

## Role of "handle" region of prorenin prosegment in the non-proteolytic activation of prorenin by binding to membrane anchored (pro)renin receptor

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## 1. ABSTRACT

A role of the "handle" region in the prorenin prosegment sequence was investigated to demonstrate the crucial non-proteolytic activation of prorenin by binding to the recombinant (pro)renin receptor on the COS-7 cell membrane. The plasmid DNA containing either rat or human (pro)renin receptor was transfected into the COS-7 cells. The highest amount of receptor was observed on the COS-7 cell membrane after 18h transfection. Of the total rat and human prorenin, 90% and 50% were bound to each of the respective receptors, respectively. The  $K_d$  values were 0.89 and 1.8 nM, respectively. Rat prorenin was activated non-proteolytically by the receptor. The  $K_m$  was determined 1.0  $\mu$ M when sheep angiotensinogen was used as the substrate. Human prorenin was also activated by the receptor. The  $K_m$  was 0.71  $\mu$ M. Additionally, decapeptides (10P-19P) known as "decoy" peptide and pentapeptides (11P-15P) named "handle" region peptide, were observed to inhibit the binding of both prorenins to receptors, respectively. The  $K_i$  were similar around 7 nM for both the peptides. Other two region peptides in the prosegment did not interfere the binding. These results show that the "handle" region probably plays a crucial role in prorenin binding to the receptor and in its enzymic activity by non-proteolytic activation.

## 2. INTRODUCTION

Prorenin is the inactive precursor of the protease renin that plays the critical role in initiating a cascade of reactions leading to the formation of angiotensin II (Ang II) from angiotensinogen via angiotensin I (Ang I), which regulates the blood pressure, electrolyte balance, cardiovascular hypertrophy and many other physiologically essential functions (1). This inactive proenzyme, synthesized in the juxtaglomerular cells of kidney and other renin producing tissues, has a 43 amino acid residue extension called prosegment at the N-terminus of mature active renin. This prosegment, which is folded in the active site cleft of renin, prevents its interaction with angiotensinogen (2-5). Activation of prorenin requires either proteolytic or non-proteolytic removal of this prosegment from the active site cleft. The proteolytic activation involves hydrolysis of a specific scissile peptide bond e.g., Arg<sup>43</sup> - Leu<sup>44</sup> of human prorenin, by any of the known renin-prorenin processing enzyme e.g., cathepsin (6), kallikrein (7), and prohormone convertases (8), thereby, irreversibly removing the prosegment. The proteolytic activation was considered to be a physiological activation mechanism. The non-proteolytic activation such as treatment under acidic pH or low temperature (cryo-activation) reversibly altered the stereo structure of intact prorenin molecule into a catalytically active form (4, 9-11).

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It has been considered that the proteolytic activation of prorenin was a natural process, whereas the non-proteolytic activation was as artifacts of low temperature, acidification or antibody binding *in vitro*. Prorenin was actually measurable in the blood circulation and amniotic fluid. Of note are reports that plasma prorenin is markedly increased in diabetes (12). However, whether it is activated by an extracellular/intracellular substance such as protease had not been known. Possible pathophysiological roles of prorenin and its activation were not clear. In 2002, Nguyen *et al.* found a (pro)renin receptor, which binds prorenin, and renin (13). In 2003, we showed that antibodies, bound to the region 11P-15P (NH<sub>2</sub>-IFLKR-COOH) in the prorenin prosegment, *in vitro* activated human prorenin (14). The pentapeptide region should be exposed in the prorenin and may serve as binding handle to the antibodies to loose the prosegment part. Thus, we termed it the "handle" region. We postulated that the receptor may bind the "handle" region in the prorenin prosegment to activate circulating prorenin. This hypothesis might provide a persuasive pathological role of locally activated prorenin in end-organ damages of certain specific organs as shown previously (15-20).

In this study, we showed a novel paradigm of non-proteolytic activation by binding to the (pro)renin receptor anchored in the membrane. We have also investigated the role of the "handle" region peptide from the prosegment sequences of both the rat and human prorenin in the receptor-ligand interaction mechanism□

### 3. MATERIALS AND METHODS

#### 3.1. Culture of COS-7 cells

An aliquot of COS-7 cells was thawed and washed with the medium. The cells were cultured to confluence in 75-cm<sup>2</sup> tissue culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 5% undialyzed fetal bovine serum and thymidine/pyrimidine, 0.1 mM non-essential amino acids, 2 mM glutamine, 100U penicillin and 100 µg streptomycin per ml at 37°C under humidified atmosphere of 5% CO<sub>2</sub> and 95% air. The cells were trypsinized and seeded in 12 well non-coated plates (1.2 x 10<sup>6</sup> cells/ml) containing 2.0 ml medium. Experiments were carried out for about 1 day after 85-90% confluence had been reached. Before the start of each experiment, cells were washed with 3.0 ml of warmed PBS with 140 mM NaCl, 2.6 mM KCl, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, and 8.1 mM Na<sub>2</sub>HPO<sub>4</sub> at pH 7.4.

#### 3.2. Preparation of rat and human (pro)renin receptor cDNA and expression on the surface of the COS-7 cells

The rat and human (pro)renin receptor genes were cloned (AB188298 and AF291814 in DDJB, respectively), fused with FLAG cDNA and subcloned into pcDNA3. The plasmids containing receptor cDNA were then amplified in *E. coli* and purified by using the mini-prep plasmid isolation kit according to the instruction of the manufacturer (BioRad, USA). Isolation of intact plasmid DNA was confirmed by gel-electrophoresis.

COS-7 cells were transiently transfected with the expression vector containing the cDNA of the (pro)renin receptor with FuGENE™ (Boeringer Mannheim, Germany). For assay, 3.0 µg of plasmid DNA was mixed with 6.0 µl of FuGENE™ and incubated for 20 minutes at room temperature and then, mixed with 1.0 ml of cell culture medium. Cells were transfected with 500 µl of the prepared plasmid DNA and allowed to express the receptors on their surface at 37°C in a CO<sub>2</sub> incubator. Different concentrations of plasmid DNA (0.5, 1.0, 2.0, 2.5, 4.0 µg/ml) tested to determine optimal transfection conditions. COS-7 cells, transfected only with the expression vector lacking the cDNA, were used as control cells. After the transfection, the cells were incubated for 0, 6, 12, 18 and 24 h at 37°C in the CO<sub>2</sub> incubator.

#### 3.3. Preparation of rat and human prorenin

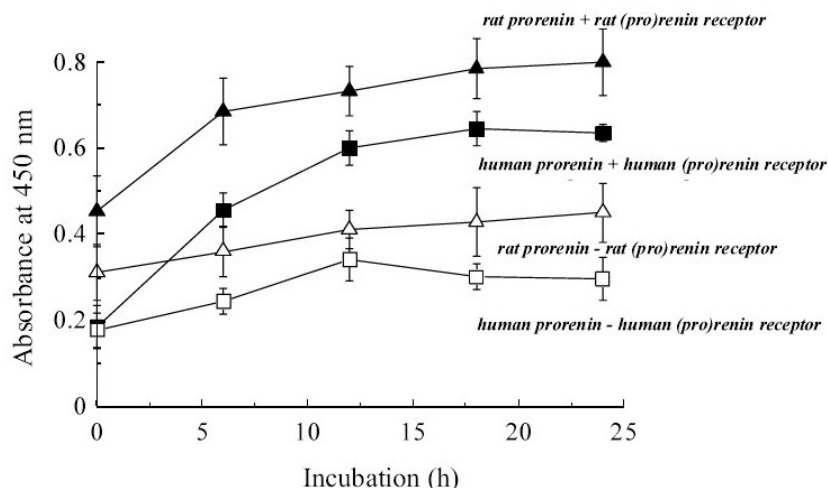
Chinese hamster ovary cell lines harboring rat and human prorenin cDNA was maintained (21,22) under humidified atmosphere of 5% CO<sub>2</sub> and 95% air in 25-cm<sup>2</sup> cell culture flasks (CELLSTAR, Greiner, Bio-One Germany) till achieving 100% confluent monolayer in the DMEM containing 0.1 mM non-essential amino acids, 2 mM glutamine, 100U penicillin, 100 µg streptomycin per ml, 200 nM methotrexate supplemented with 5% dialyzed FBS. The media containing wild type prorenin preparations secreted from the cells were collected and then stored at -80°C until further study.

#### 3.4. Determination of the binding of rat and human prorenin to the receptor expressing COS-7 cells

The stored rat and human prorenin preparations were incubated at 37°C for 1 h to avoid possible cryo-activation. By this treatment, variation in the inactive prorenin level in each experiment was minimized to less than 2% of its total potential renin activity attainable by trypsin treatment. The rat prorenin and human prorenin preparations at 2.0 nM were incubated in 1.0 ml medium containing the receptor-expressing COS-7 cells for 0, 6, 12, 18 and 24 h. Mannose-6-phosphate (100 mM) was used to minimize cross binding to mannose-6-phosphate receptors. The corresponding decoy peptides as well as the synthetic pentapeptides, termed as the "handle" region of the prosegment sequences from both the prorenin molecules were incubated at a concentration of 4.0 nM with 2.0 nM rat and human prorenin in wells containing their analogous (pro)renin receptor-expressing COS-7 cells, respectively for 0, 6, 12, 18 and 24 h at 37°C to determine the effect of the peptides on the binding of prorenin to their specific receptors. Specific binding of prorenin to the cells was estimated by subtracting prorenin concentration remaining in the medium after incubation from the initial prorenin, with the receptor-expressing COS-7 cells. Percentage of the binding of rat prorenin to the receptor was determined by using the following equation number 1:

$$[\text{Pren}]_{\text{b}}\% = \frac{[\text{Pren}]_{\text{i}} - [\text{Pren}]_{\text{r}}}{[\text{Pren}]_{\text{i}}} \times 100 \quad \text{----- (1)}$$

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**Figure 1.** Binding of rat and human prorenin to the receptor on the COS-7 cell membranes. After incubating recombinant rat and human prorenin (2.0 nM) in 1.0 ml medium with the (pro)renin receptors expressing COS-7 cells at 37°C for different time intervals (0, 6, 12, 18 and 24 h), their bindings were observed using anti-prosegment antibody [for rat (-△-) and for human (-■-) prorenin] through the activity of horse radish peroxidase conjugated with anti-rabbit IgG measured at 450 nm. Control cells (-△-) and (-□-) were tested only by transfection with the expression vector lacking the receptor cDNA. Each data represents mean  $\pm$  S.D. (n = 6).

where [Pren]<sub>b</sub> % is the percentage of prorenin bound to the receptor of COS-7 cells, [Pren]<sub>i</sub> represents the inactive rat or human prorenin and [Pren]<sub>r</sub> denotes the remaining unbound prorenin in culture medium. Renin activity of receptor-bound activated prorenin was measured by angiotensin I ELISA (23) after incubation of prorenin with receptor expressing COS-7 cells with a recombinant sheep angiotensinogen preparation (24) under standard assay conditions as described previously (25).

### 3.5. Determination of $K_d$ for prorenin-receptor binding and $K_m$ of the receptor-bound prorenin

The  $K_d$  value for the binding of prorenin to the receptor on COS-7 cells was calculated by determining the concentration-dependent binding to the receptor. Rat prorenin concentrations used were 0.5, 0.8, 1.0, 2.5, 5.0 nM and human prorenin concentrations used were 0.8, 1.0, 2.8, 5.0, 10.0 nM. Varying concentrations of rat prorenin (0.5, 0.8, 1.0, 2.5 and 5.0 nM) as well as human prorenin (0.8, 1.0, 2.8, 5.0, and 10.0 nM) with the fixed concentrations (4.0 nM) of the corresponding “handle” region pentapeptides were used to determine the inhibition of the prorenin-receptor binding. The inhibition of the prorenin-receptor binding was calculated by using the following equation number 2:

$$K_{dapp} = K_d \times (1 + [i]/K_i) \text{ ----- (2)}$$

Where  $K_{dapp}$  = apparent receptor-ligand affinity;  $K_d$  = prorenin-receptor binding affinity;  $[i]$  = concentration of inhibitor (the “handle” region pentapeptide);  $K_i$  = binding affinity of inhibitor to the receptor.

The  $K_m$  value of the enzyme activity of the receptor-activated prorenin was calculated from the rate of Ang I production at sheep angiotensinogen concentrations of 0.30, 0.35, 0.5, 0.8, 1.0, 1.2 and 1.5  $\mu$ M.

## 4. RESULTS

### 4.1. Time dependency of expression of the (pro)renin receptor on the COS-7 cell membrane

The optimum concentration of plasmid DNA harboring the receptor cDNAs was 0.5  $\mu$ g/ml for the optimum expression of both the rat and human (pro)renin receptor on the surface of the COS-7 cells under the standard assay conditions. The receptor could be expressed on the surface of the cells in a time-dependent manner and the highest level of the receptor was expressed in 18 h. These conditions were used as the standard conditions in this study.

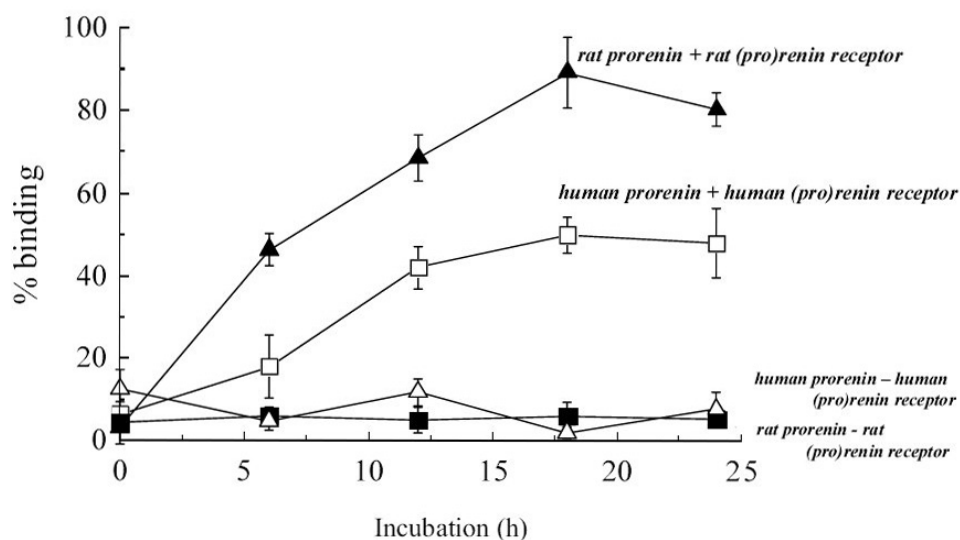
### 4.2. Binding assay of prorenin to the receptor

That prorenin is bound to the receptors on the cell surfaces was confirmed indirectly by observing the positive signal using the anti-prosegment antibody (Figure 1). The highest binding percentage, 90%, was observed after 18h incubation of prorenin at 2.0 nM with the receptor-expressing COS-7 cells under the standard binding conditions (Figure 2). Under the same condition, the highest binding percent, 50%, of human prorenin at 2.0 nM to its corresponding receptor on the cell membrane was observed (Figure 2). The  $K_d$  values for the rat and human prorenin binding to the respective (pro)renin receptors were estimated at 0.89 and 1.8 nM, respectively (Figure 3A, B).

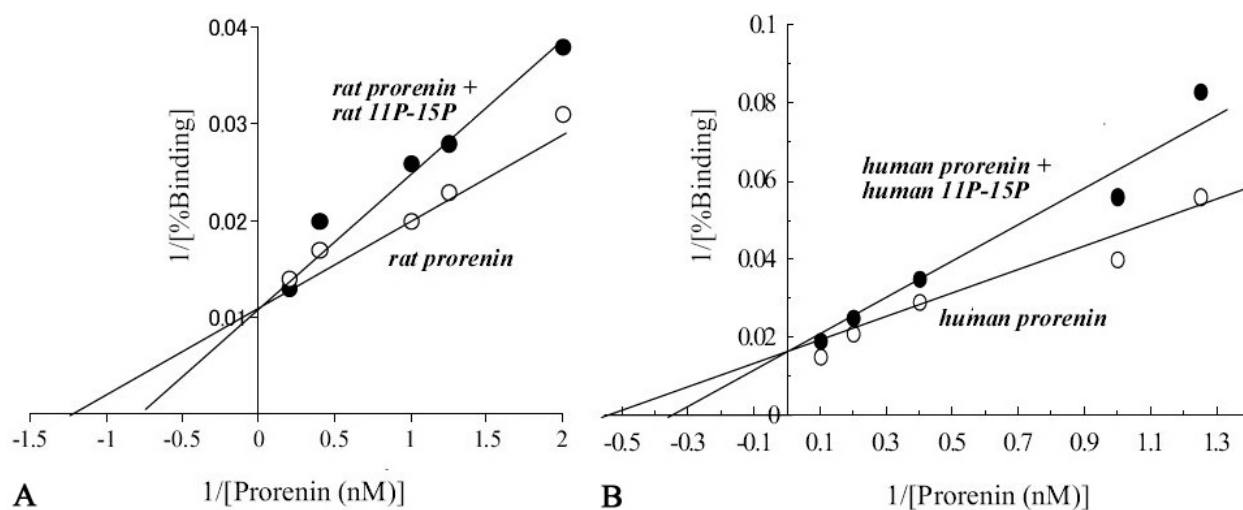
### 4.3. Inhibition of the prorenin binding by the “handle” region peptide 11P-15P

By co-incubation with synthetic pentapeptide, 11P-15P (NH<sub>2</sub>-ILLKK-COOH) at 4 nM, with rat prorenin (2.0 nM), its receptor binding was inhibited by almost 40% of the total binding (Figure 4A). When human prorenin at 2.0 nM was incubated with the corresponding “handle” region pentapeptide (NH<sub>2</sub>-IFLKR-COOH), its receptor binding was inhibited by almost 30% of the total binding

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**Figure 2.** Time dependency for binding of rat and human prorenin to the receptors expressed on COS-7 cells. Recombinant rat and human prorenin (2.0 nM) were incubated at 37°C for 0, 6, 12, 18 and 24 h. The amount of the total prorenin and unbound prorenin were determined as the renin activity after trypsinization of the prorenin media during the incubating. The binding percentages [rat prorenin (-▲-) and human prorenin (-□-)] were estimated by subtracting the percentages of receptor bound prorenin from the non-specific binding. For control, rat (-△-) and human (-■-), prorenin preparations at 2.0 nM were incubated with the cells transfected only with the expression vector lacking the receptor cDNA. Each data represents mean ± S.D. (n = 6).

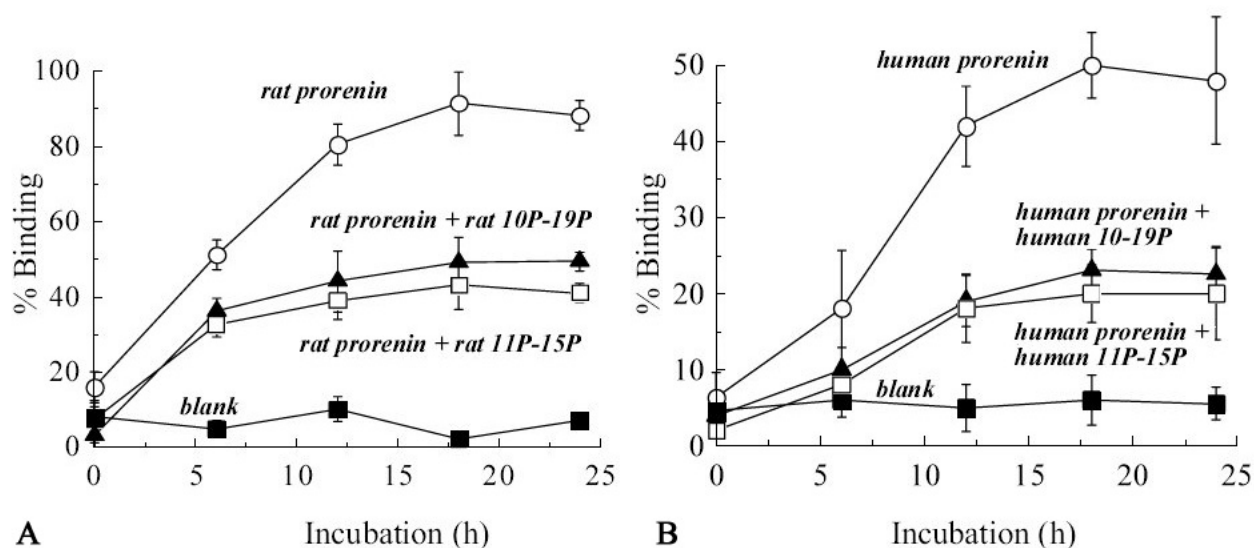


**Figure 3.** Determination of  $K_d$  and  $K_i$  of receptors and synthetic peptide in rat (Figure A) and human (Figure B). In Figure A for the determination of the  $K_d$  of rat prorenin (-○-) and  $K_i$  of the synthetic pentapeptide (-●-), 11P-15P (NH<sub>2</sub>-ILLKK-COOH), for the binding of prorenin to the rat (pro)renin receptor. Figure B represents the reciprocal plots for the determination of the  $K_d$  of human prorenin (-○-) and  $K_i$  of the synthetic pentapeptide, 11P-15P (NH<sub>2</sub>-IFLKR-COOH), for the binding of prorenin (-●-) to the human (pro)renin receptor. They showed that the  $K_d$  values of rat and human prorenin were 0.89 and 1.8 nM, respectively. The  $K_i$  for both the pentapeptides were estimated at 6.6 nM. Each data represents mean ± S.D. (n = 4). The values were determined by using the following equation:  $K_{dapp} = K_d \times (1 + [i]/K_i)$ , where  $K_{dapp}$  for rat and human prorenin were estimated to be 1.43 and 2.86 nM, respectively.

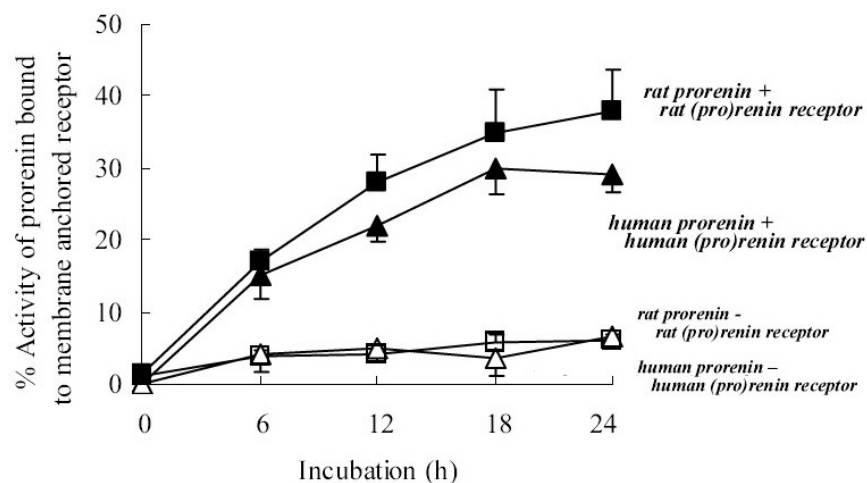
(Figure 4B). Both the peptides inhibited the binding of the corresponding prorenin molecules as efficiently as their respective 10P-19P, decoy peptides did. It was observed

that the  $K_i$  value for both the “handle” region pentapeptides, 11P-15P, was estimated to be 6.6 nM (Figure 3A, B). By contrast, other synthetic peptides, 7P-

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**Figure 4.** Interferences of the binding of rat and human prorenin to their corresponding receptors on COS-7 cells with the respective synthetic pentapeptides. In Figure A, the 4.0 nM of the synthetic peptide 11P-15P (-□-) inhibited 2 nM of rat prorenin binding to the receptor by almost 40% of the total binding (-○-), whereas the 4.0 nM of the synthetic peptide 11P-15P (-□-) inhibited 2 nM of human prorenin binding to the receptor by almost 30% of the total binding (-○-) as shown in Figure B. In both the cases, the synthetic pentapeptides inhibited binding of their corresponding prorenin molecules as efficiently as their respective decoy peptides, 10P-19P (-▲-). For controls, rat and human prorenin preparations (2.0 nM) were incubated with the cells transfected only with the expression vector lacking the receptor cDNA (-■-). Each data represents mean  $\pm$  S.D. (n = 4).



**Figure 5.** The activation of rat and human prorenin by binding with their corresponding (pro)renin receptor on COS-7 cells. Recombinant rat (-▲-) and human (-■-) prorenin preparations were incubated with the receptor-expressing COS-7 cells for 0, 6, 12, 18 and 24 h at 37°C. For controls, recombinant rat (-△-) and human (-□-) prorenin preparations were also incubated with receptor-lacking COS-7 cells. The renin activity of the receptor bound prorenin was determined under the standard assay conditions. The activities of mature renin obtained by trypsinization of the 2 nM of rat and human prorenin molecules were 350 and 450 ng Ang-I/ml/h, respectively. These activities were represented as 100%. Each data represents mean  $\pm$  S.D. (n = 6).

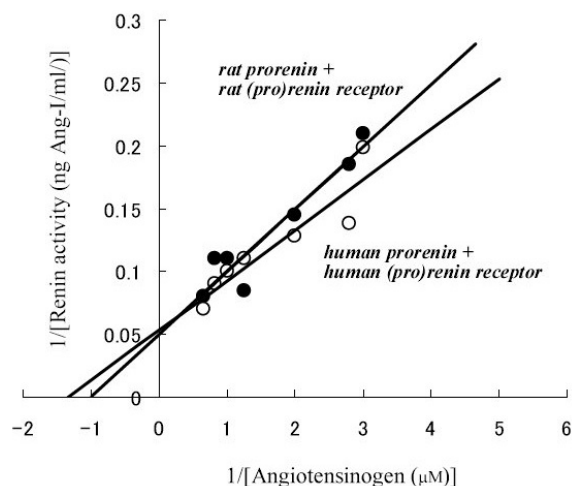
10P and 30P-36P of the prosegment sequence did not inhibit the binding of prorenin to the receptor on the cell membrane.

### 4.4. The renin activity of receptor-bound rat and human prorenin

After incubation of the corresponding receptor expressing COS-7 cells with 2.0 nM rat and human

prorenin for various time intervals (36, 24, 18, 12, 6 and 0 h), the highest activation was observed at 18h and at 24 h, respectively as shown in Figure 5. The receptor bound rat prorenin had 30% of the renin activity which can be obtained by trypsin treatment (350 ng AngI/ml/h). The receptor bound human prorenin had almost 40% of the renin activity which can be obtained by trypsin treatment (450 ng AngI/ml/h).

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**Figure 6.** The determination of the  $K_m$  of the receptor-bound recombinant rat and human prorenin. The  $K_m$  values were estimated at 1.0  $\mu\text{M}$  for receptor-bound rat prorenin ( $\bullet$ ) and 0.71  $\mu\text{M}$  for receptor-bound human prorenin ( $\circ$ ) against the substrate sheep angiotensinogen. Each data represents mean  $\pm$  S.D. ( $n = 4$ ).

### 4.5. The $K_m$ of rat and human prorenin to sheep angiotensinogen

The  $K_m$  values of the renin enzyme activities of rat and human prorenin bound to the receptor were estimated to be 1.0  $\mu\text{M}$  and 0.71  $\mu\text{M}$ , respectively, by the double reciprocal plots (Figure 6).

## 5. DISCUSSION

In this study, we observed that rat prorenin as well as human prorenin bound to each of receptor for the enzymic activation on the cell membrane (Figure 1-6), and indicated that the "handle" region peptide potentially inhibited the prorenin binding to the receptor (Figure 3, 4) and the binding properties were different in rat and human (Figure 1-6).

As shown in Figure 1 and 2, the bound amount of prorenin by the membrane-anchored receptors on the cells transfected with the vector harboring (pro)renin receptor cDNA was significantly higher than that with the vector lacking the cDNA. Usually, mannose-6-phosphate receptor is well known to localize on animal cell membranes to uptake renin and prorenin (27). In this study, such receptor binding was blocked by addition of excess amounts of mannose-6-phosphate in the medium. In addition, the plateau level for binding of human prorenin to human (pro)renin receptor was in the same level as that for the % renin activity of the bound human prorenin (Figure 2, 5). These data indicate that the complex probably little internalizes under the standard assay conditions in this study. These observations illustrate that prorenin is actually bound to the recombinant (pro)renin receptor expressed on the cell membrane.

The activation level of rat prorenin by the rat (pro)renin receptor was lower than that of the combination

of human (Figure 2, 5). The acid-activation rate of rat prorenin has been previously observed much slower than that of human (11). We have evidenced that the rate for acid-activation only depend on the amino acid sequence of the prorenin prosegment at least in rat and human (11). As we can also exclude the possibility of the internalization of the ligand-receptor in rat like human, such difference of the activation level of the bound prorenin is probably owing to the difference of their amino acid sequences. By binding of rat prorenin to the rat (pro)renin receptor, the prosegment part may not be topologically changed into the level that provides the sufficient space for the catalytic reaction as observed in the case of human.

Rat prorenin has been recently observed to bind to the rat recombinant (pro)renin receptor obtained by the baculovirus expression system (26). The bound form of prorenin expressed the renin activity. But the  $K_d$  was estimated 10 times higher than that on the cell membrane as shown in Figure 3. These results indicate that the receptor anchored on the membrane can be more active for the interaction with prorenin rather than that randomly adsorbed to the synthetic walls.

It was also observed that bindings of rat and human prorenin to the receptor were inhibited by their respective peptides (11P-15P) as shown in Figure 4. The pentapeptide regions within the prosegment sequences of prorenin molecules termed as the "handle" peptides (14). Such inhibition was not observed by using the "gate" region (7P-10P) and 30P-36P peptides, which were also parts of the prosegment sequences of prorenin molecules. Though the binding affinity for the synthetic peptide inhibitor (11P-15P) was lower ( $K_i = 6.6 \text{ nM}$ ) than that of the prorenin, its capability as inhibitor is clearly determined (Figure 3, 4). Thus, the present study demonstrates that the "handle" region plays an important role in the paradigm of prorenin binding to the (pro)renin receptor.

The use of short peptides containing the "handle region", named as "decoy peptide" in previous papers on *in vivo* study related (pro)renin receptor (15-20), provided strong evidence that the receptor is responsible for non-proteolytic activation of prorenin as shown in Figure 2, 3, 4 and 5. Thus, the receptor mediated prorenin activation on the cell membrane is distinct from the mannose-6-phosphate receptor mediated prorenin internalization (27), as already described above, and subsequent proteolytic activation as reported by van Kesteren *et al.* (28). The present results are particularly noteworthy as the  $K_d$  values of prorenin binding to the (pro)renin receptor is in nanomolar level which exclude the possibility that the binding is due to enzyme-substrate binding with micromolar level for the  $K_m$ .

It must be noted that the membrane anchored activated prorenin with a catalytic activity comparable with mature renin as shown in Figure 6 is highly effective in the local renin angiotensin system for producing Ang I. In a two-dimensional system, angiotensin I converting enzyme and angiotensin II receptor has been considerable to localize in a closed and concentrated membrane region

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which co-localizes (pro)renin receptor (13, 15-20, 27). In the blood circulation prorenin is known to observe at the 10 times higher level than mature renin. The prorenin level circulated has been reported to increase much higher level (12, 29). This is the reason why prorenin has been proposed as a marker for some diseases (12, 29). The (pro)renin receptor must play important role at least for the local renin-angiotensin system, while the receptor may have another physiological role (20, 27, 30).

In conclusion, present study shows that binding of inactive intact prorenin to the (pro)renin receptor induces a conformational change in the prorenin molecule and probably leads to change topologically the prosegment which ultimately may open active cleft on the surface of the COS-7 cells. Furthermore, the "handle" region of the prosegment part of prorenin plays a crucial role in prorenin binding and activation.

## 6. ACKNOWLEDGEMENTS

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