Pivotal role of the renin/prorenin receptor in angiotensin II production and cellular responses to renin

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Renin is an aspartyl protease essential for the control of blood pressure and was long suspected to have cellular receptors. We report the expression cloning of the human renin receptor complementary DNA encoding a 350–amino acid protein with a single transmembrane domain and no homology with any known membrane protein. Transfected cells stably expressing the receptor showed renin- and prorenin-specific binding. The binding of renin induced a fourfold increase of the catalytic efficiency of angiotensinogen conversion to angiotensin I and induced an intracellular signal with phosphorylation of serine and tyrosine residues associated to an activation of MAP kinases ERK1 and ERK2. High levels of the receptor mRNA are detected in the heart, brain, placenta, and lower levels in the kidney and liver. By confocal microscopy the receptor is localized in the mesangium of glomeruli and in the subendothelium of coronary and kidney artery, associated to smooth muscle cells and colocalized with renin. The renin receptor is the first described for an aspartyl protease. This discovery emphasizes the role of the cell surface in angiotensin II generation and opens new perspectives on the tissue renin-angiotensin system and on renin effects independent of angiotensin II.

The receptor-bound enzyme has a much more efficient catalytic activity on the substrate (24). The receptor of a protease may also activate an intracellular signaling pathway leading to a change of cell phenotype and proliferation, as for the receptors of urokinase (25) and thrombin (26).

In this report we describe the identification of a renin receptor by expression cloning. Transfection of renin receptor cDNA into cells lacking specific binding of renin results in the expression of a 45-kDa membrane protein that specifically binds renin and prorenin. The binding of renin to its receptor induces: (a) an increase of the catalytic efficiency of angiotensinogen conversion into Ang I by renin bound to the receptor compared with renin in soluble phase, and (b) a rapid phosphorylation of the receptor on serine and tyrosine residues associated with an activation of MAP kinases ERK1 and ERK2. Immunofluorescence and confocal analyses on normal human kidney and cardiac biopsies show that the receptor is localized within the mesangial area of glomeruli and in the subendothelium of kidney and coronary artery. Double-staining with anti-renin and anti-smooth muscle α-actin showed that, in the arteries, the receptor is present on smooth muscle cells. Furthermore, the staining of mature fetal placenta showed that renin and the receptor were colocalized in vascular structures and in syncytiotrophoblast cells.

Taken together, our results provide evidence for the existence of a functional renin receptor. This receptor is responsible for renin cellular effects, independent of the generation of Ang II. On the other hand, by increasing the efficiency of angiotensinogen cleavage by membrane-bound renin, this receptor also plays a role in the renin effects dependent on Ang II generation.

**Methods**

**Reagents.** Human recombinant renin, prorenin (27), and the polyclonal Ab to human renin were a gift of Walter Fischli (Hoffmann-La Roche Ltd., Basel, Switzerland). The polyclonal Ab to Ang I was a gift from Jean-Baptiste Michel (INSERM U460, Paris, France).

**Expression cloning and 5′ rapid amplification of cDNA ends PCR.** A human kidney cDNA-phage library (CLONTECH Laboratories Inc., Palo Alto, California, USA) was screened with 125I-renin. Twelve positive clones were subcloned in PGEM-T Easy Vector (Promega Corp., Madison, Wisconsin, USA) and sequenced. One clone, N14F (GenBank accession number: AF291814), was selected, and 5′ rapid amplification of cDNA ends (RACE) was performed using the 5′ RACE PCR kit of Life Technologies Inc. (Rockville, Maryland, USA). Specific RT was performed on polyA′ mRNA from primary mesangial cells with the antisense primer 5′-GAGCGTCAACAAGGATCT-TAGAA-3′ complementary to nucleotides 785–819. Nested PCR was done using the antisense primers 5′-GGGGAGTGAAGCTGACA-3′ and 5′-GCATTCTCC-AAGGTTACGA-3′ complementary to nucleotides 577–597 and 371–391. The nucleotide and the deduced amino-acid sequences were analyzed using NetPhos 2.0 prediction server (Center for Biological Sequence Analysis, Technical University of Denmark, Copenhagen, Denmark) and PSORT II prediction server (prediction of protein sorting signals and localization sites in amino acid sequence, Human Genome Center, Institute of Medical Science, University of Tokyo, Japan).

**In vitro translation of N14F and coprecipitation experiments.** The coding region of N14F with the FLAG tag was translated in vitro (TnT-Coupled Reticulocyte Lysate; Promega Corp.) in the presence of 35S-methionine and was analyzed by SDS-PAGE and fluorography. For coprecipitation experiments, the translated product was incubated with 25 nM renin for 2 hours at 37°C and precipitated with anti-renin Ab and protein G-Sepharose (Sigma-Aldrich, St. Louis, Missouri, USA). The proteins were eluted and analyzed by SDS-PAGE and fluorography.

**Transfection of mesangial cells and renin-binding assay.** A human fetal mesangial cell line (HMC) (28) that did not express N14F either by PCR or Northern blot analysis was selected and transfected by the Eugene method (Roche Diagnostics, Meylan, France) and with the coding region of N14F subcloned in pcDNA 3.1 Zeo (Promega Corp.) tagged at the 3′ end with the FLAG peptide (Asp-Tyr-Lys-Asp-Asp-Asp-Lys) by PCR, introducing GACTACAAGGAGCGATGACAG before the stop codon. Stable transfomants were selected with Zeocine. Two clones, HMC2 and HMC4, were selected. The binding experiments were performed as described (21), and the results were analyzed by the Scatchard method.

**Ang I generation by cell-associated renin and prorenin or in soluble phase.** Membranes from HMC and HMC2 were prepared as described (21). Five or ten micrograms of membrane was incubated with 5 nM renin for 1 hour at 37°C. After washing, the membranes were resuspended in 10 µl Krebs buffer containing 2% BSA and incubated 1 hour at 37°C with increasing concentrations (1 nM–1 µM) of angiotensinogen (Sigma-Aldrich) in 110 µl acetate buffer, pH 5.7. The membranes were centrifuged, and the supernatant was assayed for Ang I (29). At the end of the incubation time, aliquots of membranes were treated with 100 mM glycin buffer, pH 5.7, and the renin eluted was measured with the Renin III Generation kit (Bio-Rad Laboratories Inc., Hercules, California, USA). Identical amounts of renin in solution were incubated alone or with 10 µg of HMC membranes with angiotensinogen. Similar experiments were performed with prorenin bound to HMC2 membranes in solution, except the incubation was performed in Kreb’s buffer, pH 7.4, for 4 hours to prevent a possible activation of prorenin. These experimental conditions were determined previously for fully active renin to obtain identical Ang I generation by renin incubated in buffer at pH 5.7 and pH 7.4. Experiments with prorenin were performed twice in duplicate.

**Analysis of the receptor expressed by transfected cells and cross-linking with renin.** Control cells and HMC2 cells were metabolically labeled with 35S-methionine for 24
hours, lysed with 10 mM Tris buffer, pH 8.0, 140 mM NaCl (TSA) containing Triton X-100 1%, 1 mM PMSF, 500 U/ml aprotinin (TSA-TX). The receptor was immunoprecipitated, and the proteins eluted were analyzed by SDS-PAGE and fluorography. For cross-linking experiments, the cells were incubated with or without 25 nM renin for 1 hour at 37°C and cross-linked as described (21). The cells were lysed, and the renin-receptor complex was immunoprecipitated with anti-receptor Ab. The proteins eluted were analyzed by SDS-PAGE and fluorography.

Intracellular free [Ca\textsuperscript{2+}] and cyclic AMP changes induced by renin. Briefly, subconfluent cells in a 10-mm Petri dish were loaded with 5 µM Fura-2 for 45 minutes at 37°C, washed, and detached by mild trypsinization. Cells were resuspended in PBS buffer containing 1% BSA and transferred into a quartz cuvette under constant stirring at 37°C. Fluorescence was monitored in a Quanta Master 1 spectrofluorometer, and [Ca\textsuperscript{2+}] was calculated using Felix 1.1 software program (Photon Technology International, Burlington, New Jersey, USA).

For cAMP study, cells in 24-well plates were serum deprived for 18 hours and incubated 30 minutes in culture medium containing 100 µM isobutyl-methyl-xanthine. After addition of 10 nM renin or 1 µM PGE\textsubscript{2} or 1 µM of isoproterenol, the reaction was stopped after 5 minutes by addition of 250 µl of ethanol 95%-formic acid 5%. The supernatant was evaporated overnight, and the cAMP content of each well was measured with cAMP-[\textsuperscript{125}I] assay (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). Measurements of cAMP activity were made two times in triplicate, and statistical analysis was performed using the Wilcoxon nonparametric test.

Analysis of the pattern of the receptor phosphorylation and MAP kinases ERK1/ERK2 activation induced by renin. HMC2 cells were incubated with 10 nM renin for 0, 3, and 10 minutes and in the presence of 100 nM Captopril. The cells were lysed with TSA-TX containing 2 mM Na\textsubscript{3}VO\textsubscript{4}, 50 mM NaF, and 5 mM EDTA. The receptor was immunoprecipitated with anti-FLAG agarose. Bound proteins were analyzed by Western blotting with anti-phosphotyrosine (Chemicon International, Temecula, California, USA), anti-phosphoserine, or anti-phosphothreonine Ab’s (Sigma-Aldrich) and revealed by the ECL kit (Amersham Pharmacia Biotech). Control cells were incubated with renin, lysed, and analyzed by Western blotting with anti-phosphotyrosine and anti-phosphoserine Ab’s without prior immunoprecipitation of the receptor with anti-FLAG agarose.

To study MAP kinases ERK1 (p44) and ERK2 (p42) activation, cells were serum deprived for 18 hours and stimulated with 10 nM renin for various times in the presence of 1 µM Losartan. The cells were lysed with 100 µl lysis buffer as described. Proteins were measured with the Bio-Rad Laboratories Inc. DC protein assay and analyzed by Western blotting using Ab’s to total and active ERK1/ERK2 (Promega Corp.) and revealed by the ECL kit. The blots were scanned and plotted to obtain a bar graph representation of the ratio of active-to-total ERK1/ERK2 activation using the NIH IMAGE program (Bethesda, Maryland, USA). To confirm MAP kinases ERK1/ERK2 activation, MAP kinase activity of cell lysate was also measured using the Biotrak p42/p44 MAP kinase enzyme assay system (Amersham Pharmacia Biotech). Measurements of MAP kinase activity were made two times in triplicate, and statistical analysis was performed using the Wilcoxon nonparametric test.

Northern blot analysis. A cDNA fragment containing the Not1-Xba1 coding region of N14F subcloned into pcDNA 3.1 was radiolabeled (PrimeIt labeling kit; Stratagene, La Jolla, California, USA) and hybridized to a membrane containing the polyA’ mRNA from human tissues (CLONTECH Laboratories Inc.). The amount of RNA was adjusted so that the β-actin hybridization signal was of comparable intensity in every lane. The blots were hybridized and washed using ExpressHyb (CLONTECH Laboratories Inc.).

Immunofluorescence studies on transfected cells, on human normal kidney and heart, and on mature placenta. Rabbits were immunized by coinjection of synthetic peptides corresponding to amino acids 221–235 and 337–350 of the receptor (Eurogentec SA, Herstal, Belgium). Specimens of kidney and heart were obtained from the operating room after total nephrectomy for tumor and during mitral valve replacement, respectively, with informed consent of the patient. Mature placenta was obtained from the delivery room, with informed consent of the patient. Permeabilized cells and the cryopreserved tissues were fixed in 4% paraformaldehyde and incubated with rabbit antiserum to the receptor (1:1,000 dilution) with mAb’s to CD31 or to smooth muscle α-actin (Sigma-Aldrich). FITC or Texas red (TRITC) secondary Ab’s were used to visualize the staining. The specificity of the staining for the renin receptor was assessed by preincubation of the primary Ab with the peptides. To study the colocalization of renin and renin receptor, staining was performed on sequential sections of placenta using the polyclonal Ab to the receptor (1:1,000 dilution) revealed by FITC-secondary anti-rabbit Ig and the polyclonal Ab to human renin (1:50 dilution) revealed by TRITC-secondary anti-rabbit Ig.

Results

Expression cloning of renin receptor. We have screened 1,000,000 clones of a commercial human kidney expression library in λgt11 with recombinant human renin labeled with \textsuperscript{125}I. The positive clones were amplified by PCR using λgt11 primers and subcloned in pGEM-T Easy for sequencing. The sequence of one clone (N14F, 2,034 bp) represented a new protein with no homology with any known membrane-receptor family (Figure 1a). The initiation codon was surrounded by the optimal Kozak nucleotide consensus sequence (GCCACC), and the open reading frame encoded a 350-amino acid protein (molecular weight 39,008 kDa). The integrity of the 5’ end was assessed by rapid amplification of complementary cDNA 5’ end by PCR and by comparison with an expressed sequence tag data base. The deduced
amino acid sequence and the analysis of hydrophobicity of the translated protein (30) predicts that the protein has two hydrophobic regions, one located in the amino terminus from amino acids 1–16, which likely represents a signal peptide, and another in the carboxy terminus from amino acids 306–326, which likely represents a transmembrane region (Figure 1b). Of the 24 amino acids of the cytoplasmic tail, Ser 337, Thr 343, Tyr 335, and Tyr 340 represent possible sites for regulatory phosphorylation, of which Tyr 335 appears to be the most likely phosphorylated site (NetPhos 2.0 prediction). The predicted structure of this protein was characterized by the absence of a N-linked glycosylation site, a pI of 5.76, no PEST (proline, glutamic acid, serine, and threonine) sequence, and an estimated half-life of 30 hours (PSORT II prediction).

In vitro translation and coprecipitation of the FLAG receptor with renin. The construct N14F-FLAG was translated in vitro using a rabbit reticulocyte lysate system and labeled with 35S-methionine. Analysis by SDS-PAGE and fluorography showed one band at the expected molecular weight of 39 kDa (Figure 2a, lane A). Prior to performing cell transfection, we tested the interaction of N14F with renin by coprecipitation experiments. The translated product labeled with 35S-methionine was incubated with renin (25 nM) for 2 hours at 37°C, and the complexes precipitated with anti-renin Ab. SDS-PAGE and fluorography analysis of the precipitate showed that the receptor was recovered in the precipitate, confirming that N14F was able to interact with renin (Figure 2b, lane B). In the absence of renin, anti-renin Ab did not react with in vitro–translated receptor (Figure 2b, lane C) or by Western blot analysis (Figure 2c).

Renin binding and membrane expression of N14F protein by HMC cells transformed with N14F. Next, the identity of the cloned cDNA was demonstrated by stable transfection of immortalized fetal HMCs (28) that did not express N14F either by PCR using primers specific for N14F or by Northern blot analysis (not shown). Two clones of stable transformants, HMC2 and HMC4, were analyzed.
by binding assays. The binding of renin on transformed cells is saturable and characterized by a $K_d$ of 5.0 nM and a maximal binding site ($B_{max}$) of 2.8 fmol/100,000 cells, corresponding to approximately 17,000 sites/cell for HMC2, and a $K_d$ of 7.8 nM and a $B_{max}$ of 1.8 fmol/100,000 cells, approximately 13,000 sites/cell for HMC4. In contrast, HMC control cells do not display any specific renin binding (Figure 3a). Renin-specific binding to HMC2 cells was inhibited by an excess of cold prorenin (100 nM), suggesting that the receptor also binds prorenin. In contrast, renin binding was not inhibited by an excess of other aspartyl proteases, such as pepsin and cathepsin D, or modified by nonrelated proteins, such as hemoglobin, hepatocyte growth factor, or tissue-plasminogen activator (not shown). To further assess the membrane expression of the renin receptor on HMC2 cells we performed immunofluorescence staining with Ab to the receptor and analysis by confocal microscopy. The results showed that the renin receptor was cell surface associated and that no staining could be detected in control cells transfected with empty vector (Figure 3b).

Increased catalytic efficiency of angiotensinogen cleavage by membrane-bound renin and prorenin. The kinetics of angiotensinogen cleavage by HMC2 membrane-bound renin and renin in solution were compared. The results showed that the amount of Ang I generated by membrane-bound renin was much higher than that by renin in solution (Figure 4a). The double-reciprocal (Lineweaver-Burk) transformation of data resulted in $K_m$ values of 1 µM and 0.15 µM for renin in soluble-phase and membrane-bound renin, respectively (Figure 4b). From the slope of the individual double-reciprocal plots and the values of renin eluted from the membranes, the catalytic constant ($k_{cat}$) values were 2.4 s$^{-1}$ and 1.4 s$^{-1}$ with 0.5 nM renin for solution phase and membrane-bound renin, respectively. For 1 nM renin immobilized on the membranes or in solution, the $k_{cat}$ values were 2.6 s$^{-1}$ and 1.8 s$^{-1}$ for solution-phase and membrane-bound renin, respectively. Therefore the catalytic efficiency of the reaction of renin with angiotensinogen measured by the ratio $k_{cat}/K_m$ was lower for renin in solution, 2.2 to 2.4 µM$^{-1}$ s$^{-1}$, than for renin bound to the receptor, 9.3 to 11.2 µM$^{-1}$ s$^{-1}$ (Figure 4c). These results indicate that angiotensinogen cleavage by membrane-bound renin is five times more efficient than by renin in solution. When membranes from HMC control cells were tested for renin binding, an elution of bound renin, the amount of renin eluted from HMC membranes, was under the limit of detection of the renin antigen assay, precluding the comparison of angiotensinogen conversion by HMC membrane-bound renin to renin in solution. In addition, Ang I generation was identical in buffer and in the presence of 10 µg of HMC membrane (not shown), indicating that phospholipids alone, in the absence of receptor, do not

![Figure 2](http://www.jci.org)

**Figure 2**

In vitro transcription/translation of N14F/FLAG protein and coprecipitation with renin. (a) N14F/FLAG fusion protein (lane A) or control empty vector (lane B) transcribed in vitro and labeled with $^{35}$S-methionine are analyzed by SDS-PAGE and fluorography. (b) Coprecipitation of the N14F/FLAG fusion protein incubated with renin and immunoprecipitated with anti-renin Ab. The proteins were eluted and analyzed by SDS-PAGE and fluorography. Lane A: N14F/FLAG fusion protein immunoprecipitated with anti-FLAG agarose; lane B: elution of N14F/FLAG protein bound to renin; lane C: N14F/FLAG protein immunoprecipitated with anti-renin Ab. (c) Renin alone (lane A) was immunoprecipitated with Ab to the renin receptor and analyzed by Western blotting with anti-renin Ab. Lane B: recombinant renin.

![Figure 3](http://www.jci.org)

**Figure 3**

Membrane expression of N14F protein and renin binding by HMC cells transformed with N14F. (a) Saturation of binding on the HMC clone 2 (HMC2) expressing the receptor (squares, total; circles, nonspecific) and on HMC control cells (+, total; x, nonspecific). The nonspecific binding was determined in the presence of 100 nM cold renin. Inset: Scatchard plot of the binding of renin to HMC2. (b) Immunofluorescence and confocal analysis of the expression of N14F on HMC2 cells stably expressing N14F (upper panel) and by HMC control cells transfected with empty vector (lower panel). The cells were stained with anti-receptor Ab (dilution 1:1,000) and with TRITC–conjugated secondary Ab.
alter renin activity. However, we cannot exclude the possibility that, since renin is more closely apposed to membrane phospholipids, they may be important for the increase activity of renin. Next, we investigated the capacity of membrane-bound prorenin to activate angiotensinogen. The determination of recombinant prorenin antigen in our preparation of prorenin with the RIA Renin III generation kit, which uses an anti-renin active site Ab as secondary Ab, showed that about 25% of prorenin could be detected, indicating that the active site of prorenin was accessible in about 25% of the molecules. This prorenin preparation was used for binding to HMC2 membranes and for measuring the extent of Ang I generation as described. Prorenin bound to membranes was eluted by pH 3.5 treatment and measured with the RIA Renin III generation kit. Acidification resulted in a total activation of prorenin (not shown). Accordingly, in the control experiments identical amounts of prorenin were incubated with angiotensinogen (Agen) 1 µM under different conditions. Bars 1, 2, and 3 are renin in solution, pH 5.7; for 1 hour; pH 7.4 for 1 hour; and pH 7.4 for 4 hours, respectively. Bars 4 and 5 are prorenin pH 7.4 for 4 hours, in solution or membrane bound, respectively. Experiments were performed twice in duplicate, and the results represent the mean of the two experiments.

Immunoprecipitation of the receptor from transfected cells labeled with 35S-methionine and with the polyclonal anti-receptor Ab showed that the receptor has an apparent molecular weight of 45 kDa, greater than the in vitro translation product (39 kDa), indicating that posttranslational modifications have occurred in the eukaryotic cell (Figure 5a, lane A). Our previous results have suggested a molecular weight of 70–80 kDa for the receptor on primary human mesangial cells (21). Therefore, we performed cross-linking experiments of renin bound to transformed cells. The results showed the appearance of a 110- to 120-kDa band, confirming our previous study (Figure 5a, lane C). Precipitation of cell lysate with anti-FLAG Ab during the phosphorylation experiments confirmed a molecular weight of 45 kDa for the receptor as found by immunoprecipitation of 35S-methionine–labeled cell lysate with the anti-receptor Ab. Renin stimulation induced a phosphorylation of serine and tyrosine residues of the receptor, but not of threonine (not shown). The phosphorylation of serine residues occurred more rapidly than for tyrosine and was already detectable after 3 minutes in the presence of renin, whereas the phosphorylation of tyrosine residues appeared after 10 minutes (Figure 5b, upper panel). No phosphorylation corresponding to a 45-kDa band could be observed in control cell lysate (Figure 5b, lower panel).

Cross-linking with renin and phosphorylation of the receptor. Immunoprecipitation of the receptor from transfected cells labeled with 35S-methionine and with the polyclonal anti-receptor Ab showed that the receptor has an apparent molecular weight of 45 kDa, greater than the in vitro translation product (39 kDa), indicating that posttranslational modifications have occurred in the eukaryotic cell (Figure 5a, lane A). Our previous results have suggested a molecular weight of 70–80 kDa for the receptor on primary human mesangial cells (21). Therefore, we performed cross-linking experiments of renin bound to transformed cells. The results showed the appearance of a 110- to 120-kDa band, confirming our previous study (Figure 5a, lane C). Precipitation of cell lysate with anti-FLAG Ab during the phosphorylation experiments confirmed a molecular weight of 45 kDa for the receptor as found by immunoprecipitation of 35S-methionine–labeled cell lysate with the anti-receptor Ab. Renin stimulation induced a phosphorylation of serine and tyrosine residues of the receptor, but not of threonine (not shown). The phosphorylation of serine residues occurred more rapidly than for tyrosine and was already detectable after 3 minutes in the presence of renin, whereas the phosphorylation of tyrosine residues appeared after 10 minutes (Figure 5b, upper panel). No phosphorylation corresponding to a 45-kDa band could be observed in control cell lysate (Figure 5b, lower panel).

Intracellular [Ca2+] and cAMP changes, MAP kinases ERK1(p44)/ERK2(p42) activation induced by renin. To further support the receptor function of the cloned renin receptor, we studied the effect of renin binding on [Ca2+] concentration change, on cyclic AMP, and on MAP kinases ERK1(p44)/ERK2(p42) activation using...
two different techniques: Western blot analysis of total and active phosphorylated ERK1 and ERK2 and a MAP kinase activity assay. No changes in [Ca²⁺] or in cyclic AMP could be evidenced after renin addition (Figure 6a). On the contrary, renin addition induced a rapid activation of ERK1/ERK2 resulting in a twofold increase of active ERK1/ERK2 after 0.5 min (P < 0.05) and a three- to fourfold increase after 10 minutes and for at least 60 minutes (Figure 6b, left panel). The blot of total ERK1/ERK2 confirmed an even loading of the gels and the ratio of active-to-total ERK1/ERK2 was made using the NIH IMAGE program (Figure 6b, left). These data were confirmed by a MAP kinase activity assay (Figure 6b, left bottom). No activation of ERK1/ERK2 was observed in cells not expressing the receptor (Figure 6b, right panel).

Expression of renin receptor mRNA in human adult tissues by Northern blot analysis. A single transcript of 2.4 kb was found in the brain, placenta, heart, liver, kidney, and pancreas. Because the amount of polyA⁺ mRNA immobilized on the membrane was adjusted for β-actin, the very strong signal observed in the brain, heart, and placenta indicates very high levels of expression of the receptor mRNA in those tissues. The mRNA of the renin receptor was barely detectable in lung and skeletal muscle (Figure 7).

Immunofluorescence staining and confocal microscopy on normal human kidney, heart, and placenta. Immunofluorescence staining of kidney cortex with the anti-receptor Ab showed labeling of glomeruli and vascular structures and no staining of the tubules and, at higher magnification, confirmed that the labeling was restricted to the mesangium (Figure 8a, left). The specificity of the staining was assessed by preincubation of the immune serum with the peptides (Figure 8a, right). Double-labeling with anti-CD31 Ab specific for endothelial cells and with anti-renin receptor Ab and analysis by confocal microscopy of kidney cortex showed that the renin receptor did not colocalize with CD31 in endothelial cells in glomeruli but was localized in the mesangial area. In kidney arteries, the renin receptor was not associated with the endothelium either (Figure 8, middle panel). To compare the vascular localization of the renin receptor in another tissue, we also studied the distribution of the receptor in the heart. The results showed that in coronary arteries, as in kidney arteries, the renin receptor did not colocalize with CD31 on endothelial cells but was found in the subendothelium (Figure 8, middle panel). To better localize the renin receptor in the subendothelium of kidney and coronary arteries, we performed a double-staining with anti-receptor and anti-smooth muscle α-actin Ab. The results showed that the renin receptor colocalized with smooth muscle α-actin, indicating that renin receptor was present on smooth muscle cells (Figure 8, lower panel). The absence of evidence for a colocalization of both renin receptor and smooth muscle α-actin in normal glomeruli can be attributed to the absence of smooth muscle α-actin expression by human mesangial cells in normal, noninflammatory glomeruli (31). Finally, we looked for a colocalization of renin and of the receptor in normal tissue. Placenta is known to secrete large amounts of renin and prorenin throughout gestation (7), and Northern blot analysis also showed abundant expression of the receptor in placenta. Immunofluorescence staining showed that the receptor was found in mature placenta, associated to vascular structures and to syncytiotrophoblast cells (Figure 9). In addition, staining of sequential section of placenta with renin Ab showed that renin and renin receptor had a similar pattern of distribution in arteries and in syncytiotrophoblast cells (Figure 9).

Figure 5
Cross-linking with renin and phosphorylation of the receptor. (a) HMC and control cells were labeled with 35S-methionine, and the receptor was immunoprecipitated with anti-receptor Ab (lane A and lane B for HMC and control cells, respectively) or after incubation with renin and cross-linking (lane C and lane D for HMC and control cells, respectively). In lane E, HMC cells cross-linked in the absence of renin. (b) In the upper panel HMC2 cells were stimulated with renin in the presence of 100 nM Captopril. The receptor was immunoprecipitated with anti-FLAG agarose, and the eluate was analyzed by Western blotting using Ab’s to phosphotyrosine or to phosphoserine. In the lower panel control cells were stimulated with renin, and the cell lysate was analyzed by Western blotting using Ab’s to phosphotyrosine or to phosphoserine. The right lanes of the two lower blots are the receptor immunoprecipitated from HMC cells stimulated by renin.
Discussion
This study reports, we believe for the first time, the existence of a functional receptor of renin. The evidence that N14F cDNA is identical to the receptor of renin is as follows: (a) The N14F protein expressed in vitro is able to bind renin in coprecipitation experiments; (b) cells transfected with N14F cDNA construct and expressing the protein bind renin with high affinity, and the binding is specific for renin and prorenin; (c) renin binding induces the activation of MAP kinases ERK1 and ERK2 associated with a phosphorylation of tyrosine and serine residues.

The localization of the receptor in the mesangium of glomeruli suggests that this receptor is the one we described on human mesangial cells in culture. In primary mesangial cells (21) we have observed a molecular weight of 70–80 kDa for the receptor, whereas the cloned renin receptor here is 45 kDa. This discrepancy may be explained by a dimerization of the receptor induced by renin binding as described for receptors of the tyrosine-kinase family, which are also single-transmembrane domain receptors (32), or a complex formation with another, yet-unidentified protein. Part of this clone was identical to the previously reported “M8-9,” a truncated protein of 8.9 kDa that copurified with a proton-ATPase of chromaffin granule membranes (33). While the sequencing of our N14F clone was completed, two identical cDNA sequences from human fetal brain and human hypothalamus appeared in Genbank (DKFZ p56400582, accession number AL049929.1 and 1424...
Figure 8
Expression of the renin receptor on human kidney and heart. (a) At the left, immunoﬂuorescence staining of kidney cortex with anti-receptor Ab (upper panel magniﬁcation ×20) and a glomerulus at higher magniﬁcation (lower panel, ×40). Yellow staining is due to autoﬂuorescence of degradation products in tubular cells. The immune serum was preincubated with the peptides (right). (b–m) Double-staining and analysis by immunoﬂuorescence and confocal microscopy of kidney cortex labeled with anti-renin receptor (b, e, h), anti-CD31 (c, f, i), and anti-receptor plus anti-CD 31 (d, g, j). e–g represent the glomerulus in b–d, and h–j represent the artery in b–d at higher magniﬁcation. (k–m) A coronary artery labeled with anti-receptor, anti-CD31, anti-receptor plus anti-CD 31 Ab, respectively. (n–v) Double-staining and analysis by immunoﬂuorescence and confocal microscopy of kidney cortex labeled with anti-renin receptor (n and q) or smooth muscle α-actin Ab (SM-α-actin; o and r) and anti-receptor and anti–SM-α-actin (p and s); q–s represent the kidney cortex artery in panels n and q at higher magniﬁcation. (t–v) A coronary artery stained with anti-receptor, anti–SM-α-actin, and anti-receptor plus anti–SM-α-actin Ab, respectively.
AF248966), confirming the high level of expression of the receptor in the human CNS.

The physiological plasma concentration of renin is in the picomolar range, but it is admitted that in tissues, especially in interstitial fluids, the renin concentration may be 100-fold greater. Since we found that the dissociation constant of the renin-receptor complex was in the nanomolar range, we postulated that it could not be considered as a binding protein responsible for retaining renin in the interstitium because only about 1% of the interstitial renin (10 pM) would be bound by the receptor. Alternatively, it could function as an effective receptor. Therefore, we looked for [Ca²⁺] change and for cyclic AMP and MAP kinases ERK1(p44)/ERK2(p42) activation. Our results showed that renin binding did not modify either intracellular calcium or cAMP, but provoked a rapid activation of the ERK1/ERK2 pathway, associated with a phosphorylation of serine and tyrosine residues. The presence of either an ACE inhibitor or an AT1 receptor antagonist during the incubation of transformed cells with renin confirms that these events are independent of a possible generation or action of Ang II. The pathway involved in the tyrosine phosphorylation of the receptor, the respective roles of serine and tyrosine phosphorylation in the receptor function, and the early phase of renin signal are actually under investigation.

We have shown that receptor-bound renin activates angiotensinogen with kinetics different from those observed for renin in solution and, in particular, we showed a reduction in the $K_m$ for angiotensinogen from 1 µM in solution to 0.15 µM with receptor-bound renin. This $K_m$ is significantly below the normal plasma concentration of angiotensinogen, approximately 1 µM (29), suggesting that conversion of angiotensinogen by receptor-bound renin may be of physiological importance, especially in tissues in which the concentration of angiotensinogen is much lower than in plasma. We hypothesize that receptor-bound renin is able to activate angiotensinogen while it is actually bound to the cell surface and with higher efficiency than renin in solution. The slight decrease of $k_{cat}$ (1.5-fold reduction) observed concomitantly might reflect the constraints imposed by the immobilization of renin on the receptor. The overall catalytic efficiency $k_{cat}/K_m$ increased four to five times, indicating that the cell surface is an important site for angiotensinogen activation. Taken together with the recent report of the existence of a vascular smooth muscle chymase (34), our results suggest that the smooth muscle cell surface may play an essential role in tissue generation of Ang II. The binding of renin to its receptor, thereby increasing angiotensinogen cleavage efficiency, would facilitate Ang I cleavage in Ang II by vascular smooth muscle chymase.

We also hypothesized that bound prorenin would activate angiotensinogen. Although our prorenin preparation was reactive against Ab’s to the active site of renin, suggesting that some molecules have an accessible active site, prorenin showed very little Ang I–forming activity when incubated at pH 7.4 for 4 hours. However, the extent of Ang I generated by prorenin bound to membranes was comparable to the extent of Ang I generated by fully active renin under these experimental conditions. Whether the increase of the catalytic activity of membrane-bound prorenin should be attributed to the fraction of prorenin with an already exposed active site or to inactive prorenin that underwent conformational changes induced by receptor binding is an important issue to investigate in the near future. Supporting evidence for catalytically active prorenin in vivo, in the absence of prorenin cleavage, exists in the literature (35). Mmethot et al. generated double-transgenic mice expressing human angiotensinogen and a mutated, noncleavable human prorenin in the pituitary gland (35). These animals have elevated pituitary Ang I content in the

Figure 9
Expression and colocalization of renin and of the renin receptor in placenta. Sequential sections of placenta were stained with the Ab to the receptor and FITC-coupled secondary anti-rabbit Ab (a–c) or with anti-renin Ab and Texas-red–coupled secondary Ab (e–g). (d) FITC-secondary Ab alone. (h) TRITC–coupled secondary Ab alone. The receptor and renin have similar distribution in vascular structures and in syncytiotrophoblast cells.
absence of prorenin cleavage as shown by Western blot analysis, indicating that prorenin was enzymatically active even with the presequence still in place.

Knockout mice for angiotensinogen, Ang converting enzyme, Ang II receptors, and nullizygotes for the renin gene, display low blood pressure and severe renal vascular lesions associated with high or low plasma renin concentrations (36–39). The vascular lesions have been attributed to the absence of Ang II during development, but these data do not exclude a role of renin in the pathogenesis of vascular lesions. Moreover, rats expressing the prorenin transgene exclusively in the liver suffer severe nephroangiosclerosis, cardiac and aortic hypertrophy, and liver fibrosis in the absence of hypertension (40). This observation suggests that the effects observed in vitro on the activation of ERK1/ERK2 pathway involved in cell hypertrophy and proliferation, as well as the cellular hypertrophy and the increase of PAI1 (21) synthesis, may be relevant in vivo.

In conclusion, our results show that renin receptor exerts dual effects. The renin receptor is able to trigger intracellular signal by activating the ERK1/ERK2 pathway, and it also acts as a cofactor by increasing the efficiency of angiotensinogen cleavage by receptor-bound renin, therefore facilitating Ang II generation and action on a cell surface. This novel concept may provide a new approach to a better understanding of the pathogenesis of vascular diseases associated with RAS activation.

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