Soluble Form of the (Pro)Renin Receptor Generated by Intracellular Cleavage by Furin Is Secreted in Plasma

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Abstract—The (pro)renin receptor [(P)RR] is a 35-kDa transmembrane protein that plays a pivotal role in angiotensin tissue generation and in nonproteolytic prorenin activation. We detected a soluble form of (P)RR [s(P)RR; 28 kDa] in the conditioned medium of cultured cells. The aims of our study were to identify the protease responsible for the generation of s(P)RR, the site of shedding, and to establish the existence of circulating s(P)RR in plasma. We identified furin as the protease responsible for the shedding of endogenous (P)RR based on the following: LoVo colon carcinoma cells devoid of active furin synthesize full-length (P)RR but do not secrete s(P)RR; transfection of Chinese hamster ovary cells with a plasmid coding for α1-antitrypsin Portland variant, an inhibitor of furin, completely inhibited the generation of s(P)RR, whereas addition of GM6001, an inhibitor of metalloproteases or of tumor necrosis factor-α protease inhibitor-1, an inhibitor of ADAM17, in the culture medium has no effect; when the cDNA coding for (P)RR was translated in vitro and incubated with recombinant furin or ADAM17, only furin was able to generate the 28 kDa-s(P)RR, and mutagenesis in the potential furin cleavage R275A/KT/R278A site abolished s(P)RR generation. Immunofluorescence study in glomerular epithelial cells showed that (P)RR was cleaved in the trans-Golgi, and coprecipitation with the membrane sector of the vacuolar ATPase accessory protein 2. Because there is only one gene the gene coding for the M8.9 was then called ATP6ap2, for ATPase accessory protein 2. A receptor specific for renin and prorenin, named (P)RR for (pro)renin receptor, was first identified in human mesangial cells1 and cloned in 2002.2 Studies so far have focused on the functions of the full-length transmembrane protein because of the potential role of (P)RR in cardiovascular and renal diseases.3 However, 2 studies reported that the majority of (P)RR was found in the cytosol of cardiacmyocytes4 and in the endoplasmic reticulum in cells transfected and overexpressing tagged (P)RR.5 Shortly before (P)RR was described as an integral membrane protein of 39 kDa, a truncated form of 8.9 kDa called M8.9 was reported to coprecipitate with the membrane sector of the vacuolar H+ -ATPase (V-ATPase) from bovine chromaffin granules.6 The V-ATPase is a complex molecule made of the assembly of 13 subunits that plays an essential role in the cellular pH homeostasis and acidification of intracellular vesicles,7 and the gene coding for the M8.9 was then called ATP6ap2, for ATPase accessory protein 2. Because there is only one gene coding for (P)RR and M8.9,8 we reasoned that M8.9 must derive from the full-length (P)RR by an intracellular cleavage suggested to take place at a furin-like cleavage site9,10 predicted by the search for a conserved pattern in the linear amino-acid sequence analysis of (P)RR amino-acid sequence. Western blot analysis of the different molecular forms of endogenous (P)RR in human, mouse, rat, and Chinese hamster ovary (CHO) cells with antibodies directed against different epitopes of (P)RR revealed the presence of a truncated soluble (P)RR [s(P)RR] in the conditioned medium. This observation prompted us to identify the protease responsible for the cleavage of (P)RR and therefore the generation of s(P)RR, the site of (P)RR shedding, and to assess the presence of s(P)RR in plasma.

Key Words: (pro)renin receptor ■ soluble (pro)renin receptor ■ plasma ■ furin ■ renin-angiotensin system

Materials and Methods

Cell Culture, Cell Lysate, and Conditioned Media
Mouse embryonic stem cells E14 and RST 307 were from Mutant Mouse Regional Resource Center (University of California, Davis). RST 307 cells were generated from E14 cells by insertion of a genetrap in exon 9 of (P)RR gene, precluding full-length (P)RR synthesis. E14 and RST 307 cells were cultured without feeder layer in Glasgow minimal essential medium (Sigma), supplemented with 1000 U/mL leukemia inhibitory factor (ESGRO; Chemicon), 2 mmol/L L-glutamine (Invitrogen), 12.5% FCS, 1 mmol/L sodium pyruvate and nonessential amino acids (Invitrogen), 50 U/mL penicillin, 50 µg/mL streptomycin, and 0.1 mmol/L β-mercaptoethanol. (P)RR expression in the E14 and RST 307 cells was studied by polymerase chain reaction (PCR) with the following couples of mouse primers: forward 197-5’GCTTGCCGTGGGTAACCTATT3’...
were collected 24 hours later and analyzed by Western blotting.

Lipofectamine 2000 (Invitrogen). But GECs were extremely sensitive to transfection reagents and procedures, and the cell mortality 24 hours after transfection was 95%. To determine the relative intensity of the bands was quantified using Quantity One software.

Membranes were revealed with alkaline phosphatase–conjugated secondary rabbit antibody (1:7000; GE Healthcare) and AttoPhos (1:1500; Stressgen), anti-58 K, a marker of Golgi apparatus (1:75; Abcam), or with anti–protein disulfoisomerase, a marker of endoplasmic reticulum (1/100; Abcam). After washing with PBS, cells were stained with secondary Alexa 488–conjugated goat anti-rabbit IgG or Alexa 555–conjugated goat anti-mouse IgG (1:5000; Invitrogen). In some experiments, endosomes were also labeled by transferrin by incubating cells with transferrin coupled to Alexa 555 (1/750; Invitrogen) for 40 minutes at 37°C before fixing. Cells were mounted in PVA-DABCO (Fluka). Slides were analyzed with a Leica SP2 confocal microscope.

Immunoblot Analysis of (P)RR

Purified rabbit IgG to human (P)RR were obtained against an epitope of the ectodomain (1623) and against a sequence encompassing the furin cleavage site (1623) and against a sequence encompassing the furin cleavage site (1623) and against a sequence encompassing the furin cleavage site (1623). Eighty to 100 μg of protein was separated on an SDS–PAGE and transferred to Immobilon-P membrane (Millipore). Membranes were blocked in Tris (10 mmol/L, pH 7.4) NaCl (150 mmol/L) buffer (TBS) containing 10% nonfat milk for 30 minutes at room temperature followed by overnight incubation at 4°C with primary antibodies. Membranes were revealed with alkaline phosphatase– conjugated second rabbit antibody (1:7000; GE Healthcare) and AttoPhos substrate (Promega).

Site-Directed Mutagenesis, In Vitro Translation, and Incubation With Recombinant Furin and ADAM17

(P)RR cDNA was cloned in pcDNA 3.1, and mutagenesis in the furin cleavage site R275A/KT/R278A was performed with Quick Change II site-directed mutagenesis kit (Stratagene). Wild-type and mutated (P)RR cDNA were translated in vitro using TNT-coupled reticulocyte lysate translation system (Promega) in a total volume of 50 μL and the translation product labeled with 35S methionine. Four microilters of the translation product was incubated with 10 U of recombinant human furin (Sigma) or 2 μg of recombinant human ADAM17 (R & D Systems) for 16 hours at 37°C. Samples were separated on an SDS–16% PAGE, the gel fixed for 15 minutes and incubated in Amplify (GE Healthcare), and dried and exposed to x-ray films.

Immunofluorescence Studies

Human GECs seeded on glass cover slips were washed in PBS, fixed in 4% paraformaldehyde for 15 minutes at room temperature, and permeabilized in Tris 50 mmol/L, pH 7.4, containing 0.5% Triton X-100 for 15 minutes on ice. Nonspecific labeling was blocked for 30 minutes at room temperature with PBS containing 1% normal goat serum. Cells were then incubated with IgG 1645 or with anti–protein disulfoisomerase, a marker of endoplasmic reticulum (1:1500; Stressgen), anti-58 K, a marker of Golgi apparatus (1:75; Abcam), or with anti–lysosome associated membrane protein-1 (1/100; Abcam). After washing with PBS, cells were stained with secondary Alexa 488–conjugated goat anti-rabbit IgG or Alexa 555–conjugated goat anti-mouse IgG antibodies (1:5000; Invitrogen). In some experiments, endosomes were also labeled with transferrin by incubating cells with transferrin coupled to Alexa 555 (1/750; Invitrogen) for 40 minutes at 37°C before fixing. Cells were mounted in PVA-DABCO (Fluka). Slides were analyzed with a Leica SP2 confocal microscope.

Coprecipitation of s(P)RR and Renin in Human and Rat Plasma

Because 1623 IgG precipitated poorly, we coprecipitated s(P)RR after incubation of plasma with human His-tagged renin. Briefly, human and rat plasma was dialyzed overnight at 4°C against 20 mmol/L phosphate buffer, pH 7.4, containing a cocktail of protease inhibitors (Complete EDTA-free; Roche Applied Science). Albumin was removed by passing plasma through Hi Trap Blue HP affinity column (GE Healthcare), the fractions were collected, and their protein content measured at 280 nm. The fractions enriched in protein (optical density >1) were pooled and 2 mL incubated with 100 mmol/L His-tagged human renin (Proteos; Kalamazoo, MI) or His-tagged mouse netrin (R & D Systems) as negative control for 2 hours at room temperature. Fifteen microliters of Ni-NTA resin (Qiagen) was then added to immunoprecipitate His-protein/s(P)RR complexes, and the incubation was prolonged for 1 hour at room temperature. After extensive washing in 50 mmol/L Tris, pH 8, containing 150 mmol/L NaCl (TBS), resins-bound proteins were eluted with TBS containing 300 mmol/L imidazole. Samples were analyzed by Western blotting with 1623 IgG and compared with recombinant s(P)RR generated from a plasmid coding for the first 278 amino acids of (P)RR.

Statistical Analysis

The statistical significance was determined by use of the 1-way ANOVA and Student t test.
mouse embryonic stem cells with a genetrap in the last intron of the (P)RR gene. The PCR study performed with forward primer in exon 2 and reverse primer in exon 9 failed to detect any (P)RR mRNA as well as PCR using primers in exon 8 and exon 9, confirming that the genetrap is in exon 9. In contrast, using primers in exon 2 and exon 8, a (P)RR mRNA could be detected (Figure 2). However, no truncated (P)RR protein was ever detected in the conditioned medium or even in the cell lysate of the RST cells, either with 1623 IgG or 1645 IgG, suggesting that the mRNA or the truncated protein was degraded very rapidly. Altogether, these results confirm the specificity of the 1623 IgG and 1645 IgG in Western blotting as well as the inactivation of the (P)RR gene and the absence of (P)RR protein in these cells. Moreover, these results indicate a cross-reactivity for 1623 IgG and 1645 IgG between different species and showed that (P)RR was cleaved intracellularly by furin to generate 28-kDa s(P)RR.

Effect of Protease Inhibitors on s(P)RR Generation

Furin is an intracellular serine protease; therefore, to confirm the role of furin in (P)RR processing, we transiently transfected CHO cells with a plasmid coding for α1-PDX, a specific furin inhibitor, to ensure an effective inhibition of furin inside the cells. Indeed, transfection of α1-PDX in CHO cells almost completely inhibited s(P)RR accumulation in the conditioned medium of CHO cells (Figure 3A). Furthermore, to exclude a potential role of other proteases in (P)RR shedding, we analyzed by Western blotting with 1623 IgG the conditioned media from GECs obtained in the presence of GM6001, an inhibitor of matrix metalloproteinase types 1, 2, 3, 8, and 9, and of tumor necrosis factor-α protease inhibitor-1, an inhibitor of ADAM17/TACE. The results showed that, in contrast to α1-PDX, neither GM6001 nor tumor necrosis factor-α protease inhibitor-1 inhibitors were able to inhibit s(P)RR accumulation in the conditioned medium of GECs (Figure 3B and 3C).

Effects of Protease Inhibitors on s(P)RR Generation in Cell Lysate and Conditioned Medium

Molecular Forms of (P)RR in Cell Lysate and Conditioned Medium

Western blotting of GECs, E14 cells, rat vascular smooth muscle cells, and CHO cells with anti-(P)RR 1623 IgG showed in the cell lysate 2 bands of 35 kDa and of 28 kDa. In the conditioned media, only the 28-kDa band was visible. In contrast, in the lysate of LoVo cells devoid of active furin, only the 35-kDa band could be detected, and no band was visible in their conditioned medium (Figure 1A). These results show that (P)RR is detected under 2 molecular forms: a-35 kDa band representing full-length (P)RR, and a 28-kDa band representing s(P)RR. Western blotting with 1645 IgG directed against the furin cleavage site showed only the 35-kDa band in all lysates tested. GECs, LoVo cells, E14 cells, rat vascular smooth muscle cells, and CHO cells (Figure 1B), indicating that this IgG specifically recognizes the full-length (P)RR with an intact R275/KT/R278 sequence. RST cells are

Figure 3. Effect of protease inhibitors on the secretion of s(P)RR. A, CHO cells were transfected with a plasmid coding for α1-PDX, a furin inhibitor, and the conditioned medium (CM) analyzed by Western blotting with 1623 IgG. Conditioned media of GECs were obtained in the presence-increasing concentration of GM6001, a metalloprotease inhibitor (B), or of tumor necrosis factor-α protease inhibitor-1 (TAPI-1), an inhibitor of ADAM17 (C), and were analyzed by Western blotting with 1623 IgG. The Western blot is a representative experiment, and the bar graphs represent the mean ± SD of 5 independent experiments. *p < 0.05 Student t test (A); 1-way ANOVA (B and C).
Recombinant Furin and ADAM17 Effects on the In Vitro Translation Product of (P)RR

Wild-type (P)RR and (P)RR mutated at the furin cleavage site R275A/KT/R278A were translated in vitro and labeled with $^{35}$S methionine. The products of in vitro translation were incubated for 16 hours at 37°C with purified furin (10 U) or ADAM17 (2 μg) and analyzed by SDS-16%PAGE and autoradiography. The results showed that furin cleaved wild-type (P)RR to generate a 28-kDa band, corresponding exactly to the band of s(P)RR in the conditioned media and an additional band of approximately 10 kDa, which may correspond to the M8.9 described by Ludwig et al.6 Incubation of the translation product of (P)RR mutated in the furin site produced no fragments. These results also indicate that in a pure in vitro noncellular system, excess of ADAM17 is able to cleave wild-type and mutated (P)RR and to generate 2 fragments, a faint band of $\approx$10 kDa and one of 32 kDa, but not the 28-kDa s(P)RR found in the conditioned medium (Figure 4).

Immunofluorescence Study on Endogenous (P)RR in GECs

Studies by others have reported that endogenous4 or transiently transfected5 (P)RR was, in majority, intracellular. Our immunofluorescence study of endogenous (P)RR synthesized by GECs confirm that the staining is mainly intracellular. When we performed double staining of GECs with 1645 IgG, which recognizes only full-length (P)RR, and with markers of the endoplasmic reticulum, the Golgi apparatus, lysosomes, and endosomes, the results showed that the staining observed was mainly colocalized with the marker of the Golgi apparatus (Figure 5). These results suggest that (P)RR is cleaved after the Golgi compartment, probably in the trans-Golgi by furin13 to generate s(P)RR, leaving a small proportion of full-length (P)RR. The full-length (P)RR is addressed to the plasma membrane, whereas s(P)RR is secreted in the medium.

Evidence for s(P)RR in Human and Rat Plasma

Coprecipitation experiments with human renin indeed showed that human and rat plasma contained s(P)RR (Figure 6).

Discussion

(P)RR is a single transmembrane protein that binds renin and its inactive proenzyme form prorenin equally well. The 2 major characteristics of (pro)renin binding, nonproteolytic activation of prorenin and activation of the mitogen-activated protein kinase–extracellular signal-regulated kinase 1/2 pathway leading to the upregulation of profibrotic gene expression, explain why the interest has focused on the potential role of (P)RR in hypertension and organ damage.3 However, this receptor has unusual features for a receptor and for a component of the renin-angiotensin system. The majority of (P)RR is found intracellularly. If this feature was already described for other receptors, this was because of their function of transporting their ligand from plasma membrane to the intracellular vesicles, such as the receptor for transferrin that permanently recycles from plasma membrane to intracellular vesicles.14–16 This was not the case for (P)RR, which is not a transport receptor, and this was further
confirmed by showing that renin bound to (P)RR was neither internalized nor degraded.\(^1,17,18\)

Because it was described that a truncated form of (P)RR, consisting of its transmembrane and cytoplasmic domain, was found in the chromaffin granules, one possibility would be that part of (P)RR is processed in the cell and that most of (P)RR detected intracellularly is composed of truncated forms of (P)RR. Although there is strong evidence that furin is the enzyme responsible for (P)RR processing, especially because they share essential characteristics such as an expression at very early stages of embryonic development\(^19\) (personal observation) and ubiquitous expression at adult age, unlike other proconvertases, we cannot totally exclude that other proconvertases may be involved in (P)RR processing. Our results show that (P)RR is detected mainly in the Golgi apparatus, and that it is probably cleaved in the trans-Golgi to generate a soluble form. They totally exclude the plasma membrane as the site of shedding and any role of metalloproteases and ADAM17 in the shedding of (P)RR.

We were unable to demonstrate the M.8.9 fragment in cell lysate, even when we transfected cells with a (P)RR tagged at the C terminus with either a c-myc or a V5 epitope, suggesting that this fragment is unstable in our experimental model (data not shown).

The impossibility of a total ablation of the (P)RR gene in mice\(^8\) (M. Bader, personal communication, 2006) is another unexpected feature for a component of the renin-angiotensin system and may be attributable to a functional relationship of the truncated membrane form of (P)RR with the V-ATPase, which is essential for control of cellular pH homeostasis and for intracellular vesicle acidification. In zebrafish, a genetrap disrupting (P)RR gene provoked the death of the fish embryo, leading to the postulate that (P)RR is an essential embryonic gene.\(^20\) Strikingly, the phenotype of the (P)RR\(-/-\) fish embryo is similar to the one observed with a genetrap in several V-ATPase subunits, and is essentially characterized by the absence of pigmentation of the mutated fish, smaller head, and necrosis of the central nervous system. The absence of pigmentation could be regarded as a defect in melanocyte function related to impaired acidification of the melanosome that are primary endosomes. It was observed that genes essential in zebrafish are highly conserved in evolution and are also genetically essential in other species, most of them probably being required for cell viability or more specific developmental processes, including patterning, differentiation, and physiology. And, indeed, the (P)RR gene is a highly conserved protein among a large number of species, and multispecies protein sequence comparison revealed the existence of homologues to the human receptor in a variety of species, including rat, mouse, chicken, frog, zebrafish, mosquito, drosophila, or even in species as remotely related to humans as Caenorhabditis elegans.\(^5-10\) The sequence alignment shows that the highest degree of homology encompasses the transmembrane and cytoplasmic regions of the protein corresponding to M8.9, the fragment of (P)RR that was reported to coprecipitate with the membrane sector V-ATPase.\(^6\) These observations suggest that the C-terminus region is the one endowed with the highly conserved cellular function, which might be related to the V-ATPase. However, to date, there is no study establishing a clear link between the lethality attributable to (P)RR gene ablation and impaired activity or assembly of the V-ATPase subunits.

In contrast to the species specificity for angiotensinogen cleavage, the binding of renin and prorenin to (P)RR shows a surprising absence of species specificity. Indeed, we found that rat mesangial cells were able to bind human renin (G. Nguyen, unpublished observation, 2006), and Feldt et al\(^21\) have shown that mouse (P)RR was able to bind human renin and prorenin.

Several studies have indicated that plasma membrane (P)RR is in dimer form.\(^1,5\) However, the fact that (P)RR is detected as a 35-kDa band in Western blots indicated that (P)RR dimer on the cell membrane is not covalently bound. Further, it was necessary to stabilize renin and (P)RR with covalent cross-linking reagents to show the existence of (P)RR dimers,\(^1\) and coprecipitation experiments always showed (P)RR in a form of a 39- to 35-kDa band.\(^5\) Most soluble receptors compete with their membrane-associated counterparts for their ligand binding; in other words, the soluble receptor behaves like a natural antagonist of the membrane receptor.\(^22,23\) In contrast, some soluble receptors behave like agonists and, in this case, the complexes of ligand and soluble receptor bind on target cells to second receptor subunit and initiate intracellular signaling. Previous studies reported in plasma the existence of a high–molecular weight renin of \(\approx 60\) kDa or higher by size exclusion chromatography.\(^24,25\) Our demonstration of the existence in plasma of an s(P)RR able to bind renin suggests that this high–molecular weight renin might represent renin–s(P)RR complexes, and further studies are needed to confirm this hypothesis and to understand the functions of this complex.

The production and purification of the (P)RR in monomeric and dimeric forms will be a fundamental step, allowing to solve the issue of s(P)RR being an agonist or antagonist of membrane (P)RR and to study the function of s(P)RR. In particular, if s(P)RR can also bind and activate prorenin in a nonproteolytic manner, as described for the membrane
(P)RR, thereby if s(P)RR also plays a role in the control of circulating renin activity.

Perspectives

(P)RR is now accepted to play a pivotal role in the generation of angiotensin peptides, and the fact that receptor-bound prorenin is able to display a catalytic activity has shed new light on the physiology of the renin-angiotensin system at a tissue level. The existence of a soluble form of (P)RR in plasma is now drawing our attention back to the circulating renin-angiotensin system. Demonstration of s(P)RR is only the first step. Production of recombinant s(P)RR will allow us to study whether s(P)RR can modulate the enzymatic activity of renin and prorenin when in solution and if s(P)RR can compete for the binding of renin and of prorenin to membrane–(P)RR by complexing with renin and prorenin. In parallel, it will be important to determine the existence of s(P)RR in other biological fluids, such as the interstitial fluid and urine, and to study its variations in physiological and pathological situations known to be associated with high renin or prorenin, such as pregnancy and diabetes.

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Disclosures

None.

References

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