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## High-affinity prorenin binding to cardiac man-6-P/IGF-II receptors precedes proteolytic activation to renin

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**Saris, Jasper J., Frans H. M. Derkx, René J. A. De Bruin, Dick H. W. Dekkers, Jos M. J. Lamers, Pramod R. Saxena, Maarten A. D. H. Schalekamp, and A. H. Jan Danser.** High-affinity prorenin binding to cardiac man-6-P/IGF-II receptors precedes proteolytic activation to renin. *Am J Physiol Heart Circ Physiol* 280: H1706–H1715, 2001.—Mannose-6-phosphate (man-6-P)/insulin-like growth factor-II (man-6-P/IGF-II) receptors are involved in the activation of recombinant human prorenin by cardiomyocytes. To investigate the kinetics of this process, the nature of activation, the existence of other prorenin receptors, and binding of native prorenin, neonatal rat cardiomyocytes were incubated with recombinant, renal, or amniotic fluid prorenin with or without man-6-P. Intact and activated prorenin were measured in cell lysates with prosegment- and renin-specific antibodies, respectively. The dissociation constant ( $K_d$ ) and maximum number of binding sites ( $B_{max}$ ) for prorenin binding to man-6-P/IGF-II receptors were  $0.6 \pm 0.1$  nM and  $3,840 \pm 510$  receptors/myocyte, respectively. The capacity for prorenin internalization was greater than 10 times  $B_{max}$ . Levels of internalized intact prorenin decreased rapidly (half-life =  $5 \pm 3$  min) indicating proteolytic prosegment removal. Prorenin subdivision into man-6-P-free and man-6-P-containing fractions revealed that only the latter was bound. Cells also bound and activated renal but not amniotic fluid prorenin. We concluded that cardiomyocytes display high-affinity binding of renal but not extrarenal prorenin exclusively via man-6-P/IGF-II receptors. Binding precedes internalization and proteolytic activation to renin thereby supporting the concept of cardiac angiotensin formation by renal prorenin.

local renin-angiotensin system; cardiomyocytes; fibroblasts; heart; kidney

THE BENEFICIAL CARDIAC EFFECTS of angiotensin-converting enzyme (ACE) inhibitors in subjects with heart failure are usually attributed to interference of these drugs with ANG II generation at cardiac tissue sites. Although initially it was thought that this generation depends on the de novo synthesis of renin in the heart, it is now well established that such synthesis does not occur either under normal circumstances (13, 24, 34,

35, 40, 56) or under pathological conditions (14, 31, 50). Therefore, the heart must sequester renin from the circulation to synthesize ANG II locally. Renin may diffuse into the interstitial space (18, 28) or bind to renin receptors and/or renin-binding proteins (8, 46, 53). Because renin in blood is predominantly present in the form of its inactive precursor prorenin (11), it is also conceivable that the heart sequesters prorenin instead of renin. This prorenin must then be activated locally. In support of this concept we recently demonstrated that endothelial cells and cardiac myocytes and fibroblasts, which do not synthesize (pro)renin (1, 59), are capable of binding recombinant human prorenin to cell-surface mannose-6-phosphate (man-6-P)/insulin-like growth factor-II (man-6-P/IGF-II) receptors (1, 58). Binding precedes rapid internalization and appearance of renin-specific enzymatic activity. Furthermore, the receptors also bound and internalized recombinant human prorenin produced in Chinese hamster ovary (CHO) cells contains the man-6-P signal that is required to bind to man-6-P/IGF-II receptors (2, 25). This is not a unique property because many other prohormones [e.g., latent transforming growth factor- $\beta$  (19), procathepsin D (29), and proliferin (39)] also carry the man-6-P recognition marker, and these prohormones (like prorenin) are activated after binding and internalization via man-6-P/IGF-II receptors.

At present it is unlikely that the man-6-P/IGF-II receptor is the only renin- and prorenin-binding receptor, because excess man-6-P did not block prorenin binding to microsomal membrane fractions prepared from various rat tissues (53). In addition, it is not known whether intracellular prorenin activation occurs proteolytically or nonproteolytically. Proteolytic activation involves the actual removal of the prosegment by any of the known prorenin-renin convertases [e.g., cathepsin B (45), kallikrein (37), and prohormone convertases (51)]. Nonproteolytic activation implies transient unfolding of the prosegment so that it no longer folds over the enzymatic cleft, thereby allowing

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prorenin to cleave angiotensinogen. In vitro, acid pH or cold storage favor the latter type of activation (21). Moreover, a recent study in mice has shown that non-proteolytically activated prorenin is capable of generating ANG I at tissue sites in vivo (43).

The aim of the present study was to investigate in neonatal rat cardiomyocytes and fibroblasts 1) the kinetics of man-6-P/IGF-II receptor-dependent prorenin binding [dissociation constant ( $K_d$ ), maximum number of binding sites ( $B_{max}$ )] and activation; 2) the possibility of prorenin binding independent of the man-6-P/IGF-II receptor; 3) the nature of the prorenin activation (proteolytic or nonproteolytic) and, in case of proteolytic activation, the nature of the prorenin-activating enzyme; and 4) possible differences between binding of recombinant human prorenin and binding of native human prorenin from renal and extrarenal sources.

## METHODS

**Cell culture.** All experiments were performed according to the regulations of the Animal Care Committee of Erasmus University Rotterdam, Rotterdam, The Netherlands, and in accordance with the "Guiding Principles in the Care and Use of Laboratory Animals" as approved by the Council of the American Physiological Society.

Primary cultures of rat neonatal cardiac cells were prepared as previously described (57). Briefly, ventricles of Wistar strain rat pups (age 1–3 days) were minced and cells were dispersed by eight subsequent trypsinization steps. Nonmyocytes were separated from myocytes by differential preplating. Myocytes were seeded in noncoated 12-well plates (Corning Costar Europe; Badhoevedorp, The Netherlands) yielding a confluent monolayer of spontaneously beating cells at  $1.5 \times 10^5$  cells/cm<sup>2</sup> after 24 h. The preplated cells (fibroblast fraction) were passaged after 4 days to noncoated 12-well plates yielding a confluent monolayer of  $0.75 \times 10^5$  cells/cm<sup>2</sup> after 2 days. The cells were maintained for 72 h in a humidified incubator at 37°C with 5% CO<sub>2</sub> in air and 1.5 ml of growth medium consisting of a 4:1 (vol/vol) ratio of DMEM (GIBCO Life Technologies; Breda, The Netherlands) and medium 199 (GIBCO) supplemented with 5% FCS (Roche Diagnostics; Almere, The Netherlands), 5% horse serum (Sigma-Aldrich; Zwijndrecht, The Netherlands), 100 U/ml of penicillin (Roche), and 100 mg/ml of streptomycin (Roche). The incubations with prorenin (see *Incubation of cells with prorenin at 4 or 37°C*) were carried out under serum-free conditions. Before the start of each experiment, cells were washed with 1 ml of warm (37°C) PBS consisting of (in mM) 140 NaCl, 2.6 KCl, 1.4 KH<sub>2</sub>PO<sub>4</sub>, and 8.1 Na<sub>2</sub>HPO<sub>4</sub> (pH 7.4). The cells were then preincubated either at 4 or 37°C for 30 min with 0.4 ml of incubation medium consisting of a 4:1 (vol/vol) ratio of DMEM and medium 199 supplemented with 1% (wt/vol) BSA (Sigma-Aldrich).

**Prorenin preparation.** Recombinant human prorenin was a kind gift of Dr. S. Mathews (Hoffmann-LaRoche; Basel, Switzerland). It was secreted by CHO cells transfected with a vector containing human prorenin cDNA. To remove traces of renin, the prorenin was partially purified by Cibacron blue Sepharose affinity chromatography (Amersham Pharmacia Biotech; Roosendaal, The Netherlands). The intrinsic renin activity of the prorenin preparation before proteolytic activation was <2% of the activity after complete proteolytic activation when the prorenin preparation contained  $\sim 2 \times 10^5$  U/l (4  $\mu$ M) renin.

**Man-6-P receptor affinity chromatography.** To separate prorenin into fractions that do or do not contain the man-6-P signal, recombinant human prorenin was applied to a 0.5-ml bovine liver man-6-P/IGF-II receptor column (kindly provided by Dr. S. Kornfeld, St. Louis, MO). The column was equilibrated with column buffer containing 50 mM imidazole at pH 6.5, 150 mM NaCl, 5 mM Na- $\beta$ -glycerophosphate, 0.1% (wt/vol) BSA, 6  $\mu$ M antipain, 8  $\mu$ M leupeptin, 6  $\mu$ M pepstatin A, 7  $\mu$ M chymostatin (all from Sigma-Aldrich), and 10 kallikrein inhibitor U/ml of aprotinin (Bayer; Mijdrecht, The Netherlands) (26). All manipulations were performed at 4°C. After the application of recombinant human prorenin (500 units in 0.1 ml of column buffer), the column was washed with column buffer ("column runthrough"). Subsequently, 10 mM man-6-P was added to the column buffer and man-6-P-containing prorenin was eluted. Prorenin was measured in 0.5-ml fractions. Fractions corresponding to column runthrough (i.e., man-6-P-free prorenin) and man-6-P-containing prorenin were separately pooled, concentrated, and adjusted to contain PBS (pH 7.4) by Centricon C-30 ultrafiltration (Amicon Bioseparations; Bedford, MA).

**Incubation of cells with prorenin at 4 or 37°C.** After preincubation at 4 or 37°C for 30 min under serum-free conditions (see *Cell culture*), experiments were started by replacing the incubation medium by 4 or 37°C incubation medium containing either recombinant human prorenin (final concentration 3–1,000 U/l), man-6-P-free recombinant human prorenin (10 U/l), or man-6-P-containing recombinant human prorenin (10 U/l). Incubations at 37°C were also performed with pools of human plasma and human amniotic fluid diluted to a 1:3 ratio with incubation medium. Plasma was obtained from subjects with renal artery stenosis (one man and two women, age 41–66 yr). Amniotic fluid was obtained from three women (age 19–38 yr) after natural delivery. All incubations at 4 or 37°C lasted 4 h and were performed in both the presence and absence of 10 mM man-6-P to determine man-6-P/IGF-II receptor-specific prorenin binding (58). To investigate the intracellular presence of man-6-P/IGF-II receptors in myocytes, incubations with recombinant human prorenin (1,000 U/l) at 4°C were also performed after prior permeabilization of the cells with PBS containing 0.2% saponin (Merck; Amsterdam, The Netherlands) (52). Finally, to determine what proteases are responsible for prorenin activation, incubations at 37°C were performed in the presence of the following five protease inhibitors: 0.04 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF, Calbiochem; LaJolla, CA); 0.1 mM leupeptin; 0.14 mM L-trans-3-carboxyoxiran-2-carboxyl-L-leucylagmatine (E64, Sigma-Aldrich); 1.0 mM 1,10-phenanthroline (Merck); or 0.1 mM pepstatin A.

At the end of the incubation period the culture medium was removed. Each well was washed three times with 1 ml of ice-cold PBS. Prorenin was not detectable in the last PBS wash. Cells were then lysed in 0.2 ml of ice-cold PBS containing 0.2% Triton X-100 (Merck), and the cell lysates were quickly frozen on dry ice. Cell lysates were stored at –70°C until assays for total and cell-activated prorenin were performed.

To determine whether prorenin had been internalized, the acid-wash method was used (58). At low pH surface-bound prorenin dissociates from the cells; internalized prorenin, however, is acid resistant. Briefly, after the cells had been washed three times with ice-cold PBS, cells were incubated at 4°C with 0.4 ml of an acid solution containing 50 mM glycine and 150 mM NaCl at pH 3.0. After 10 min the acid solution was removed, the cells were washed and lysed as described above, and the cell lysates were stored at –70°C until being assayed.



*Incubation of cells at 4°C followed by incubation at 37°C.* To study the kinetics of prorenin activation in more detail, cells cultured in six-well plates (Corning Costar) were loaded with 1 ml of recombinant human prorenin-containing incubation medium (final concentration 100 U/l) for 2 h at 4°C. After this period, free prorenin was removed by washing the cells three times with 3 ml of ice-cold PBS. After the last wash, 1 ml of fresh incubation medium without prorenin at 37°C was added, and the cells were incubated at 37°C. The incubation was terminated after 15, 30, 60, 120, 180, or 240 min by removing the culture medium and subsequently washing the cells three times with 3 ml of ice-cold PBS. The cells were then lysed in 0.5 ml of ice-cold PBS containing 0.2% Triton X-100 as described above. Culture medium and cell lysate were stored at -70°C until assays for total prorenin, cell-activated prorenin, and intact propeptide-containing prorenin were performed.

*Prorenin measurement.* In the experiments with recombinant human prorenin in myocytes, cell-activated prorenin and total prorenin (i.e., cell-activated plus nonactivated prorenin) were measured by immunoradiometric assay (IRMA). The proteolytic activation of recombinant human prorenin by myocytes was monitored with an IRMA specific for intact prorenin, i.e., prorenin in which the propeptide was still bound to the renin part of the molecule. The IRMAs are not sensitive enough to measure the low levels of cell-activated and total recombinant human prorenin in fibroblasts. The prorenin measurements in these cells were therefore performed by enzyme-kinetic assay. The renin and prorenin measurements in the experiments with human plasma and human amniotic fluid were also performed by enzyme-kinetic assay.

*Enzyme-kinetic assay.* Cell-activated recombinant prorenin and native renin were measured by incubating a 100- $\mu$ l sample for 3 h with a saturating amount of sheep renin substrate at 37°C and pH 7.4 in the presence of serine protease and angiotensinase inhibitors (58). The generated ANG I was quantified by RIA. Results were expressed as microunits per 1,000,000 cells or microunits per milliliter medium using plasmin-activated recombinant human prorenin as a reference. The lower limit of detection was 1  $\mu$ U/10<sup>6</sup> cells or 1  $\mu$ U/ml of medium. To measure total recombinant prorenin and native prorenin, the samples were first incubated for 48 h at 4°C with plasmin (0.5 caseinolytic U/ml, obtained from Chromogenix; Mölndal, Sweden). This preincubation with plasmin causes complete proteolytic activation of prorenin. The serine protease inhibitor aprotinin (final concentration 100 kallikrein-inhibiting U/ml) was added to the incubation medium of the ANG I-generating step to inactivate plasmin.

*Immunoradiometric assays.* The monoclonal antibodies (MAb) used in these assays were MAb R3-36-16 (Nichols Institute; Wychen, The Netherlands), which reacts equally well with activated and nonactivated prorenin ( $K_d = 0.6$  pM)

(30), MAb R1-20-5 (Nichols Institute), which recognizes the active site of renin ( $K_d = 250$  nM) (60), and MAb F258-37-B1 (a kind gift of Dr. S. Mathews, Hoffmann-LaRoche), which is directed against the COOH-terminal part (P20-P43) of the propeptide (Fig. 1) and does not react (<0.1%) with renin (F. H. M. Derkx, unpublished observation). MAbs R1-20-5 and F258-37-B1 do not react (<0.1%) with intact inactive prorenin. However, they do react with prorenin after the treatment of prorenin with the renin inhibitor remikiren (0.1 mM; a kind gift of Dr. W. Fischli, Hoffmann-LaRoche; Basel, Switzerland) for 48 h at 4°C (16). The renin inhibitor enters the enzymatic cleft in which the active site is located, thereby inducing a slow conformational change of the inactive ("closed") form of the prorenin molecule into the active ("open") form. This nonproteolytic conformational change not only allows subsequent recognition of the active site by MAb R1-20-5, it also causes the propeptide to move to the surface of the molecule so that it can react with MAb F258-37-B1. In the IRMA for cell-activated and total prorenin, 200  $\mu$ l of untreated and remikiren-treated sample (diluted to a 1:9 ratio in heat-inactivated sheep serum, obtained from Biotrading; Mijdrecht, The Netherlands), respectively, were incubated for 6 h at 37°C with biotinylated MAb R3-36-16, <sup>125</sup>I-labeled R1-20-5 (250,000 counts/min), and an avidin-coated bead as described (15). In the IRMA for intact propeptide-containing prorenin, 250  $\mu$ l of remikiren-treated sample (diluted to a 1:4 ratio in heat-inactivated sheep serum) were incubated for 6 h at 37°C with an avidin-coated bead to which 1.6  $\mu$ g of biotinylated MAb F258-37-B1 had been bound (1). The bead was then washed three times with 2 ml of PBS containing 0.1% (wt/vol) BSA and subsequently incubated with 100  $\mu$ l (250,000 counts/min) of <sup>125</sup>I-labeled MAb R1-20-5 and 200  $\mu$ l of heat-inactivated sheep serum containing 0.1 mM remikiren for 24 h at room temperature. After the 6-h and 24-h incubation periods, the beads in both IRMAs were washed three times with 2 ml of PBS containing 0.01% (vol/vol) Triton X-100, and bound radioactivity was measured in a gamma-counter. The results of these assays were expressed as microunits per 1,000,000 cells using intact recombinant human prorenin as a reference. The lower limit of detection was 5  $\mu$ U/10<sup>6</sup> cells.

*Statistical analysis.* Results are expressed as means  $\pm$  SE. Data were compared using Student's *t*-test for paired observations or ANOVA. A value of  $P < 0.05$  was considered to be significant. Binding data were analyzed by nonlinear regression analysis using the GraphPad Prism (version 3) computer program (GraphPad; San Diego, CA).

## RESULTS

*Incubation of myocytes and fibroblasts with recombinant human prorenin at 4 or 37°C.* Myocytes (Fig. 2) and fibroblasts (Fig. 3) bound recombinant human pro-

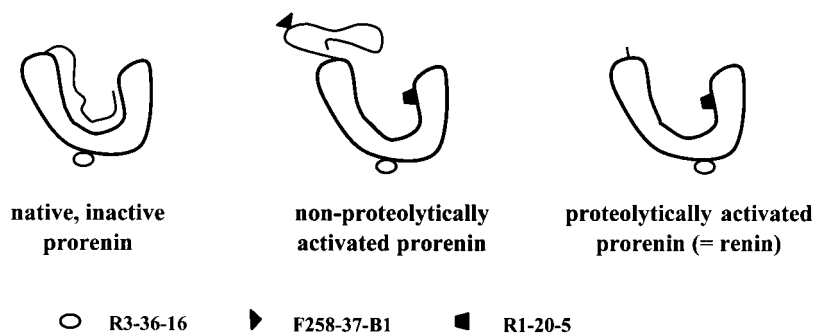


Fig. 1. Prorenin isoforms and recognition sites of the monoclonal antibodies used in the present study.

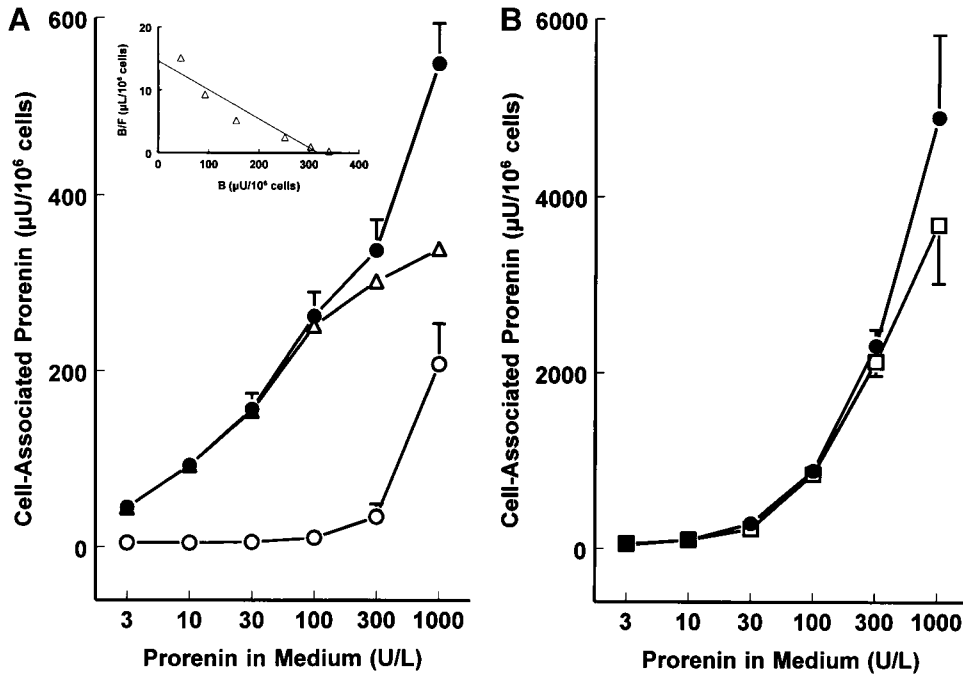


Fig. 2. A: levels of cell-associated total prorenin after incubation of myocytes with recombinant human prorenin at 4°C for 4 h in the absence (●) or presence (○) of 10 mM mannose-6-phosphate (man-6-P). Man-6-P/insulin-like growth factor-II (IGF-II) receptor-specific binding (Δ) was taken as the difference between levels of cell-associated prorenin with and without man-6-P. Data are means ± SE; n = 8. Dissociation constant ( $K_d$ ) and maximum number of binding sites ( $B_{max}$ ) were calculated from a plot according to Scatchard (inset; B/F represents the ratio of bound to free prorenin). B: levels of cell-associated total (●) and cell-activated (□) prorenin after incubation of myocytes with recombinant human prorenin at 37°C for 4 h. Data are means ± SE; n = 7.

renin in a concentration-dependent manner at both 4 and 37°C. Cell-associated prorenin at 37°C but not at 4°C was acid resistant (data not shown), indicating that prorenin internalization occurred at 37°C only. For a given prorenin concentration in the medium, the level of cell-associated total prorenin after 4 h of incubation was 10–15 times higher at 37°C than at 4°C.

**Binding of recombinant human prorenin to man-6-P/IGF-II receptors:  $K_d$  and  $B_{max}$ .** Man-6-P significantly reduced recombinant human prorenin binding in both myocytes (Fig. 2A) and fibroblasts (Fig. 3A). The reduction was much smaller in fibroblasts than in myocytes,

suggesting that fibroblasts may contain a second prorenin binding site that cannot be blocked by man-6-P. However, because the levels of cell-associated prorenin after 4 h of incubation in the presence of man-6-P were similar in myocytes and fibroblasts, a more likely explanation is that non-man-6-P/IGF-II receptor-mediated prorenin binding represents nonspecific binding and that the man-6-P-induced reduction in prorenin binding is smaller in fibroblasts because these cells contain less cell-surface man-6-P/IGF-II receptors. Indeed, Scatchard analysis revealed that binding of prorenin to man-6-P/IGF-II receptors occurred with simi-

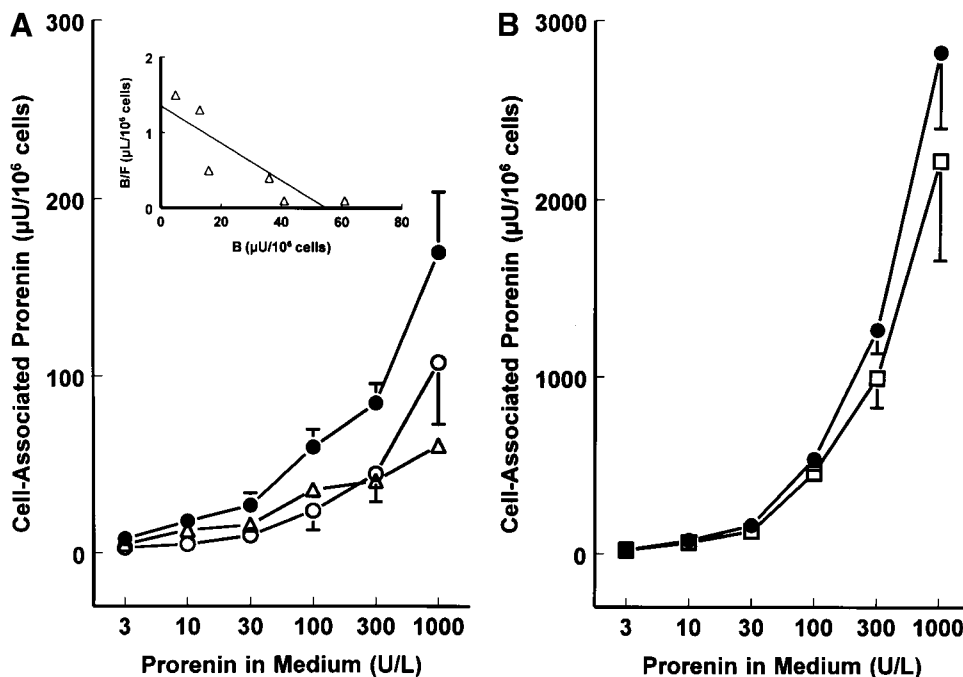


Fig. 3. A: levels of cell-associated total prorenin after incubation of fibroblasts with recombinant human prorenin at 4°C for 4 h in the absence (●) or presence (○) of 10 mM mannose-6-phosphate (man-6-P). Man-6-P/IGF-II-receptor-specific binding (Δ) was taken as the difference between levels of cell-associated prorenin with and without man-6-P. Data are means ± SE; n = 4.  $K_d$  and  $B_{max}$  were calculated from a plot according to Scatchard (inset; B/F represents the ratio of bound to free prorenin). B: levels of cell-associated total (●) and cell-activated (□) prorenin after incubation of fibroblasts with recombinant human prorenin at 37°C for 4 h. Data are means ± SE; n = 4.

lar affinity in both myocytes ( $K_d = 0.6 \pm 0.1$  nM;  $n = 8$ ) and fibroblasts ( $K_d = 0.8 \pm 0.2$  nM;  $n = 4$ ) and that  $B_{max}$  was  $3,840 \pm 510$  sites/myocyte and  $650 \pm 150$  sites/fibroblast. Prior permeabilization of myocytes with saponin increased man-6-P/IGF-II receptor-dependent prorenin binding  $7.7 \pm 0.4$ -fold ( $n = 4$ ), indicating that  $>85\%$  of the man-6-P/IGF-II receptors is located intracellularly. Recycling of these intracellular receptors to the cell membrane most likely explains why the levels of cell-associated prorenin at  $37^\circ\text{C}$  are much higher than at  $4^\circ\text{C}$ .

*Does prorenin binding occur independently of man-6-P/IGF-II receptors?* To investigate whether prorenin binding occurs independently of man-6-P/IGF-II receptors, recombinant human prorenin was separated into man-6-P-free and man-6-P-containing fractions with the help of a bovine man-6-P/IGF-II receptor-affinity column (Fig. 4). The amount of recombinant human prorenin ( $38 \pm 1\%$ ;  $n = 3$ ) that was not bound by this column (which did not contain the man-6-P signal) resembled the amount of prorenin ( $38 \pm 2\%$ ) that eluted only after the addition of man-6-P to the elution buffer (which did contain the man-6-P signal). The remaining prorenin eluted shortly after the first runthrough peak (before the addition of man-6-P) and was not investigated further.

Incubation of myocytes and fibroblasts with man-6-P-containing prorenin resulted in cellular prorenin levels that were  $\approx 1.5$ – $2.5$  times higher than observed after incubation with nonfractionated prorenin (Fig. 5). Assuming that only man-6-P/IGF-II receptors are involved in prorenin binding, this is exactly what one would predict when exposing cardiac cells to a prorenin solution in which either all or only  $\approx 40\%$  of the prorenin molecules carry the man-6-P signal.

Incubation with man-6-P-free prorenin resulted in cellular prorenin levels that were equal to or lower than the levels observed after incubation with nonfrac-

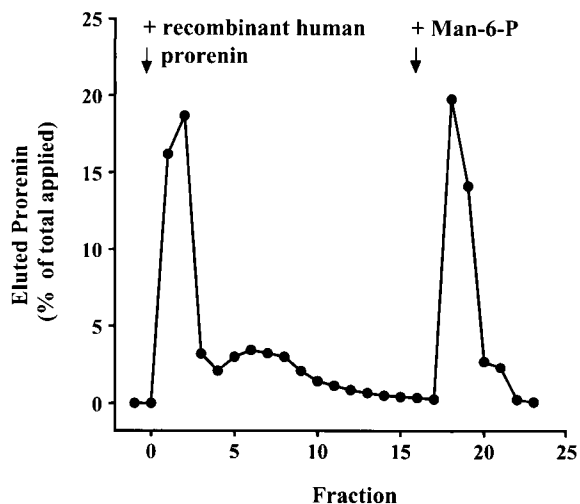


Fig. 4. Bovine liver man-6-P/IGF-II receptor-affinity chromatography of recombinant human prorenin. Representative example is shown. After application of prorenin (500 units), the column was washed with column buffer (see METHODS). Man-6-P-containing prorenin was eluted with 10 mM man-6-P in column buffer.

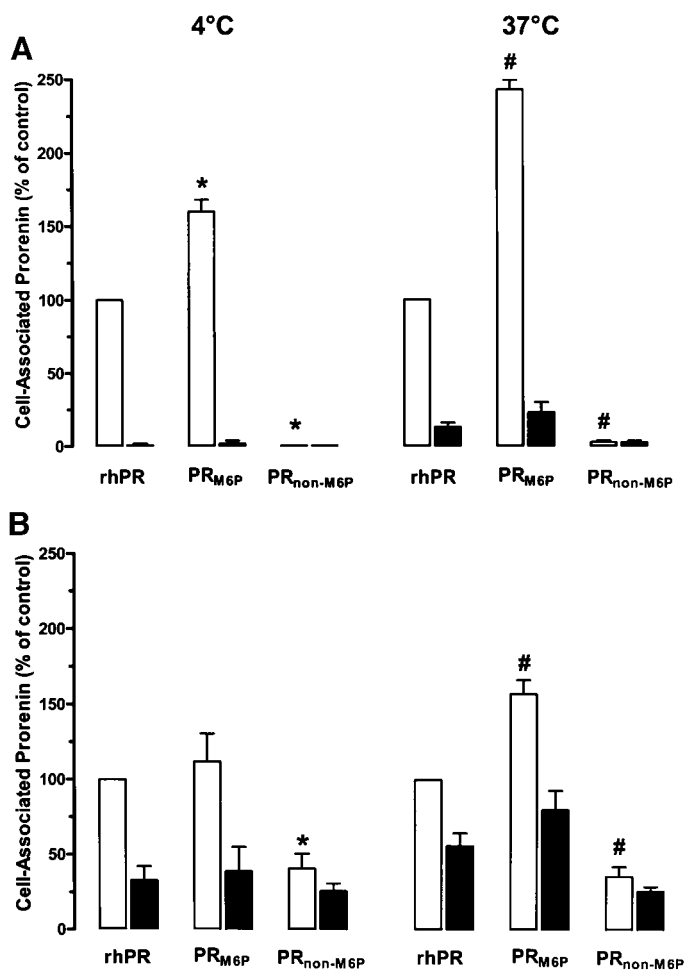


Fig. 5. Levels of cell-associated total prorenin after incubation of myocytes (A) or fibroblasts (B) with nonfractionated recombinant human prorenin (rhPR, 10 U/l; control), man-6-P-containing recombinant human prorenin (PR<sub>M6P</sub>, 10 U/l), or man-6-P-free recombinant human prorenin (PR<sub>non-M6P</sub>, 10 U/l) at 4 or  $37^\circ\text{C}$  for 4 h in the absence (open bars) or presence (filled bars) of 10 mM man-6-P. Data are means  $\pm$  SE ( $n = 3$ ) expressed as a percentage of the cellular levels of control. \* $P < 0.05$  vs. control at  $4^\circ\text{C}$ ; # $P < 0.05$  vs. control at  $37^\circ\text{C}$ .

tionated prorenin in the presence of man-6-P (Fig. 5). Because the latter level was similar for myocytes and fibroblasts (Figs. 2A and 3A), this level most likely represents nonspecific prorenin binding. Thus our findings do not provide evidence for prorenin binding to receptors other than the man-6-P/IGF-II receptor.

*Activation of recombinant human prorenin: effect of protease inhibitors.* Activation of recombinant human prorenin was detectable at  $37^\circ\text{C}$  only (Figs. 2B and 3B). Saturation of the activation process did not occur because the percentage of cell-associated prorenin that was activated was similar at all concentrations of prorenin to which the cells were exposed [ranging from  $88 \pm 15\%$  at 3 U/l to  $78 \pm 8\%$  at 1,000 U/l in myocytes ( $n = 7$ ) and from  $83 \pm 9\%$  at 3 U/l to  $75 \pm 9\%$  at 1,000 U/l in fibroblasts ( $n = 4$ )]. The serine protease inhibitor AEBSF partially blocked the activation of prorenin in myocytes but had no effect in fibroblasts (Table 1). None of the other protease inhibitors that were tested



Table 1. Effect of protease inhibitors on cellular levels of total (i.e., cell-activated and nonactivated) prorenin and cell-activated prorenin after incubating myocytes and fibroblasts for 4 h at 37°C with 100 U/l recombinant human prorenin

Protease Inhibitor	Type Protease Inhibited	Myocytes			Fibroblasts		
		n	Total prorenin, % of Control	Activated prorenin, % of Total prorenin	n	Total prorenin, % of Control	Activated prorenin, % of Total prorenin
None		7	100 ± 18	75 ± 6	5	100 ± 24	77 ± 6
Pepstatin A	Aspartic	4	109 ± 12	87 ± 3	4	115 ± 10	87 ± 7
Leupeptin	Serine and cysteine	5	143 ± 5*	85 ± 8	4	179 ± 18†	85 ± 6
AEBSF	Serine	5	118 ± 17	52 ± 4*	4	73 ± 6	71 ± 10
E64	Cysteine	5	136 ± 10	70 ± 11	3	136 ± 15	82 ± 12
1,10-Phenanthroline	Metallo	5	111 ± 17	69 ± 9	4	72 ± 20	64 ± 6

Data are means ± SE; n, no. of myocytes or fibroblasts. AEBSF, 4-(2-aminoethyl)-benzenesulfonylfluoride hydrochloride; E64, L-trans-3-carboxyoxiran-2-carbonyl-L-leucylagmatine. \*P < 0.05 vs. control; †P < 0.01 vs. control.

blocked prorenin activation in either myocytes or fibroblasts. The cysteine protease inhibitor E64 and the mixed serine-cysteine protease inhibitor leupeptin increased the level of cell-associated total prorenin by 40–50% in myocytes and by 40–80% in fibroblasts, thereby indicating that cysteine proteases contribute to (pro)renin degradation in cardiac cells.

*Proteolytic or nonproteolytic activation of prorenin in myocytes.* After 2 h of incubation at 4°C with 100 U/l prorenin and repeated washing with ice-cold PBS, the level of cell-associated total prorenin was 350 ± 45 μU/10<sup>6</sup> cells (n = 7). Acid wash confirmed that at that time, all cell-associated prorenin was located on the cell surface. Immediately after the temperature was raised to 37°C, the level of cell-associated intact (i.e., prosegment-containing) prorenin started to decrease. The decrease followed a biphasic pattern (Fig. 6). The first phase [half-life (t<sub>1/2</sub>) = 5 ± 3 min] corresponds with

the release of cell-surface-bound intact prorenin into the medium and did not differ from the first phase that was observed for total prorenin (t<sub>1/2</sub> = 4 ± 1 min). This phase is determined by the rapidity of the internalization process. The second phase represents the proteolytic removal of the prosegment from internalized prorenin (t<sub>1/2</sub> = 21 ± 4 min), because a rise in the cellular levels of activated prorenin was simultaneously observed. These levels reached a maximum after 60 min and then started to decrease, with a t<sub>1/2</sub> (67 ± 8 min) similar to that of the second phase of total prorenin (t<sub>1/2</sub> = 74 ± 5 min). Release of activated prorenin into the medium could not be demonstrated during the 6-h observation period (data not shown). Taken together, these findings suggest that prorenin, after its internalization, is rapidly activated by proteolytic cleavage of the prosegment and that activated prorenin is subsequently metabolized by degrading enzymes without being released into the medium.

*Incubation of myocytes with native human (pro)renin at 37°C.* To verify man-6-P/IGF-II receptor-dependent binding, internalization, and activation of native human prorenin of renal and nonrenal origin, myocytes were incubated at 37°C during 4 h with human plasma or human amniotic fluid (diluted to a 1:3 ratio with incubation medium) in the presence or absence of man-6-P. Expressed as a percentage of the sum of renin and prorenin, plasma and amniotic fluid contained 79 ± 10% and 94 ± 3% prorenin, respectively (Fig. 7). Incubation with plasma resulted in man-6-P/IGF-II receptor-mediated (pro)renin uptake by myocytes. After incubation with plasma, myocytes contained predominantly (>75%) renin (Fig. 7). Because man-6-P/IGF-II receptors bind and internalize man-6-P-containing renin and prorenin equally well (58), this is not due to selective uptake of plasma renin. The increased renin-to-prorenin ratio in cell lysates therefore suggests that internalized plasma prorenin, like internalized recombinant human prorenin, is activated to renin by myocytes. The cellular (pro)renin levels after incubation with amniotic fluid were close to the detection limit and did not differ with or without man-6-P (Fig. 7). Thus amniotic fluid does not contain prorenin that carries the man-6-P signal.

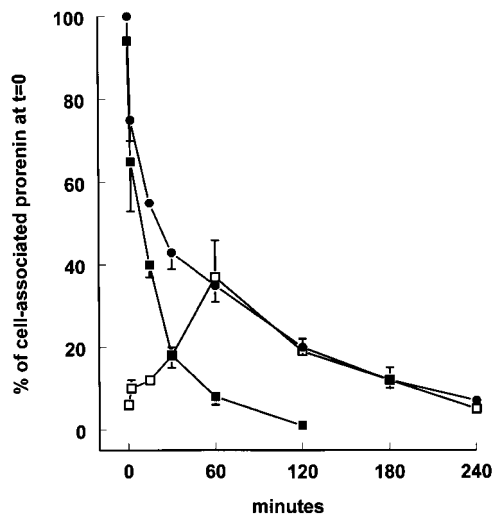


Fig. 6. Time course of internalization and proteolytic activation of prorenin by myocytes. Cells were preincubated with recombinant human prorenin (100 U/l) for 2 h at 4°C and were washed with ice-cold PBS. Fresh culture medium free of prorenin was added and the incubation was continued at 37°C. Data are means ± SE (n = 7) expressed as a percentage of the cellular levels of prorenin at the end of the 2-h preincubation period (t = 0). Total prorenin (●), cell-activated prorenin (□), and intact prosegment-containing prorenin (■) are shown.

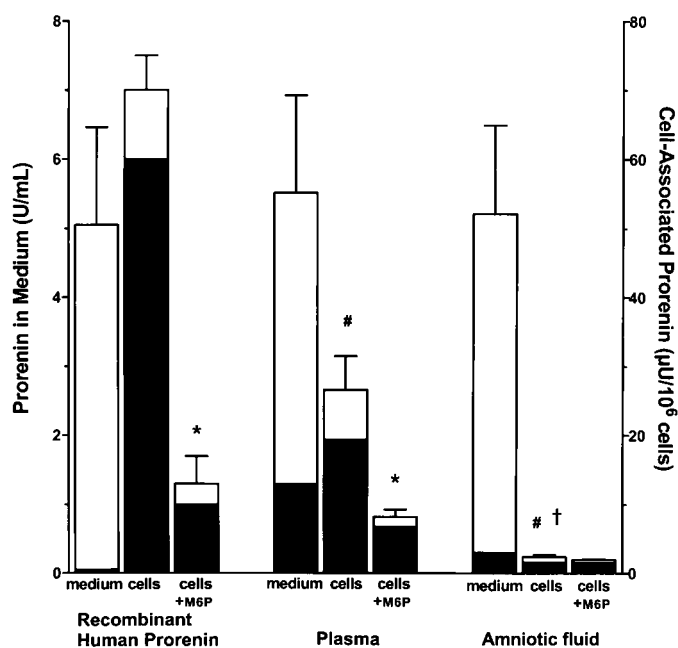


Fig. 7. Levels of total prorenin in medium and cell lysate after incubation of myocytes with recombinant human prorenin, human plasma (diluted to 1:3 ratio in incubation medium), or human amniotic fluid (diluted to 1:3 ratio in incubation medium) at 37°C for 4 h in the absence or presence of 10 mM man-6-P. Solid region represents activated prorenin (i.e., renin). Data are means  $\pm$  SE ( $n = 4$ ). \* $P < 0.05$  vs. without man-6-P; # $P < 0.05$  vs. recombinant human prorenin; † $P < 0.01$  vs. plasma.

## DISCUSSION

The data of the present study show that man-6-P-containing prorenin binds with high affinity to man-6-P/IGF-II receptors on neonatal rat myocytes and fibroblasts. Binding is followed by internalization and subsequent proteolytic activation to renin, possibly by a serine protease. Internalization is greatly enhanced by receptor recycling. Obtained with recombinant human prorenin, these results could be fully reproduced with native human prorenin of renal origin (i.e., prorenin in human plasma) but not with native human prorenin of extrarenal origin (i.e., prorenin in human amniotic fluid), suggesting that local angiotensin production by nonrenin-producing cells such as myocytes and fibroblasts (59) depends on renin of renal origin.

Evidence for prorenin binding to receptors other than the man-6-P/IGF-II receptor was not obtained. First, man-6-P significantly reduced prorenin binding in myocytes and fibroblasts. Although the reduction was more modest in fibroblasts, prorenin binding in the presence of man-6-P (i.e., "nonspecific" prorenin binding) was similar in myocytes and fibroblasts and did not differ from prorenin binding to human umbilical vein endothelial cells in the presence of man-6-P (data not shown). These findings may point to the existence of a second unidentified prorenin receptor. However, in view of the similarity of the non-man-6-P/IGF-II receptor-mediated prorenin binding in cardiac and endothelial cells, a more likely explanation is that binding in the presence of man-6-P represents nonspecific bind-

ing. Second, when exposing cardiac cells to a recombinant human prorenin fraction in which all molecules contain the man-6-P signal (as opposed to only 40% of the molecules in the nonfractionated recombinant human prorenin preparation), binding and internalization increased 1.5- to 2.5-fold. This is within the range expected when increasing the level of man-6-P-containing prorenin  $\approx$ 2.5-fold and also indicates that the true  $K_d$  is  $\approx$ 2.5-fold lower than the values reported here. The increases were somewhat smaller in fibroblasts, which is most likely due to the fact that man-6-P/IGF-II receptor-specific prorenin binding in these cells was comparable to prorenin binding not mediated via man-6-P/IGF-II receptors (i.e., nonspecific prorenin binding). A low number of cell-surface man-6-P/IGF-II receptors also explains why the decrease in prorenin binding in the presence of excess man-6-P was more modest in fibroblasts than in myocytes.

Binding of prorenin to man-6-P/IGF-II receptors occurred with high affinity ( $K_d < 1$  nM), which suggests that prorenin occupies both man-6-P binding sites of this receptor (22, 38). In both fibroblasts and myocytes, the levels of cell-associated prorenin increased 10–15-fold when the cells were incubated with prorenin at 37°C instead of 4°C. This can be explained on the basis of the internalization and continuous recycling of man-6-P/IGF-II receptors among the cell surface and intracellular compartments (e.g., Golgi and endosomes) that are known to occur at 37°C but not at 4°C (10). In myocytes, >85% of the man-6-P/IGF-II receptors were found to be located in the cells. Internalization of the receptor-prorenin complex appeared to occur rapidly with a half-life of <5 min and was followed by activation of prorenin. The rapid decline in the levels of prorenin still containing the carboxy terminal part of its prosegment [as measured with monoclonal antibody F258–37-B1 (Fig. 6)] confirms that prorenin activation was a proteolytic process and that cleavage occurred in the correct manner. The cells did not release proteolytically activated prorenin (i.e., renin) into the medium. Instead, renin was degraded intracellularly with a half-life of  $\sim$ 1 h. This raises the possibility that the man-6-P/IGF-II receptor is involved in (pro)renin clearance. Alternatively and perhaps more likely in view of the rapid activation process, cell-activated prorenin may contribute to intracellular angiotensin generation before its destruction. Several studies have provided evidence for intracellular angiotensin generation (17, 32, 42), and ANG II is known to activate intracellular AT<sub>1</sub> receptors in the cytosol and nucleus (23, 27). In the absence of local renin synthesis, such intracellular angiotensin generation will depend on the uptake of circulating renin or prorenin. Moreover, because in previous studies we were unable to demonstrate angiotensinogen synthesis in neonatal rat cardiac myocytes and fibroblasts (59), it seems that renin substrate also has to be internalized to allow intracellular angiotensin generation in the neonatal heart. This may be different in the adult heart or under pathological conditions (40). In addition, age as well as pathological conditions may affect the density of cardiac man-6-P/



IGF-II receptors and/or give rise to the appearance of alternative (pro)renin receptors or uptake mechanisms (4, 8, 46, 48, 53).

At present it cannot be concluded what enzymes are responsible for the intracellular prorenin activation. With the exception of the serine protease inhibitor AEBSF, none of the protease inhibitors used in this study exerted an inhibitory effect on prorenin activation. This is not due to the inability of these blockers to reach the proper intracellular compartment because others have demonstrated efficacy in the same setup (7, 44). Moreover, the mixed serine-cysteine protease inhibitor leupeptin and the cysteine protease inhibitor E64 increased the levels of cell-associated prorenin by >40%, indicating that cysteine proteases contribute to (pro)renin degradation and that exogenous inhibitors apparently are capable of reaching the intracellular sites where degradation occurs. Possible candidates for the prorenin-activating enzyme are kallikrein (37), prohormone convertases (51), and cathepsin B (45), although the latter seems unlikely in view of the absence of an inhibitory effect of E64. These enzymes have been demonstrated in the heart (6, 41, 49, 55). Furthermore, Baba and colleagues (3) described a serine protease (mol mass 26 kDa) capable of activating prorenin in rat adrenal explant cultures. The pH optimum for prorenin activation by this enzyme was 6.5. The rapid activation of prorenin in the present study, which is indicative for early endosomal (i.e., at pH 6.5) removal of the prosegment, as well as the inhibitory effects of AEBSF on prorenin activation in myocytes, is in agreement with the existence of a similar serine protease in the heart.

Finally, our results are not limited to recombinant human prorenin but also apply to native human plasma prorenin. Plasma prorenin is predominantly of renal origin (11), although extrarenal prorenin sources such as the eye (12), ovary (20), placenta (33), and testis (54) are also known to contribute to circulating levels of prorenin. When incubated at 37°C for 4 h with plasma containing prorenin (and low levels of renin), myocytes were found to contain predominantly renin. It is unlikely that this is due to selective uptake of plasma renin because man-6-P/IGF-II receptors do not make a distinction between man-6-P-containing renin and prorenin (58). Therefore, these findings suggest that plasma prorenin, like recombinant prorenin, is activated intracellularly to renin. In absolute terms, the levels of cell-associated renin and prorenin after incubation with plasma were two to three times lower than after incubation with equal amounts of recombinant prorenin (Fig. 7). This may have at least two explanations. First, the percentage of plasma prorenin carrying the man-6-P signal may be lower than the percentage of recombinant human prorenin carrying this signal. Second, the high (in the nanomolar range) levels of soluble man-6-P/IGF-II receptors that have been reported in plasma (9) may affect prorenin binding to cellular man-6-P/IGF-II receptors, especially because the density of the latter (expressed per million cells) is in the femtomole range. Studies reporting on

the presence of high-molecular-weight forms of prorenin in plasma (5, 47) are in agreement with the contention that plasma prorenin is in part bound to soluble man-6-P/IGF-II receptors. Further experiments are required to resolve this issue. Interestingly, prorenin present in human amniotic fluid did not bind to myocytes, which suggests that this prorenin does not contain the man-6-P signal. Amniotic fluid prorenin is derived primarily from the placental chorion laeve (33) (i.e., is synthesized extrarenally), and its isoelectric focusing pattern differs from that of plasma prorenin (36). The levels of soluble man-6-P/IGF-II receptors in amniotic fluid are ~100 times lower than in plasma (9). Whether prorenin from other extrarenal sources also lacks the man-6-P signal is currently unknown, but if so, this would imply that only prorenin of renal origin is meant to be taken up by the heart and thus that cardiac angiotensin generation is regulated by the kidney and not by other (pro)renin-producing tissues. Consequently, the release of large amounts of prorenin from extrarenal sources [e.g., during pregnancy (33)] would not necessarily result in increased cardiac angiotensin generation.

In conclusion, our data support the concept of cardiac angiotensin generation by renal (pro)renin. Prorenin is sequestered from the circulation by cardiac cells through binding to man-6-P/IGF-II receptors. Binding occurs with high affinity and is limited to prorenin containing the man-6-P signal. After binding, the prorenin-man-6-P/IGF-II receptor complex is internalized and prorenin is activated to renin, possibly by a serine protease in an early endosomal compartment. The receptor then returns to the cell surface to repeat the binding and internalization process. Activated prorenin may participate in intracellular ANG I production before its destruction by cysteine proteases in lysosomes.

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