsy (22). Besides, association between (pro)renin receptor gene polymorphisms and blood pressure levels has also been observed (23). Taking these facts into consideration, studies for explaining the pathophysiological roles of (P)RR have been carried out clinically and biochemically so far and thereby, its close association with diabetes and hypertension have been indicated (1,4,24-34). However, detail functional aspects of this receptor are still undetermined. The decoy peptide could be a better choice for elucidating the functional characteristics of (pro)renin receptor and prorenin. Thus, in this review, the functional features of decoy peptide have been discussed by means of recent *in vitro* and *in vivo* studies.

3. THE DECOY PEPTIDE AND *IN VITRO* STUDIES

Suzuki *et al.* demonstrated that both "gate" ($T^{7P}FKR^{10P}$) and "handle" region peptides are important for the non-proteolytic activation of prorenin through protein-protein interaction (5). Depending on this groundwork, it was assumed that "handle" region sequence could also play pivotal role in the interaction of prorenin to the (pro)renin receptor. This was then confirmed by many *in vitro* studies using recombinant (P)RR coupled to CM5 sensor chips (2,3), immobilized on synthetic surfaces (3) as well as COS-7 cells with over expressed (P)RR (10). We showed that both human and rat decoy peptides inhibited the bindings of human and rat prorenins to their respective (P)RR expressed on the membranes of COS-7 cells with a similar K_i of 6.6 nM (10). This peptide inhibited the bindings of not only prorenin but also renin to the pre-adsorbed receptors with the K_i values of 15.1 and 16.7 nM, respectively (3). Moreover, recent real-time bindings using surface plasmon resonance (SPR) technique in BIAcore assay system revealed evidence for the direct binding of native decoy peptide (Figures 1A and 2) to the immobilized (P)RR with K_D of 3.5 nM (2,3). The SPR technique displayed reduced resonance signal of prorenin binding when decoy peptide was co-injected (at 10 µl/min) with prorenin preparation for binding to the immobilized receptors. The higher binding affinity of prorenin for the (P)RR compared to that of renin was also attributed to the existence of decoy peptide region within prorenin prosegment (2,3). Batenburg *et al.* also found that (pro)renin receptors expressed by the vascular smooth muscle cells (VMSCs) could bind prorenin (but not renin) with a K_D of ~6.0 nM but neither rat nor human HRP was able to inhibit binding and activation of prorenin at a concentration of 1 µM (8). Other reports claimed that human HRP at 1 µM did not inhibit binding of I^{125}-renin/prorenin to (P)RR or renin- and prorenin-induced ERK 1/2 phosphorylation in human U937 monocytes (15,25,26). Together these experimental results demonstrate that so far all the *in vitro* experiments have been carried out in different cell lines using different methods.

As the (pro)renin receptor is mostly distributed intracellularly and it is thought that very few of them are available on the membrane (8,14) thus, it could be hypothesized that the distribution of this receptor on the membrane might vary from cell to cell which would certainly affect the results about efficacy of HRP. Using COS-7 cells over expressing (P)RR (10), we reported highest amount of prorenin binding at around 18 hours (Figure 3) while Batenburg *et al.* with (P)RR on VSMCs showed that plateu of prorenin binding to the membrane-anchored receptors reached within two-four hours (8). On the basis of the recycling of membrane receptors (8,14), the contrast between the two experimental data could be due to the fact that receptors might take longer time to appear on the surface in case of COS-7 cells than those of VSMCs or there might be other associated proteins which could have stabilized the (P)RR on the membrane. Moreover, soluble form of shedded (P)RR (28 kDa) in human plasma and cell culture medium has recently been reported (18). If shedding of (P)RR is a routine work of the cell, then use of HRP in the studies with cell lines (either the receptor being naturally expressed or over-expressed using transfection method) would produce uncertainty. Therefore, to close the ambiguity about the HRP related data, we should address and investigate (i) transportation mechanism of the (P)RR from cell to membrane (ii) half life of the receptor on the cell membrane (iii) amount/percentage of receptors on the cell membrane.

4. THE DECOY PEPTIDE AND *IN VIVO* STUDIES

Various transgenic models were used to testify the effectiveness of decoy (including "handle" region peptide) hypothesis *in vivo*. Ichihara *et al.* first demonstrated that administration of HRP significantly inhibited the increase in renal angiotensin II levels, the development of proteinuria and glomerulosclerosis in a model of diabetic nephropathy (1). Additionally, rat HRP completely prevented the development of diabetic

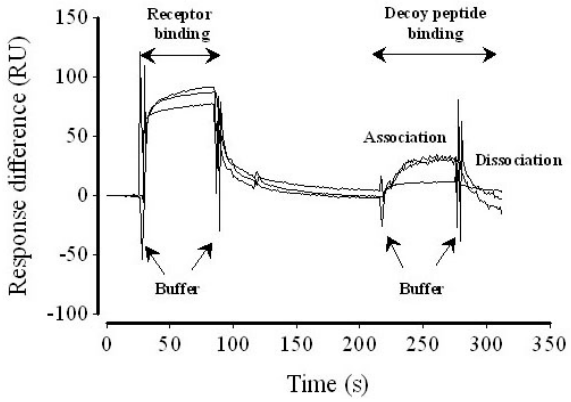

Figure 2. A typical sensogram in BIAcore assay system showing bindings of different concentrations (1000, 800, 50 nM) of native decoy peptide (RIFLKRMPSI) to the recombinant (pro)renin receptor. The receptor was immobilized with the help of anti-His tag antibody, which was coupled to the carboxymethyl (CM5) sensor chip through amine coupling as described previously (2,3). Different concentrations of the decoy were then flown at a rate of 10 µl/min and bindings response of these peptides to the antibody associated-(P)RR were indicated by the change of resonance units in sensograms. The sensograms were corrected after subtracting the specific bindings from the non-specific bindings. The surface was regenerated by injecting a mixture of glycine (10 mM) and NaCl (150 mM) at pH 2.0 to avoid repeated coupling of anti-His tag antibody.

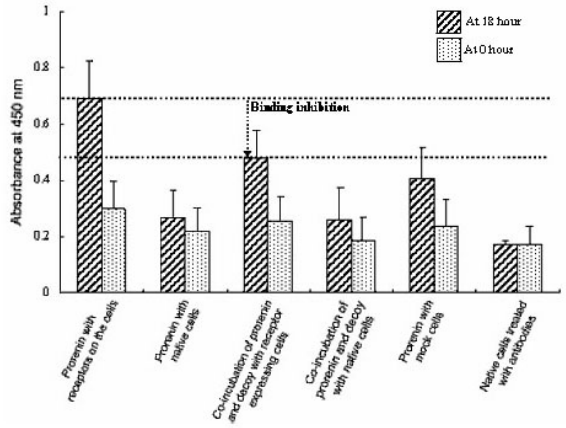

Figure 3. Binding of human prorenin to the (pro)renin receptor on the membrane of COS-7 cells. Human (pro)renin receptor was over expressed on the membrane of the COS-7 cells according to our previous protocol. Human prorenin preparation at a concentration of 2.0 nM was incubated with the COS-7 cells at 37°C for 0 and 18 h. Receptor-bound prorenin was detected using anti-prosegment antibody together with the horse radish peroxidase conjugated anti-rabbit IgG antibody (10). Intensity of the color was measured at 450 nm. Binding response reduced when prorenin (2 nM) and decoy peptide (200 nM) were co-incubated with the receptor-expressing COS-7 cells. Both native cells and cells transfected with the vector lacking plasmid containing receptor cDNA (mock cells) were used as the controls. Mannose-6-phosphate (100 mM) was used to block any possibility of cross binding of prorenin with the mannose-6-phosphate receptors. Each experiment represents mean ± SD (n = 5).

nephropathy in heminephrectomized streptozotocin-induced diabetic rats without affecting hyperglycemia (27). Urinary albumin excretion and the renal production of tumor necrosis factor-α and interleukine-1β were decreased significantly when rat HRP was administered directly into the renal cortical interstitium of diabetic rats (28). Prevention of the development of proteinuria, glomerulosclerosis, and complete inhibition of the increase of ERK1/2, p38 as well as JnK activation in the kidney of diabetic angiotensin-II type-1a receptor-deficient mice was reported and thus, the role of (P)RR via angiotensin II-independent pathway in association with prorenin was suggested (29). Other investigators also confirmed the action of prorenin and (P)RR via angiotensin-II independent pathway (12,15,25,26). Moreover, HRP inhibits the development of retinal neovascularization by inhibiting prorenin activation caused by interaction with (P)RR in experimental retinopathy model of prematurity (30). Satofuka *et al.* using the same model, showed that the HRP suppressed the pathological angiogenesis, leukocyte adhesion and retinal expression of ICAM-1 and VEGF; also, reduced retinal gene and protein expression of inflammatory mediators (31,32).

HRP administration in young spontaneously hypertensive rats (SHR) under high salt-diet, though not completely but, significantly attenuated glomerulosclerosis with proteinuria, cardiac hypertrophy with left ventricular fibrosis without affecting the development of hypertension (24,34). In addition, Susic *et al.* made a further interesting observation by reporting reduced beneficial effects of decoy (PRAM-1) in SHR rat with normal diet (34). On the contrary, Muller *et al.* revealed that chronic HRP treatment did not improve target organ damage in renovascular Goldblatt hypertensive rats with high renin, prorenin and PRA that lead to Ang II-dependent target organ damage (25).

Data of the *in vivo* studies mentioned so far are also representative of different animals with various quantitative ratios of the renin-angiotensin system components. For example, diabetic humans, rats or mice will always have high prorenin with low renin and PRA while, renovascular hypertensive rats contain high renin and prorenin (25). Thus, it could be assumed that being a part of prorenin prosegment, decoy peptide would exert its potential actions in a circumstance like high prorenin to renin ratio. In case of high renin and prorenin, though the decoy inhibits their bindings to (P)RR (alternatively inhibits non-proteolytic activation by interfering protein-protein interaction), it is the free form of active renin that may possibly be responsible for the adverse effects even after HRP treatment. Also, our recent *in vitro* study revealed that the decoy only inhibited protein-protein interaction but not the enzymatic activity (3). Consequently, this peptide only exerts its effects on non-proteolytic activation through interfering receptor-ligand interaction. Further, reduction of cardiac hypertrophy and fibrosis by lowering the levels of cardiac Ang-II after treating the SHR rats with HRP made things even more complex. Because cardiac Ang-II generation to a large extent depends on renal renin which is taken up by the

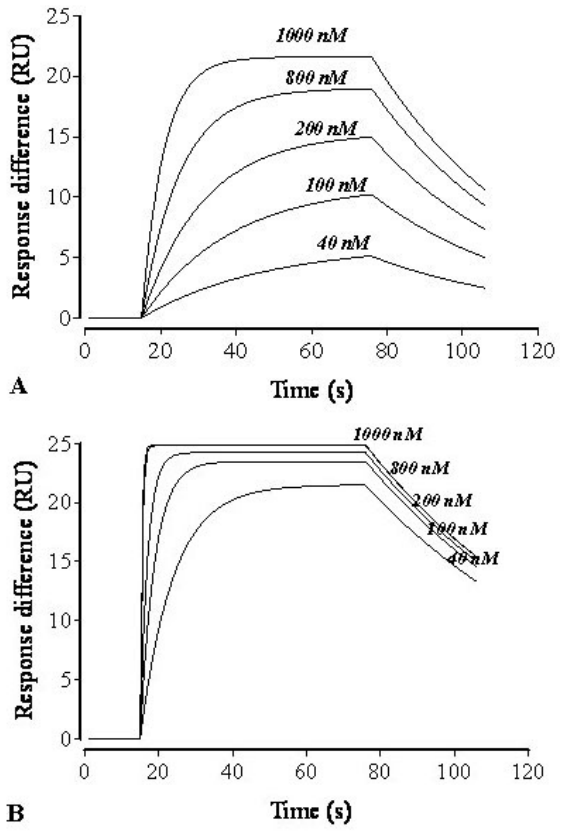

Figure 4. Real-time bindings of FITC-tagged decoy peptides (A) 5(6)-Carboxyfluorescein-NH_2-RIFLKRMPSI-COOH and (B) NH_2-RIFLKRMPSI-COOH-K-5(6)-Carboxyfluorescein to the immobilized (pro)renin receptor. The chemically modified peptides showed distinct binding patterns with different kinetics. All data were analyzed by global fitting to a 1:1 Langmuir binding model of both the association and dissociation phases for several concentrations simultaneously, using the BIAevaluation software. Both Figures (A) and (B) represent the bindings of decoy peptides to (P)RR constructed using association (k_a) and dissociation (k_d) rates by BIAsimulation software evaluated from their typical binding sensograms after background subtraction. The dissociation constants (1.26 x 10^{-7} M and 4.74 x 10^{-6} M, respectively) were determined as the ration of k_d/k_a.

heart (25) so, why the decoy, being a part of prosegment, would lower Ang-II by inhibiting renin action? For the sake of argument, if we consider that the decoy should not show its potency in the state like high renin, then we would miss to point out the accumulative action of renin/prorenin associated with (P)RR in cardiac cells (6,8). These cells express (P)RR that could act as capturing molecule for renin/prorenin. As a result, cumulative amount of active renin/prorenin would help to elevate the levels of Ang-II in cardiac cells because enzymatic activity of renin increases several folds after receptor binding (6). In this situation, only when the binding of renin/prorenin is inhibited by the decoy, the levels of active enzyme would go down which was ultimately reflected by the study of Ichihara *et al.* with lower levels of Ang-II in renovascular hypertensive rats (24). Besides these plausible events, the chance of degradation of the peptide in the circulation and/or its transportation from tissue to tissue could not be ignored. Thus, these issues could be explained after determining bioavailability of the decoy and its level in the plasma *in vivo*.

5. *IN VITRO* AND *IN VIVO* USE OF FLUORESCENT TAGGED DECOY PEPTIDE

As fluorescent (FITC)–HRP bound to not only U937 monocytes (15) but also embryonic stem cells that lack the transmembrane domain of (P)RR so, it was concluded that HRP does not specifically bind (P)RR rather it binds other binding proteins in the cell. To find out the binding mode of either native or FITC conjugated decoys *in vivo*, tissue specific (P)RR knock-out models could be very useful. Having mentioned the possibility of other decoy binding proteins in cells, we should also consider its bindings to other known proteins e.g., mannose-6-phosphate receptors and/or renin binding proteins (RBPs). But, till-to-date, there is no report to evaluate this fact. However, the K_D for the bindings of prorenin and the peptide to (P)RR was determined in the nanomolar order with a very marginal difference (1.2 and 3.5 nM, respectively). Therefore, if the decoy is suggested to bind to other binding proteins, prorenin should also bind to the same protein as this peptide mimics the N-terminus of prorenin prosegment. Furthermore, our recent findings (2,3) showed direct binding of native decoy peptide to the recombinant (pro)renin receptor using SPR technique (Figure 2). Ichihara and his group (34,35) also detected the existence of fluorescent labeled HRP in the renal glomeruli and tubular lumen even after 28 days of administration. Nevertheless, confusion persists whether the signals of fluorescent compounds are due to the intact HRP. These discrepancies led us to investigate *in vitro* binding properties of FITC-tagged decoy peptides (primary structures shown in Figures 1B, C and D) to the immobilized (pro)renin receptors. The recombinant (pro)renin receptor with six histidine residues at the C-terminal end instead of transmembrane domain was expressed in a cell free *in vitro* system based on wheat germ lysate, purified using anti-His trap column and confirmed by Western blot analysis (3). An anti-His tag antibody was used for immobilizing the (P)RR. Detail explanation of the methods for the preparation of sensor chip with coupled antibody, and reason for using antibody for immobilizing receptors have been discussed in our previous studies (2,3). Briefly, the sensor chip, which is consisted of dextran matrix, contains carboxymethyl groups. This group was activated using N-ethyl-N1-dimethyl-aminopropyl carbodiimide (EDC) and N-hydroxysuccinimide (NHS) and was allowed to couple with amino groups of proteins of interest. The (pro)renin receptor at a concentration of ~15 nM was injected (10 µl/min) and allowed to associate with the antibody. The flow cells, which were activated with EDC/NHS, treated with only buffers (HBS-EP) and finally, blocked by ethanol amine, were used as control cells. Any binding response observed in these cells were considered as non-specific

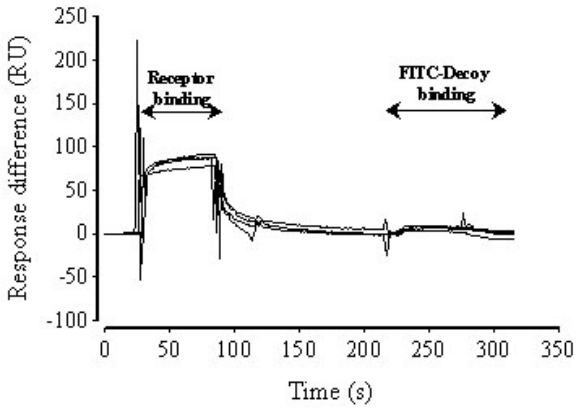

Figure 5. BIAcore analysis for the bindings of different concentrations of 5(6)-Carboxyfluorescein-Ahx-NH_2-RIFLKRMPSI-COOH peptide to the recombinant (pro)renin receptor. The binding experiment was performed as described previously (2,3). Different concentrations (1000, 800, 400 and 50 nM) of the peptide were flown at a rate of 10 µl/min and bindings response of these peptides to the antibody associated-(P)RR were indicated by the change of resonance units in sensograms. The sensogram shows the relative response in RU after background subtraction versus time in seconds. We could not determine the dissociation constant (K_D) for the binding of this FITC-tagged peptide to the (P)RR as the association and dissociation phases for the binding data did not fit with the Langmuir 1:1 kinetic binding model at least under the present assay condition.

binding and finally, subtracted from the specific binding responses of proteins. Our data illustrated that different decoy peptides had different patterns of bindings to the immobilized (P)RR (Figures 2, 4A, B and 5). Native decoy (Figures 1A and 2) peptide demonstrated very high binding affinity (3.5 x 10^{-9} M) to the (P)RR (2,3) rather than chemically modified FITC-tagged decoy. Even among the FITC-tagged peptides, the sequence RIFLKRMPSI with 5(6)-Carboxyfluorescein at its N-terminus (Figure 1B) showed higher binding affinity (Figure 4A, k_a: 1.88 x 10^5 $M^{-1}s^{-1}$, k_d: 0.0237 s^{-1} and K_D: 1.26 x 10^{-7} M) compared to that of the decoy tagged with 5(6)-Carboxyfluorescein at its C-terminal end (Figure 1C) by means of a lysine residue (Figure 4B, k_a: 5.78 x 10^4 $M^{-1}s^{-1}$, k_d: 0. 274 s^{-1} and K_D: 4.74 x 10^{-6} M). On the other hand, under the present assay condition, we could not determine the dissociation constant (K_D) for the binding of RIFLKRMPSI sequence attached to a FITC compound at the N-terminus with the help of a spacer (Figure 1D) as the association and dissociation phases for the binding data did not fit with the Langmuir 1:1 kinetic binding model (Figure 5). These data indicated that the reciprocal of dissociation constants of the FITC conjugated decoy peptides are several order of magnitude higher than that of the native decoy. It could be due to the change in configuration of the peptides attained after the conjugation. If so, then we have to investigate the function of decoy peptide (any form) in great detail.

6. CONCLUSION

These novel studies help to advance our understanding of the structure-function relationship between prorenin and the (P)RR. Considering the values of dissociation constants of prorenin and the decoy (1.2 and 3.5 nM, respectively) for their bindings to (P)RR it could be concluded that RIFLKRMPSI region of prosegment plays a dominant role for the interaction of prorenin with (P)RR. This peptide sequence could recognize and would help to illustrate the counter binding region(s) within the receptor molecule. Determination of the three-dimensional structure of (P)RR is the necessity of time to evaluate the binding mechanism of either native or FITC conjugated chemically modified decoy peptides to the receptor and thus, to explain means of complex formation of renin/prorenin with (P)RR.

7. 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)

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Key words: Decoy peptide, (Pro)renin receptor, Prorenin, Prorenin prosegment, Prorenin-(P)RR interaction, Non-proteolytic activation, Handle region peptide

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