Megalin

A Novel Endocytic Receptor for Prorenin and Renin

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Abstract —Megalin is an endocytic receptor contributing to protein reabsorption. Impaired expression or trafficking of megalin increases urinary renin and allowed the detection of prorenin, which normally is absent in urine. Here, we investigated (pro)renin uptake by megalin, using both conditionally immortalized proximal tubule epithelial cells and Brown Norway Rat yolk sac cells (BN16). To distinguish binding and internalization, cells were incubated with recombinant human (pro)renin at 4°C and 37°C, respectively. (Pro)renin levels were assessed by immunoradiometric assay. At 4°C, BN16 cells bound 3× more prorenin than renin, suggestive for a higher affinity of prorenin. Similarly, at 37°C, prorenin accumulated at 3- to 4-fold higher levels than renin in BN16 cells. Consequently, depletion of medium prorenin (but not renin) content occurred after 24 hours. No such differences were observed in conditionally immortalized proximal tubule epithelial cells, and M6P (mannose-6-phosphate) greatly reduced conditionally immortalized proximal tubule epithelial cells (pro)renin uptake, suggesting that these cells accumulate (pro)renin largely via M6P receptors. M6P did not affect (pro)renin uptake in BN16 cells. Yet, inhibiting megalin expression with siRNA greatly reduced (pro) renin binding and internalization by BN16 cells. Furthermore, treating BN16 cells with albumin, an endogenous ligand of megalin, also decreased binding and internalization of (pro)renin, while deleting the (pro)renin receptor affected the latter only. Exposing prorenin's prosegment with the renin inhibitor aliskiren dramatically increased prorenin binding, while after prosegment cleavage with trypsin prorenin binding was identical to that of renin. In conclusion, megalin might function as an endocytic receptor for (pro)renin and displays a preference for prorenin. Megalin-mediated endocytosis requires the (pro)renin receptor. (***Hypertension*****. 2020;75:1242-1250. DOI: 10.1161/HYPERTENSIONAHA.120.14845.)**

● **Data Supplement**

Key Words: mannose-6-phosphate receptor ■ prorenin receptor ■ renin ■ trypsin ■ tubular reabsorption

Components of the renin-angiotensin system (RAS) in urine have been suggested to reflect renal RAS activity, that is, to be released from renal tissue sites.[1,2] However, recent studies challenge this view and instead support the concept that urinary renin, prorenin, and angiotensinogen are blood-derived (ie, filtered through the glomerulus) and are reabsorbed in the proximal tubule.[3–5] Hence, elevated urinary RAS component levels rather reflect the net result of enhanced filtration (due to an injured glomerular barrier) and diminished reabsorption (due to tubular dysfunction). Remarkably, prorenin reabsorption is close to 100%, and thus under physiological circumstances, prorenin cannot be detected in urine.[6] Since the reabsorption is megalin-dependent, prorenin can be detected in urine of patients with Dent's disease or Lowe syndrome, in whom tubular reabsorption is disturbed due to impaired renal expression and trafficking of megalin.[7] It can also be detected in urine of women with preeclampsia.[8]

Megalin is a 600 kDa single transmembrane protein with a small intracellular tail and a large extracellular domain containing 4 ligand-binding regions, allowing it to bind >40 different ligands, including albumin.[9,10] Megalin forms a 1:1 complex with the extracellular protein cubilin. The ligand-binding regions and the gross structural composition of extracellular motifs of megalin are identical in humans and rats.[11] Megalin is the major endocytic receptor in proximal tubule cells.[10] It co-localizes with renin in the proximal convoluted tubule,[12] and disruption of megalin expression in rodents resulted in a significant rise in urinary renin levels.[4] Megalin-mediated renin reabsorption amounts to ≈95%,[5,6] and thus a reduction to 90% or 85% would already double or triple urinary renin levels. Consequently, even small variations in megalin may cause great variation in urinary renin levels for a given plasma renin level, thus explaining why plasma and urinary renin are often unrelated. Ang II (angiotensin II) negatively regulates megalin expression at both the mRNA and protein levels through its type 1 receptor.[13,14]

In the present study, we set out to compare megalin-mediated renin and prorenin uptake, making use of both human

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conditionally immortalized proximal tubule epithelial cells line (ciPTEC), which endogenously express multiple influx and efflux transporters, in addition to the entire endocytosis machinery,[15–17] and Brown Norway rat yolk sac epithelial cells (BN16), which are known to highly express megalin.[9] We hypothesized that megalin prefers prorenin over renin, thus explaining why urine normally contains renin only. A comparison was made versus other receptors capable of binding and internalizing renin and prorenin, that is, the (P)RR [(pro)renin receptor] and the M6P (mannose-6-phosphate) receptor. We distinguished binding and internalization by performing studies at 4°C and 37°C, respectively and made use of either receptor antagonists or siRNA to determine the contribution of each receptor separately.

Methods

All supporting data are available within the article and in the Data Supplement.

Human and Rat Renal Cortex

Rest material of one healthy human kidney cortex was supplied by the Department of Pathology. This occurred anonymously, in agreement with the Dutch regulations on medical research. Similarly, the kidney cortex of one healthy, 12-week old Sprague Dawley rat was obtained. Both cortical tissue pieces were frozen immediately in liquid nitrogen and stored at −70°C.

Cell Culture

ciPTEC cells were derived from a healthy donor and immortalized as described.[15] Cells were cultured in phenol red-free DMEM/F12 (Invitrogen, Breda, the Netherlands) supplemented with 10% (v/v) FCS (MP Biomedicals, Uden, the Netherlands), containing insulin (5 µg/mL), transferrin (5 µg/mL), selenium (5 ng/mL), hydrocortisone (36 ng/mL), epithelial growth factor (10 ng/mL), and triiodothyronine (40 pg/mL). P22-24 passages of ciPTEC were used. The cells were seeded at a density of 55.000 cells per cm^2 in 96- or 24-well plates (Costar, Corning, NY), cultured for 24 hours at 33°C, and matured for 7 days at 37°C, in 5% CO_2. BN16 cells[18] were cultured to confluence in a humidified incubator in 75 cm^2 flasks with Minimum Essential Media (Gibco, Thermo Fisher Scientific, Paisley, UK) supplemented with 1×GlutaMAX (Gibco) and 10% FCS (GE Healthcare, Eindhoven, the Netherlands) at 37°C with 5% CO_2 in air. Thereafter, cells were seeded into 24-well plates and cultured for 48 hours to yield 2×10^5 cells per well (1.75 cm^2). Passages 10 to 14 of BN16 cells were used.

Prorenin and Renin Binding and Uptake

To study prorenin and renin binding and internalization, cells were incubated at 4°C or 37°C with 300 µL of 1000 U/L (≈2×10^6 pg/L) recombinant human prorenin or renin (a gift from Actelion Pharmaceuticals, Allschwil, Switzerland), aliskiren-pretreated recombinant human prorenin (10 µmol/L at 4°C for 48 hours), or trypsin-activated[19] recombinant human prorenin in serum-free medium. Incubations lasted up to 24 hours and were performed in the absence or presence of 10 mmol/L M6P (Sigma, St. Louis), 10 µmol/L of aliskiren (a gift of Novartis, Basel, Switzerland), and recombinant 100 µg/L of RAP-GST (receptor-associated protein fused to glutathione-S-transferase, plasmid kindly provided by Dr de Matteis, TIGEM, Naples, Italy, recombinant protein produced as described previously[20]). To further evaluate the role of megalin and the (P)RR, identical experiments were performed after transfection of BN16 cells with negative control siRNA (siNC), LRP2 siRNA (siLRP2), or (P)RR siRNA [si(P)RR] (Invitrogen, Paisley, UK) by using RNAi max transfection reagent (Invitrogen) for 48 hours. After incubation, the medium was removed, and the cells were washed twice with ice-cold 0.5% BSA in PBS and twice with ice-cold PBS. Then, the cells were lysed with ice-cold 0.2% Triton X-100 (SEEVA, Heidelberg, Germany) in PBS with protease inhibitor (Roche, Mannheim, Germany) and centrifuged for 10 minutes at 4°C at 14.000*g*. Supernatants were collected and stored at −80°C. Renin in the supernatants was measured with the Renin III (Cisbio, Gif-sur-Yvette, France) immunoradiometric assay. Prorenin was also measured with this assay, after its conversion to the renin conformation (allowing its detection in the assay) by incubating it with 10 µmol/L aliskiren at 4°C.[21,22] Prorenin measurements were performed both before and after aliskiren incubation, with levels being detected before aliskiren exposure representing prorenin that had been activated by the cells and levels after aliskiren exposure representing the total amount of prorenin.

Albumin Uptake

To study whether M6P interferes with megalin-mediated uptake, cells were incubated in a 96-well plate setup at 37°C with 10 µg/mL fluorescently labeled BSA (BSA Alexa Fluor conjugate, Invitrogen, Carlsbad, CA) in the absence or presence of M6P (1 µmol–10 mmol/L) or RAP-GST (1.5–200 µg/mL) for 4 hours.[15] During the last 10 minutes, the Hoechst33342 stain (Life Technologies, Carlsbad, CA) was added to dye the nucleus. Then, the medium was removed, and the wells were washed with HBSS at room temperature. Florescence readings were performed using a CV7000S high-content imager (Yokogawa, Tokyo, Japan).

RNA Isolation and Quantitative Polymerase Chain Reaction Analysis

Total RNA was extracted using the Direct-zol RNA kit (Zymo Research, Irvine, CA). One microgram total RNA was reverse transcribed by QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany). SYBR Green real-time quantitative polymerase chain reaction assays were performed on a One-step Plus (Thermo Fisher, Waltham, MA) using SYBR Premix Ex TaqTM II kit (Qiagen, Venlo, the Netherlands). Primers are specified in Table S1 in the Data Supplement.

Immunoblotting

For protein expression studies, cells were homogenized in lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 3 mmol/L KCl, 1 mmol/L EDTA, 1% Triton X-100, complete protease inhibitor cocktail Roche, pH 7.4). Lysates were centrifuged at 14000*g* at 4°C for 10 minutes. Supernatants were collected, and total protein concentrations were determined by BCA assay (Pierce, Waltham, MA). Twenty micrograms of total protein was loaded and separated on 6% Tris-glycine gels and transferred to PVDF membranes using Trans-Blot Turbo Transfer System (Bio-Rad, Hercules, CA). The blots were probed with megalin (1:200; Santa Cruz, Dallas, TX) and β-Actin (1:50.000; Merck Millipore, Darmstadt, Germany) detected by Clarity Western ECL Substrate (Bio-Rad, Hercules, CA). The intensities of bands were analyzed using ImageJ software.

Data Analysis

Results are shown as mean±SEM, unless n<3. For experiments with n>3, normal distribution was verified by Kolmogorov-Smirnov test, while for experiments with n=3, the Shapiro-Wilk test was used to evaluate distribution. Once normal distribution was confirmed, differences were either tested by 1-way ANOVA, followed by Bonferroni multiple comparison test, or by Student *t* test. $P<0.05$ were considered significant. Data below detection limit were assumed to equal the detection limit.

Results

Proximal Tubule Epithelial Cells

Prorenin and Renin Binding and Internalization Largely Depend on M6P Receptors

Incubating ciPTEC with either prorenin or renin at 37°C resulted in a time-dependent increase in their cellular levels,

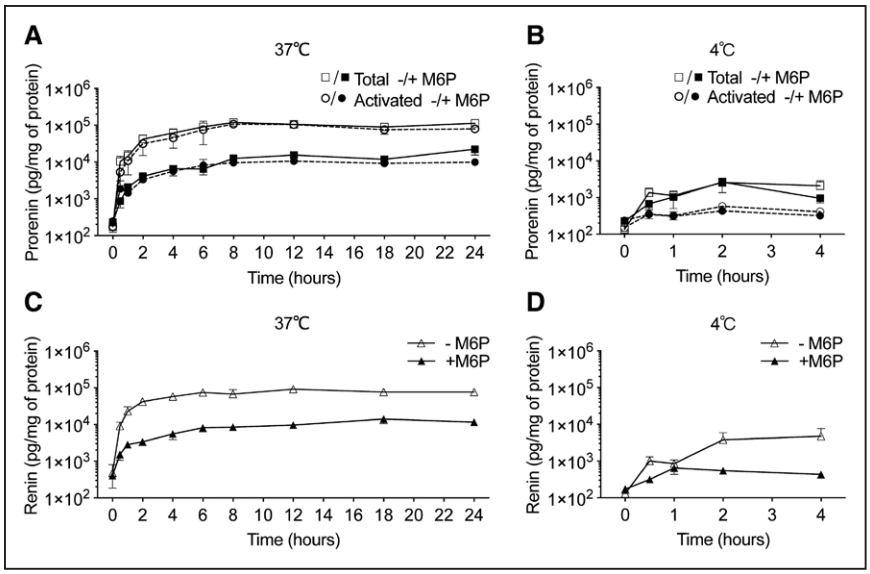

Figure 1. Cell-associated prorenin (**A** and **B**, distinguishing activated and total prorenin; n=5 and 4) and renin (**C** and **D**, n=5 and 3) levels in conditionally immortalized proximal tubule epithelial cells (ciPTEC) following their incubation at 37°C or 4°C with 2×10^6 pg/L prorenin or renin in the presence or absence of 10 mmol/L M6P (mannose-6-phosphate) for maximally 24 h. Data are mean±SEM.

a plateau being reached after 4 to 6 hours (Figure 1A and 1C). The majority of cellular prorenin (up to 90%) was recognized as renin, suggestive for prorenin activation upon binding and internalization. In contrast, following incubation at 4°C, the majority of prorenin was in its inactive form, and the steady-state levels of both prorenin and renin were ≈5- to 10-fold lower than those at 37°C (Figure 1B and 1D). At 37°C, M6P reduced the cellular accumulation of prorenin ($P<0.05$) and renin ($P<0.01$) by >80%, without altering the degree of prorenin activation. At 4°C, the blocking effect of M6P was much more modest, significance being reached only in the case of renin ($P<0.05$). In summary, these data demonstrate that ciPTEC bind and accumulate renin and prorenin largely via M6P receptors, and internalization is accompanied by prorenin activation. In agreement with this conclusion, M6P receptors were readily expressed in ciPTEC, while megalin was below detection limit (Figure S1). Human cortical tissue did express megalin and M6P receptors, while cubilin and the (P)RR were found in both cortical tissue and ciPTEC.

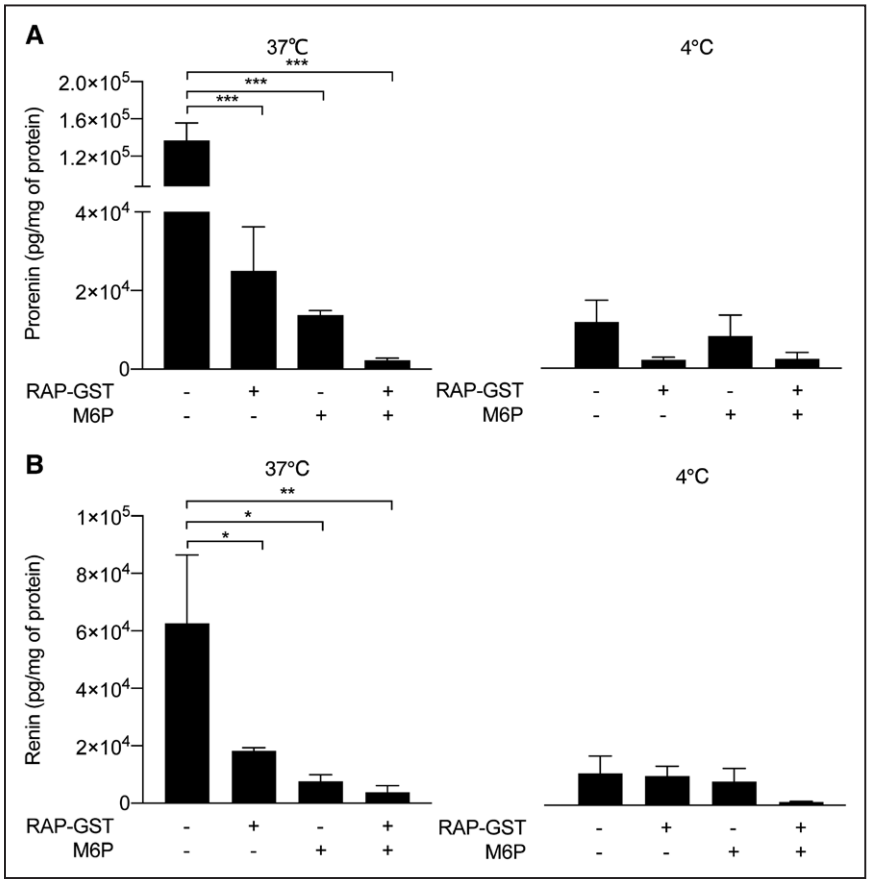

Figure 2. Cell-associated prorenin (**A**) and renin (**B**) levels in conditionally immortalized proximal tubule epithelial cells following their incubation at 37°C or 4°C with 2×10^6 pg/L prorenin or renin in the presence or absence of 10 mmol/L M6P (mannose-6-phosphate) and 100 μg/mL of RAP-GST (receptor-associated protein fused to glutathione-S-transferase) for 4 h. Data are mean±SEM of n=4. *$P<0.05$, **$P<0.005$, and ***$P<0.0005$.

RAP-GST Blocks Both (Pro)renin and Albumin Internalization

At 37°C, the megalin ligand RAP-GST reduced cellular prorenin and renin accumulation after 8 hours by >80%, both in the absence and presence of M6P (Figure 2). At 4°C, its blocking effects were more modest, particularly in the presence of M6P. The combined effects of RAP-GST and M6P were not significantly different from those of each blocker alone. RAP-GST blocked the uptake of labeled BSA at 37°C (IC_{50}=140 μg/mL, corresponding with 21 μmol/L; Figure S2). Unexpectedly, M6P blocked the uptake of labeled BSA to the same degree as RAP-GST (IC_{50}=10 mmol/L). These data suggest that either M6P is able to interfere with the megalin/cubilin-mediated uptake of BSA, or that RAP-GST, like M6P, prevents M6P receptor-mediated uptake of BSA.

Brown Norway Rat Yolk Sac Epithelial Cells

Prorenin and Renin Binding and Internalization: No Role for M6P Receptors

BN16 cells accumulated prorenin and renin in a concentration-dependent manner when incubated with increasing levels at 37°C, and all cellular prorenin was found to be in the activated form (Figure 3A). Importantly, for a given (pro)renin level in the incubation medium, cellular prorenin levels were 3- to 4-fold higher than those of renin (P<0.05), suggesting that prorenin uptake occurred more efficiently. A similar pattern was observed at 4°C, although now prorenin remained in the inactive form, and the cellular levels of both renin and prorenin were 3- to 10-fold lower than those at 37°C (Figure 3B). When incubating BN16 cells with 1000 U/L prorenin or renin at 37 or 4°C, peak levels were reached after 2 to 4 hours under all conditions (Figure 3C through 3F). At 37°C, in the case of renin, peak levels remained constant up to 24 hours, while in the case of prorenin the levels started to fall after 6 hours. This illustrates that after 6 hours the prorenin levels in the incubation medium had become too low to allow an equilibrium between internalization and degradation. Indeed, when measuring prorenin in the incubation medium after 24 hours, its levels had dropped to 30±8% of the levels measured at 4 hours, while in the case of renin the levels at 24 hours were still identical (86±4%) to those measured at 4°C (Figure 3C and 3E). M6P did not affect renin and prorenin accumulation at 37°C, nor renin binding at 4°C (Figure 3D). M6P did reduce prorenin binding at 4°C (P<0.005; Figure 3F). Taken together, these data indicate that BN16 cells bind and accumulate renin and prorenin largely via a non-M6P receptor-mediated mechanism (most likely megalin) and that this uptake mechanism prefers prorenin over renin. In agreement with this conclusion, BN16 cells readily expressed megalin, while M6P receptor expression was low (Figure S1). Rat cortical tissue did express megalin and M6P receptors, while cubilin and the (P)RR were found in both cortical tissue and BN16 cells.

Megalin Determines (Pro)renin Binding and Internalization

To clarify whether megalin truly is the main receptor mediating (pro)renin binding and internalization in BN16 cells, we transfected BN16 cells with siRNA against megalin. This approach reduced megalin mRNA and protein by >50% and >80%, respectively (Figure 4A and 4B). Parallel reductions were observed in the cellular renin and prorenin binding and

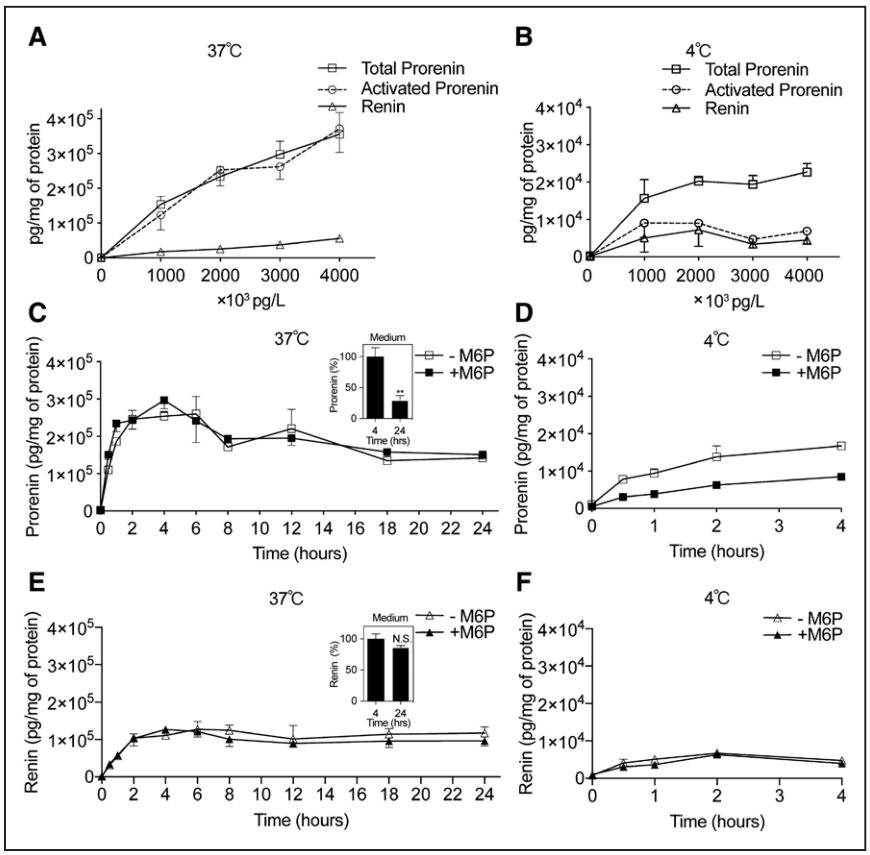

Figure 3. Cell-associated prorenin (**A**–**D**) and renin (**A** and **B**, **E** and **F**) levels in BN16 cells following their incubation at 37°C or 4°C with 2×10⁶ pg/L prorenin or renin in the presence or absence of 10 mmol/L M6P (mannose-6-phosphate) for maximally 24 h. Inserts display medium (pro)renin levels at 4 and 24 h, expressed as a percentage of the levels at 4 h. Data are mean±SEM of n=5.

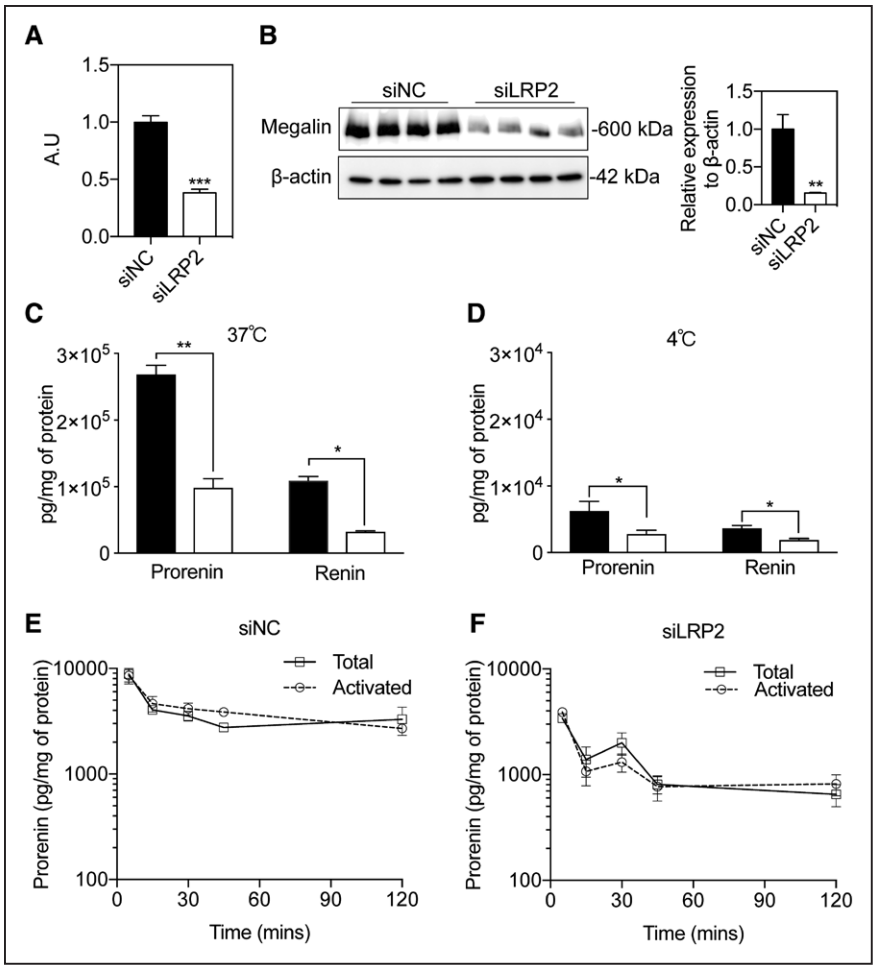

Figure 4. Megalin gene (**A**, n=5) and protein (**B**, n=5) expression levels in BN16 cells after transfection with control siRNA (siNC) or siRNA against megalin (siLRP2) for 48 h; cell-associated prorenin (**C**, n=5) and renin (**D**, n=5) levels in BN16 cells following their incubation at 37°C or 4°C with 2×10^6 pg/L prorenin or renin for 4 h after siNC (black bars) or siLRP2 (open bars) pretreatment; decrease in cell-associated prorenin levels after loading BN16 cells with prorenin (2×10^6 pg/L, 2 h 37°C) following siNC (**E**, n=3) or siLRP2 (**F**, n=3) pretreatment. Data are mean±SEM; **P*<0.05, ***P*<0.005, and ****P*<0.0005.

internalization (Figure 4C and 4D). Obviously, given the fact that megalin was not fully suppressed, this approach also did not fully suppress (pro)renin binding and internalization. Importantly, adding 50 µg/mL of BSA (a well-known megalin ligand) similarly reduced (pro)renin binding and internalization (Figure S3). Although megalin reduction largely prevented prorenin uptake at 37°C, it did not affect prorenin activation or its intracellular degradation half-life (Figure 4E and 4F). Taken together, these data confirm that megalin is the predominant receptor for (pro)renin binding and internalization in BN16 cells. Yet, megalin does not affect intracellular prorenin activation and degradation.

Prorenin Prosegment Facilitates Binding to Megalin, While Aliskiren Stabilizes Renin and Prorenin

The much greater uptake of prorenin versus renin suggests a role for the prosegment. Since aliskiren is capable of removing the prosegment from the enzymatic cleft (while not cleaving it), we studied whether co-incubating the cells with aliskiren-pretreated prorenin alters prorenin binding and internalization. For comparison, renin binding and internalization were also studied in the presence of aliskiren. As shown in Figure 5A and 5B, aliskiren facilitated renin and prorenin internalization at 37°C, but its effect on prorenin was much larger. At 4°C, aliskiren only facilitated prorenin binding. Incubation of prorenin with trypsin (cleaving off the prosegment; Figure 5A and 5B) and megalin knockdown (Figure S4) annihilated the difference between prorenin and renin. Importantly, as has been shown before,[22] aliskiren greatly increased the intracellular half-life of both renin and prorenin (from 35 to 30 minutes, respectively, to >24 hours; Figure 5C and 5D), that is, virtually preventing their degradation. In summary, these data show that the prosegment facilitates binding to megalin, while aliskiren binding stabilizes both renin and prorenin, thus reducing their intracellular degradation.

(P)RR Contributes to Prorenin Internalization but Does not Affect Prorenin Binding in BN16 Cells

The (P)RR binds renin and prorenin with low affinity.[23] To study (P)RR-megalin interaction, megalin and (P)RR expression in BN16 cells were inhibited by transfecting with their respective siRNA for 48 hours (Figure 6A). Megalin knockdown again reduced prorenin binding and internalization by about 50% (Figure 6B and 6C), while (P)RR inhibition reduced internalization only. Dual (P)RR/megalin inhibition induced the same effect of megalin inhibition alone. These data suggest that the (P)RR contributes to prorenin endocytosis, but not binding and that endocytosis involves megalin and the (P)RR simultaneously.

Discussion

This study shows that megalin is an endocytic receptor binding both renin and prorenin, displaying a preference for the latter. This preference involves the prosegment, and moving out the

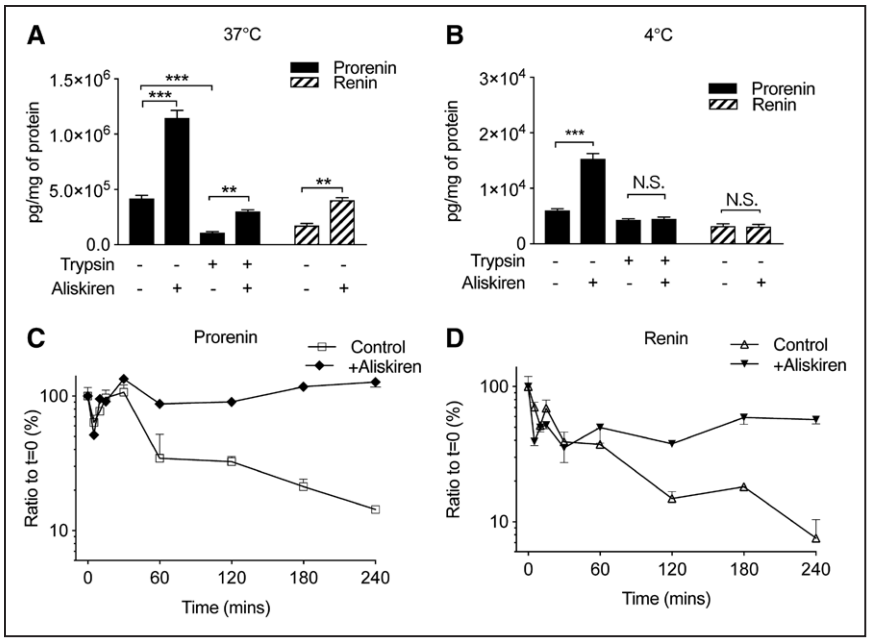

Figure 5. Cell-associated prorenin and renin levels after incubating BN16 cells with 2×10^6 pg/L prorenin or renin at 37°C (**A**, n=6) or 4°C (**B**, n=6) for 4 h with or without prior (pro)renin exposure to trypsin or 10 μmol/L aliskiren for 48 h; decrease in cell-associated prorenin (**C**, n=3) and renin (**D**, n=3) levels after loading BN16 cells with prorenin or renin (2×10^6 pg/L, 4 h 37°C) with or without prior (pro)renin exposure to 10 μmol/L aliskiren for 48 h. Data are mean±SEM; $^{**}P<0.005$ and $^{***}P<0.0005$.

prosegment from the enzymatic cleft further enhanced binding. Endocytosis, occurring at 37°C, depended on the (P)RR and was followed by intracellular degradation of both renin and prorenin. Aliskiren reduced degradation, most likely because renin inhibitor-binding stabilizes renin and prorenin.[22,24]

Megalin-dependent renin binding has been demonstrated before,[12] and recent studies have shown that this process rather than tubular (pro)renin release determines urinary renin levels. Indeed, blocking megalin with lysine,[25] impaired megalin trafficking[3] and megalin deletion[4] all greatly increased urinary renin levels, implying that normally >95% of filtered renin is reabsorbed in a megalin-dependent manner. Human urine did not contain detectable levels of prorenin, except in patients with Dent's disease and Lowe syndrome in whom megalin expression and trafficking is impaired, and in women with preeclampsia.[8] The current data now explain the absence of prorenin under normal conditions: its reabsorption is 4 to 5× more efficient than that of renin and thus might be closer to 100%. Tang et al[25] have claimed suppressed megalin expression in experimental diabetes mellitus, which, particularly in combination with the increased glomerular protein filtration in this condition, results in elevated urinary renin levels, despite

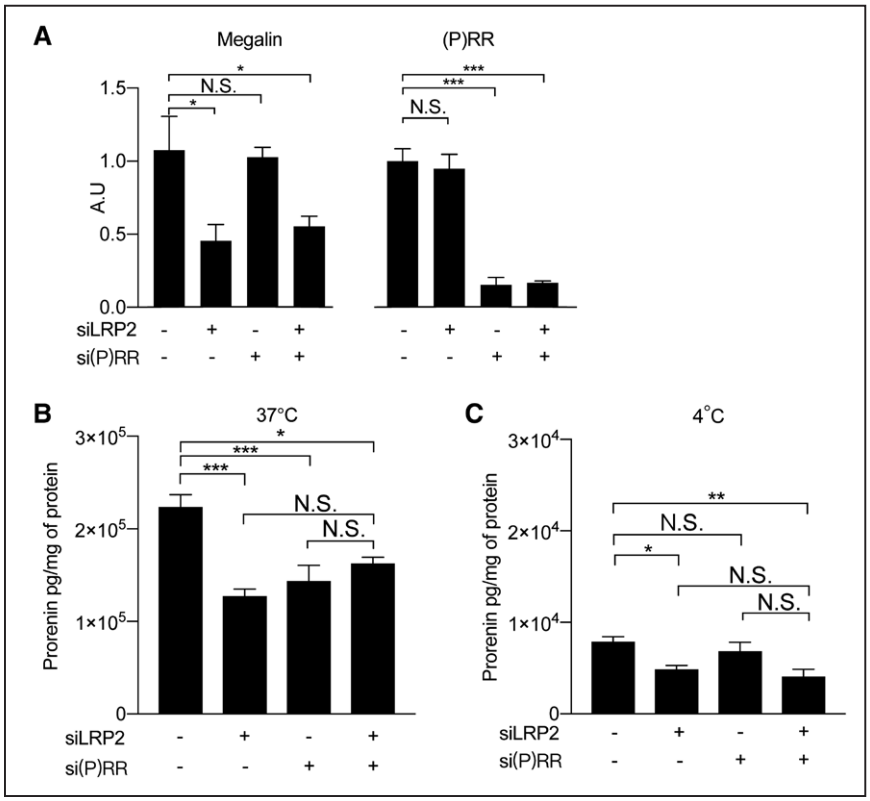

Figure 6. **A**, siRNA-induced reduction of megalin and (P)RR [(pro)renin receptor] expression levels in BN16 cells. **B** and **C**, cell-associated prorenin levels after incubating BN16 cells with 2×10^6 pg/L prorenin at 37°C (**B**) or 4°C (**C**) for 4 h in the presence or absence of megalin (*LRP2*) and the (P)RR. Data are mean±SEMs of n=5. $^{*}P<0.05$, $^{**}P<0.005$, and $^{***}P<0.0005$.

the well-known circulating RAS downregulation in diabetes mellitus.[25,26] Future studies should now evaluate whether the same is true in preeclampsia, a condition characterized by hypertension, proteinuria, and RAS downregulation.[27]

Tojo et al,[28] after infusing human prorenin in diabetic rats, reported increased renal prorenin accumulation versus nondiabetic rats, which they attributed to (P)RR and megalin binding. Similar observations were made for human angiotensinogen, and no comparison was made versus human renin. Given the reduced expression of megalin under diabetic conditions observed by Tang et al,[25] these data are most likely explained on the basis of enhanced glomerular filtration of prorenin and angiotensinogen. Combined with our current findings, they suggest that the earlier reported upregulation of prorenin immunoreactivity in the collecting duct of diabetic rodents[29] is the consequence of enhanced filtration and subsequent tubular reuptake. Furthermore, we now reveal that the (P)RR contributes to the internalization of megalin-bound prorenin. Given the fact that the (P)RR is identical to ATPA6P2, that is, accessory protein 2 of V-ATPase (vacuolar ATPase), this may not be too surprising, since V-ATPases are expressed on the membrane of intracellular compartments in virtually every cell type and play important roles in vesicle trafficking, protein degradation, and intracellular signaling.[30] Indeed, also in the case of megalin, V-ATPase ensures optimal endosomal acidification, resulting in rapid dissociation of the megalin-ligand complex and fast recycling of megalin to the cell membrane. Hence, inhibiting ATP6AP2 impairs megalin regulation.[31]

Our data subsequently illustrate that, at least under the applied cell culture conditions, megalin/(P)RR-mediated (pro)renin uptake results in intracellular degradation of both renin and prorenin, with a half-life of ≈30 minutes. In the case of prorenin, this metabolic process involves prosegment removal, that is, prorenin-renin conversion.[32] As has been shown before,[22,24] aliskiren binding stabilizes (pro)renin, thereby greatly suppressing its metabolism. Combined with aliskiren's high affinity (virtually excluding dissociation), this explains why the renin inhibitor enhances intracellular renin and prorenin accumulation at 37°C. Importantly, aliskiren also increased prorenin binding to megalin, evidenced by the elevated cell-associated prorenin levels at 4°C in the presence of the renin inhibitor. The latter did not apply to renin, suggesting that the aliskiren-induced conformational change of prorenin, resulting in the removal of the prosegment from the enzymatic cleft,[21,33] underlies this phenomenon, rather than aliskiren-induced stabilization of the (pro)renin molecule. This indicates that the prosegment, particularly when fully exposed, facilitates prorenin-megalin interaction. In summary, aliskiren facilitates binding only in the case of prorenin and slowed down degradation for both renin and prorenin. Hence, at 37°C, when both binding and internalization occur, aliskiren's effects were largest for prorenin.

ciPTEC express most of the transporters and efflux pumps physiologically present in the proximal tubule, and possess the complete endocytosis machinery, making these cells an appropriate model to study proximal tubule physiology.[17] In many studies with different cell types such as rat vascular smooth muscle cells, cardiomyocytes, fibroblasts, and human endothelial cells, it has been demonstrated that besides megalin, M6P receptors bind and internalize renin and prorenin.[32,34,35] In the current study, the >80% decrease in prorenin and renin uptake induced by M6P in ciPTEC suggests an important role for M6P receptor-mediated uptake of renin and prorenin in these cells. Not surprisingly, the effect of M6P was best seen at 37°C, since at this temperature (but not at 4°C), M6P receptors cycle continuously between the cell surface and the intracellular compartment, thus allowing substantial (pro)renin accumulation in the cells. In contrast, at 4°C, renin and prorenin can only bind to cell surface M6P receptors, without being internalized,[23] and prorenin activation will not occur. In agreement with a predominant role for M6P receptors in ciPTEC, we observed high M6P receptor expression and low to undetectable megalin expression in these cells. The cells additionally expressed cubilin and the (P)RR. Interestingly, the M6P receptor has been described as a ligand for megalin.[36,37] To what degree M6P itself acts as a ligand for the megalin receptor is unknown. Our results revealed that the megalin inhibitor RAP-GST exerted identical effects as M6P: up to 90% inhibition of renin/prorenin uptake at 37°C in ciPTEC, and a comparable, but much smaller effect at 4°C. Given the low expression of megalin in ciPTEC, the simplest explanation of these findings is that RAP-GST additionally blocks M6P receptors. Alternatively, megalin may interact with M6P receptors,[38] or M6P might block megalin-mediated pathways. To evaluate the latter, we studied the effect of M6P on the uptake of BSA by ciPTEC, a widely accepted megalin ligand.[39] It was observed that M6P not only blocked BSA uptake to the same degree as RAP-GST, but that it also decreased RAP-GST endocytosis (data not shown). Although this suggests that M6P is a megalin receptor blocker, an important caveat remains the low expression of megalin in ciPTEC. Given these observations, we focused on BN16 cells rather than ciPTEC to verify megalin-(pro)renin interaction, and we used megalin siRNA knockdown instead of RAP-GST to inhibit megalin in these cells. Conveniently, M6P receptor expression in BN16 cells was low, and M6P did not interfere with (pro)renin binding.

Perspective

Megalin is major determinant of urinary renin and prorenin levels. Given its preference for prorenin, urinary prorenin is normally undetectable. Since even filtered renin is reabsorbed by >95% via megalin, decreases in megalin expression (as occurring in diabetes) may easily result in rises in urinary renin that are entirely unrelated to plasma renin levels. These rises, therefore, reflect tubular (dys)function rather than upregulation of the intrarenal RAS, as is often advocated.[3,12] Future studies should now unravel (1) megalin-prosegment interaction, which appears to underlie the preference for prorenin; (2) renal megalin alterations in diseases other than diabetes mellitus (like preeclampsia); (3) the potential role of megalin outside the kidney, if any; (4) megalin-(P)RR and megalin-M6P receptor interaction; and (5) the destiny of megalin-internalized (pro)renin: destruction or abluminal release into the renal interstitium and a contribution to renal angiotensin production? An exciting recent study supports the latter following podocyte injury.[40] The link with the (P)RR might explain why

previous studies reported (P)RR-(pro)renin interaction in the kidney—this is unlikely to represent actual (pro)renin binding to the (P)RR, given its low affinity,[41–44] but might simply represent a role for the (P)RR in (pro)renin endocytosis.

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Disclosures

None.

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Novelty and significance

What Is New?

- Megalin is a novel endocytic receptor for renin and prorenin which displays a preference for the latter.
- Megalin-dependent (pro)renin internalization, but not binding, involves the (pro)renin receptor.

What is relevant?

- These data explains why urine normally does not contain prorenin, except under conditions where megalin trafficking is disturbed, like in patients with Dent's disease or Lowe syndrome.

Summary

To investigate (pro)renin binding and internalization by megalin, Brown Norway Rat yolk sac cells were incubated with renin and prorenin. The cells bound and internalized much more prorenin than renin, and inhibiting megalin expression with siRNA greatly reduced both binding and internalization, while inhibiting the (pro)renin receptor inhibited the latter only. Exposing prorenin's prosegment with the renin inhibitor aliskiren dramatically increased prorenin binding, while after prosegment cleavage with trypsin prorenin binding was identical to that of renin. In conclusion, megalin is a novel endocytic receptor for (pro)renin which displays a preference for prorenin, and endocytosis depends on the (pro)renin receptor.