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Loss-of-function point mutations associated with renal tubular dysgenesis provide insights about renin function and cellular trafficking

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Renal tubular dysgenesis (RTD) is a recessive autosomal disease characterized by persistent fetal anuria and perinatal death. During the systematic screening of mutations of the different genes of the renin–angiotensin system associated with RTD, two missense mutations in the renin gene were previously identified, the first affects one of the two catalytic aspartates (D38N) of renin, and the second, S69Y, is located upstream of the 'flap', a mobile β-hairpin structure which covers the substrate-binding site of renin. Here we report a novel renin mutation leading to the duplication of the tyrosine residue Y15dup, homologous to Y9 in some other aspartyl proteases, which seems to play a crucial role along the activation pathway. The biochemical and cellular mechanisms underlying renin inactivation were investigated. We expressed prorenin constructs harboring the identified point mutations in two established cell lines, able (AtT-20 cells) or unable (CHO cells) to process prorenin to renin and we evaluated the cellular localization of renin mutants and their functional properties. All three mutants were misfolded to different levels, were enzymatically inactive and exhibited abnormal intracellular trafficking. We suggest a misfolding of Y15dup renin, a partial misfolding of D38N prorenin and a misfolding of S69Y prorenin leading to complete absence of secretion. The structural consequences of the renin mutations were estimated by molecular modeling, which suggested some important structural alterations. This is the first characterization of the mechanisms underlying loss of renin function in RTD.

INTRODUCTION

The renin–angiotensin system (RAS) plays a key role in the control of water and salt metabolism and in the regulation of blood pressure. Angiotensinogen is cleaved by renin, an aspartyl protease produced in juxtaglomerular cells located in the afferent arterioles of the kidney. Angiotensin I (Ang I), a decapeptide produced by renin after cleavage of angiotensinogen, is inactive and further converted into the active octapeptide, angiotensin II (Ang II) by angiotensin-converting enzyme (ACE). Ang II exerts its action by interacting with two G-protein-coupled angiotensin receptors, AT_1 and AT_2. In humans, homozygous or compound heterozygous mutations in the genes encoding either renin, angiotensinogen, ACE or AT_1 receptor have been shown to cause familial autosomal recessive renal tubular dysgenesis (RTD), a severe disorder of renal tubular development consisting in a quasi-absence of proximal tubules and a quite marked stimulation of renin gene expression in juxtaglomerular apparatus and along the afferent renal arteries. Clinically, it is characterized by persistent fetal anuria and most often perinatal death (1,2). These mutations reveal that the RAS is critically involved in

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human kidney development. They also represent unique models for analyzing the structure–function relationship of genes encoding proteins of the RAS. During the systematic screening of RAS gene mutations responsible for autosomal recessive RTD, three renin gene mutations were identified. We were particularly interested in these mutations as they could unravel some novel functions associated with renin structure. Renin belongs to the A1 family of aspartyl proteases with a three-dimensional (3D) structure similar to that of pepsin. Analysis of 3D structures of human and mouse renins revealed that they consist of two β-sheet domains (N and C domains) related by an ∼2-fold axis (3–5). The active site consists of a deep cleft located between the two domains, each comprising one of the two catalytic aspartyl residues D38 and D226 (human renin gene numbering). All human aspartyl proteases are synthesized as inactive zymogens. In the case of human renin, an N-terminal 43 amino acid propeptide accounts for the lack of activity of prorenin. It fills the enzymatic cleft of renin and thereby obstructs access of angiotensinogen to its active site. Ionic and hydrophobic interactions (6,7) as well as hydrogen bonds between the profragment and the mature renin molecule are assumed to keep prorenin in an inactive state. Another particular feature of eukaryotic aspartyl proteases is the presence of a long β-hairpin loop structure called 'flap' in the N-terminal domain [amino acids 79–89 (3)]. This flap is a mobile segment which covers the central part of the substrate-binding site. The 'open' conformation with the tip of the flap elevated allows the entrance of the substrate and removal of hydrolytic products during the catalytic turnover. In the case of prorenin, the flap is most likely open to accommodate the profragment and closes upon substrate binding to mature renin.

Three non-truncated renin mutations were identified in RTD patients. They consisted in: (i) a homozygous tyrosine duplication in the N-terminal part of mature renin (Y15dup); (ii) a heterozygous missense mutation leading to the amino acid substitution of one of the two catalytic aspartyl residues for an asparagine residue (D38N), associated with a loss of function mutation (IVS3 + 1G → A) (1); (iii) a homozygous missense mutation leading to the amino acid substitution (S69Y) which affects the highly conserved serine located upstream of the flap (8). Because of the dramatic clinical consequences of these three point mutations and since the functional importance of these different parts of the N-terminal domain of the renin molecule had not previously been studied, we decided to investigate *in vitro* the role of these mutations in prorenin/renin expression, intracellular trafficking, enzyme secretion and enzymatic activity.

RESULTS

Renin mutations in patients affected with RTD

In a systematic study of patients with autosomal recessive RTD, three renin gene non-truncating mutations were found to be associated with the disease. A missense variant, S69Y, discovered in two unrelated families (patients I and II) and a newly reported tyrosine residue duplication (Y15dup) were present in the homozygous state. The other mutation, D38N, present in the heterozygous state was associated with a G → A transition in the obligatory splice acceptor site of IVS 3 (IVS3 + 1G → A) leading to a frameshift. Main clinical and pathological features of all patients are summarized in Table 1. A dramatic phenotype was noted before and after birth and led either to perinatal death or to major renal insufficiency requiring renal transplantation for patient I. In all patients, renal pathology showed the quasi-absence of proximal renal tubules. Renin mRNA was markedly stimulated (data not shown) and juxtaglomerular cells were strongly labeled by renin antibody, indicating a high abundance of renin in these structures (Fig. 1). However, there was no detectable mature plasma renin in the surviving patient I (8), suggesting that prorenin was synthetized, stored in the juxtaglomerular cells but could not mature and exited from the producing cells.

Alignment of the human renin sequence with further human aspartic proteases of the pepsin family A1 (Fig. 2) revealed that two mutations affected either one of the absolutely conserved two catalytic aspartates (D38), or an absolutely conserved amino acid with distinct structural function just upstream of the flap (S69Y). The third mutation (Y15dup) introduces a bulky amino acid in a region which is structurally highly conserved in eukaryotic aspartic proteinases.

In order to investigate the functional consequences of Y15dup, D38N and S69Y mutations, we stably expressed these mutated enzymes in AtT-20 and CHO cells. *In vitro* renin production can be studied in CHO cells, which do not process prorenin to renin (9), and in AtT-20 cells, which are a model of neurosecretory cells able to process endogenous pro-opiomelanocorticotropin to ACTH and prorenin to renin and to regulate renin production to some extent (10,11).

Expression of wild-type prorenin, Y15dup, D38N and S69Y mutants in AtT-20 and CHO cells

Production and secretion. As shown in Figure 3A, WT prorenin and renin were secreted in the culture medium of AtT-20 cells as a 45 and a 43 kDa protein band, respectively (Fig. 3A, lane 1). Stimulation by the secretagogue forskolin increased the release of WT renin in the medium (Fig. 3A, lane 2). Y15dup and D38N mutant prorenins and renins were also secreted in the culture medium of AtT-20 cells, but renin release in the medium was not modified by forskolin (Fig. 3A, lanes 4 and 6) showing an abnormal traffic of these two renin mutants. S69Y prorenin mutant was not detected in the culture medium but was present within the cell extract. Its production and intracellular accumulation were stimulated by forskolin (Fig. 3A, lanes 7 and 8). In CHO cells, WT prorenin, Y15dup and D38N mutants were secreted into the culture medium (Fig. 3B, lanes 1, 3 and 5); however S69Y (Fig. 3C, lane 2) was only found in the cell lysate (Fig. 3C, lane 4). It was verified that all prorenin mutants can be processed in mature 43 kDa renins by trypsin (Fig. 3B, lanes 2, 4 and 6).

To confirm these results, metabolic studies were performed and they revealed that WT prorenin, Y15dup and D38N mutants were present in the culture medium of AtT-20 (Fig. 4A and B). Under the conditions used, labeled renin was not detected, as the pulse and chase times were too short to reveal renin. The secretion rate of D38N mutant

Table 1. Clinical and renal pathology features of renin mutations in patients with RTD

Number of families	Nucleotide alteration(s)[a]	Exon	Alteration(s) in coding sequence (renin numbering)	Duration of gestation (weeks)	Age of death	Renin expression	References
1	241–243dup	2, hom	Y15dup	31	3 days	IHC +++; ISH +++	Present paper
1	310G → A; IVS3 + 1G → A	3, het	D38N; splice-site mutation	22	TP	IHC +++; ISH +++	Ref. (1)
2	404C → A	4, hom	S69Y	35	Patient I: survival after transplantation at 4 years	Biopsy; HIC +++; ISH +++	Ref. (8)
				29	Patient II: 1 h	HIC +++; ISH +++	Present paper

hom, homozygous; het, heterozygous mutation; TP, termination of pregnancy.
[a]Nucleotides are numbered with respect to the A of the ATG start codon, in accordance with standard nomenclature.

Figure 1. Kidney renin expression in patients with Y15dup, D38N and S69Y mutations. (**A**) Control fetal kidney (provided by A.L. Delezoide): renin was present in a few cells (arrow). (**B**) Y15dup mutant; (**C**) D38N mutant; (**D**) S69Y mutant (kidney autopsy specimens from patient II): strong expression of renin protein with abnormal extension of positive cells. Immunohistochemistry was performed with R15 antibody to renin (immunoperoxydase staining), with counterstaining with hematoxylin for sections (A) and (B).

was slightly delayed compared with WT. Only a small amount of S69Y mutant was detected in the culture medium although the expression level of S69Y was close to that of WT prorenin. The prorenin mutant was mostly retained inside the cell during the chase period. These results suggested that only the S69Y prorenin led to abnormal secretion and intracellular protein degradation, quite likely as a consequence of incorrect protein folding.

Intracellular distribution. Intracellular distribution was studied in AtT-20 cells by confocal fluorescent microscopy (Fig. 5A). WT prorenin, Y15dup and D38N mutants were present inside the Golgi apparatus, as shown by their co-localization with the TGN38 marker. Respective mutated proteins and ACTH were co-localized both at the tip of the cell and in some cytoplasmic vesicules, indicating that mutants were present throughout the whole secretory pathway. In contrast, the S69Y mutant was only found within the endoplasmic reticulum (ER), indicating that it was fully retained in the ER and transported neither to the Golgi complex nor to the secretory vesicules. Identical distributions were observed in CHO cells (Fig. 5B).

To determine the subcellular localization of prorenin and mature renin, we used the monoclonal antibody (mAb) R1-20-5, which detects the exposed active site of renin (12), and the polyclonal antibody R12, which binds both prorenin and mature renin. WT renin and Y15dup mutant expressed in AtT-20 cells were present at the tip of the cell, whereas the respective proforms were located in the Golgi complex (Fig. 6A). Some vesicules were detected by the antibody R1-20-5 and it was verified that it was a non-specific labeling in AtT-20 cells (data non shown). These data suggest that renin is matured during transport from the TGN to the secretory granules. The active site of D38N could be detected in cell tips but also in the Golgi complex, suggesting an improperly folded D38N prorenin. In contrast, S69Y mutant was not

Figure 2. Amino sequence of human prorenin and alignment of human prorenin with several human aspartyl proteases of the pepsin family A1. (**A**) The amino sequence of human prorenin with the underlined prosegment (1^{P}–43^{P}). The amino acids surrounding the aspartyl residues of the active sites and the flap (79–89) are framed. (**B**) The amino acid sequence of human prorenin (amino acids 1^{P}–43^{P}-1–96) was aligned with the following human aspartyl proteases: pepsinogen A (amino acid 1^{P}–46^{P}-1–88), pepsinogen C (progastriscin) (amino acid 1^{P}–42^{P}-1–88), procathepsin D (amino acid 1^{P}–46^{P}-1–91) with underlined profragments. The sequences were retrieved from the MEROPS database and aligned using the program ClustalW (http://www.uniprot.org). Note the highly conserved tyrosine and serine residues (box) corresponding to Y15 and S69 in renin sequence.

detected in the Golgi apparatus but prorenin as well as exposed active site was detected in the ER, indicating that the profragment was not occupying the active site. Similar experiments performed in CHO cells (Fig. 6B), which do not cleave prorenin, confirmed the results obtained for WT and Y15dup prorenins, indicating that their profragments properly covered the active site. D38N prorenin was partially in an open configuration in the Golgi complex with the propeptide not covering the active site. S69Y mutant was present in the ER only and could be labeled by the antibody specific against the active site, showing an improper folding of the proform which exposes the normally hidden epitope of this antibody.

Thus, these data on the secretion and distribution of the three mutants suggest an abnormal traffic of Y15dup and D38N renins and a misfolding of S69Y prorenin.

Renin quantification. Renin was quantified by using two different methods, direct ponderal measurements and enzymatic activity. The renin IRMA measures renin concentration by using a pair of mAbs, 3E8 and 4G1. These antibodies recognize either prorenin and renin or only renin, respectively. WT renin was quantified in CHO cells medium but none of the three mutants was detected even though a 200-fold more than WT were assayed (data not shown). Absence of recognition of mutants by 4G1 antibody, as found by metabolic labeling and immunoprecipitation, explains this result (data not shown). WT renin was found enzymatically active. However, renin mutant proteins, even at a concentration 200 times higher, were unable to hydrolyze angiotensinogen to produce Ang I. The S69Y mutant displayed no renin activity in the intracellular lysate (data not shown).

Rescue attempts of defective secretion of S69Y prorenin mutant

The mutation of serine 69 to tyrosine is close to one of the renin glycosylation site (Asn 75) and could have possibly affected renin glycosylation. It was verified that the S69Y mutant was glycosylated using peptide:*N*-glycosidase F (PNGase F). Both the S69Y mutant and WT prorenin were glycosylated (data not shown), allowing S69Y being directed to the secretory pathway.

Molecular chaperone association. After glycosylation, protein folding requires the help of a series of molecular chaperones like calnexin (CNX) and BiP/GRP78 (13,14). Co-immunoprecipitation of WT prorenin transfected in CHO cells with CNX and R12 antibody showed that prorenin was transiently associated with CNX (Supplementary Material, Fig. S1A) and BIP (data not shown). Similarly, the S69Y mutant could be co-precipitated with CNX and BIP. CNX was more stably associated with the S69Y mutant for up to 80 min. The association of a protein with CNX can be suppressed by pretreatment with glucosidase I and II inhibitors such as castanospermine (CST), preventing misfolded proteins from being stuck to CNX (15). In the case of S69Y mutant, CST treatment had no beneficial effect on protein secretion (data not shown).

Thermosensitivity. In an attempt to rescue the folding defect induced by the S69Y mutation on cellular trafficking, we tested whether incubation at a temperature $<37^{\circ}C$ could affect folding and exiting of the mutant from the ER and eventually prorenin secretion (16,17). WT prorenin and S69Y

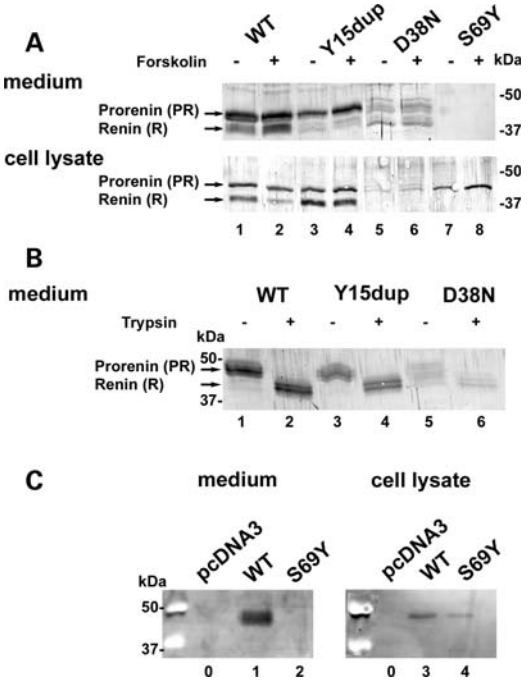

Figure 3. Immunoblot analysis of prorenin WT and Y15dup, D38N, S69Y mutants. (**A**) Prorenin (PR) and renin (R) expressed during 24 h in the AtT-20 culture medium, and cell lysates were analyzed by western blotting as described in Materials and Methods. Cells were subjected to 10 μM of forskolin (lanes 2, 4, 6 and 8). AtT-20 transfected by empty vector did not express prorenin (data not shown). (**B**) Proteins secreted in the medium during 24 h were analyzed from stably transfected CHO. Prorenin (lanes 1, 3 and 5) and renin (after activation by trypsin) (lanes 2, 4 and 6) were detected by western blotting. (**C**) Immunoprecipitations of WT and S69Y prorenins with 3E8 mAb in medium (lanes 1–2) and cell lysates (lanes 3–4). CHO cells transfected by empty vector did not express prorenin (pcDNA3).

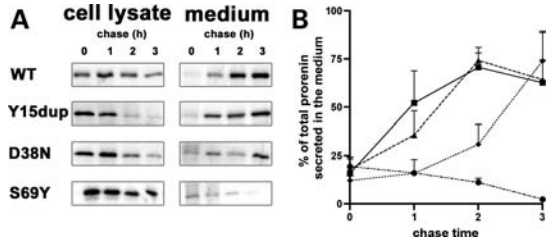

Figure 4. Pulse-chase analysis of WT prorenin and mutants. AtT-20 cells stably expressing WT, Y15dup, D38N and S69Y mutants were labeled and subjected to 1–3 h chase periods. Proteins were immunoprecipated from cell lysate and culture medium with polyclonal antibody R12 and shown in (**A**). Secreted prorenins are featured in (**B**): radioactivity associated with prorenin WT (filled square), Y15dup (filled triangle), D38N (filled diamond) and S69Y (filled circle) were quantified by densitometry and expressed as percentage of the total amount in cell and medium at t_0. Results shown are means of four experiments.

prorenin were incubated at 26°C and 37°C in CHO cells. At 26°C, the secretion of S69Y mutant into the culture medium could be partially restored (Supplementary Material, Fig. S1B), indicating a positive effect on its folding but neither enzymatic activity nor IRMA immunoreactive renin was detectable (data not shown).

Inhibition of the 26S proteasome–ubiquitin pathway. To investigate whether the degradation of the uncorrectly folded S69Y mutant is mediated by the proteasome pathway, stably transfected CHO cells were treated with the proteasome inhibitor lactacystin (Supplementary Material, Fig. S1C). Lactacystin had little or no effect on WT prorenin expression but clearly protected S69Y mutant from intracellular degradation, and allowed a minimal secretion after 20 h. Finally, lactacystin together with lowering the temperature at 26°C further increased the yield of S69Y mutant in the medium, showing that the effect of these two means were additive rather than exclusive (data not shown).

DISCUSSION

The importance of the RAS in human kidney development and perinatal function has been revealed by the discovery of the genetic cause of autosomal recessive RTD. Homozygous or compound heterozygous mutations of any of the genes coding for renin, angiotensinogen, ACE or AT1 receptor lead to the same dramatic phenotype consisting into a loss of renal function and perinatal death (1,2,18). Some renin mutations could be easily predicted to result in a loss of function of the protein as they affect renin mRNA translation, induce renin truncation or inactivate the catalytic site. Other renin gene mutations might give insight on the structure/function of the enzyme as well as on its intracellular trafficking and sorting.

In this study, we elucidated the underlying molecular and cellular mechanisms of three renin point mutations linked to RTD: a recently identified renin mutation, Y15dup, and two known renin mutations, D38N (1) and S69Y (8). Among the three different mutants studied, only the D38N mutation could have been anticipated to be lethal since it inactivates the catalytic site. There are no data about the functional importance of the region concerning the two other mutations. We have investigated the processing, intracellular trafficking, protein stability and enzymatic activity of all these mutants. Our experiments revealed the role of key amino acids in proper (pro)renin folding whose mutations result in defects in structure, function and trafficking.

Kidney specimens of the patients showed the typical morphological hallmarks of RTD in all cases and a high renin gene transcription level (1,2), resulting from the interruption of the RAS short feedback loop on juxtaglomerular cells after the production of an inactive enzyme. In patient I bearing the S69Y mutation, despite the high level of intracellular renin in the kidneys, no plasma immunoreactive renin was detected (8), indicating abnormal renin sorting.

Human prorenin follows two secretory pathways in juxtaglomerular cells, a constitutive pathway which leads to the production of inactive prorenin (19) and a regulated pathway whereby prorenin is sent to secretory granules, processed to active renin and secreted by regulated exocytosis (20,21). Constructs were expressed in AtT-20 cells, which can mimic neurosecretory cells and which share some common secretory features with juxtaglomerular cells. These cells process prorenin into mature renin to some extent and regulated secretion of renin could be stimulated by forskolin (10).

All three renin mutants (Y15 dup, D38N and S69Y) expressed in CHO cells were undetectable by direct renin

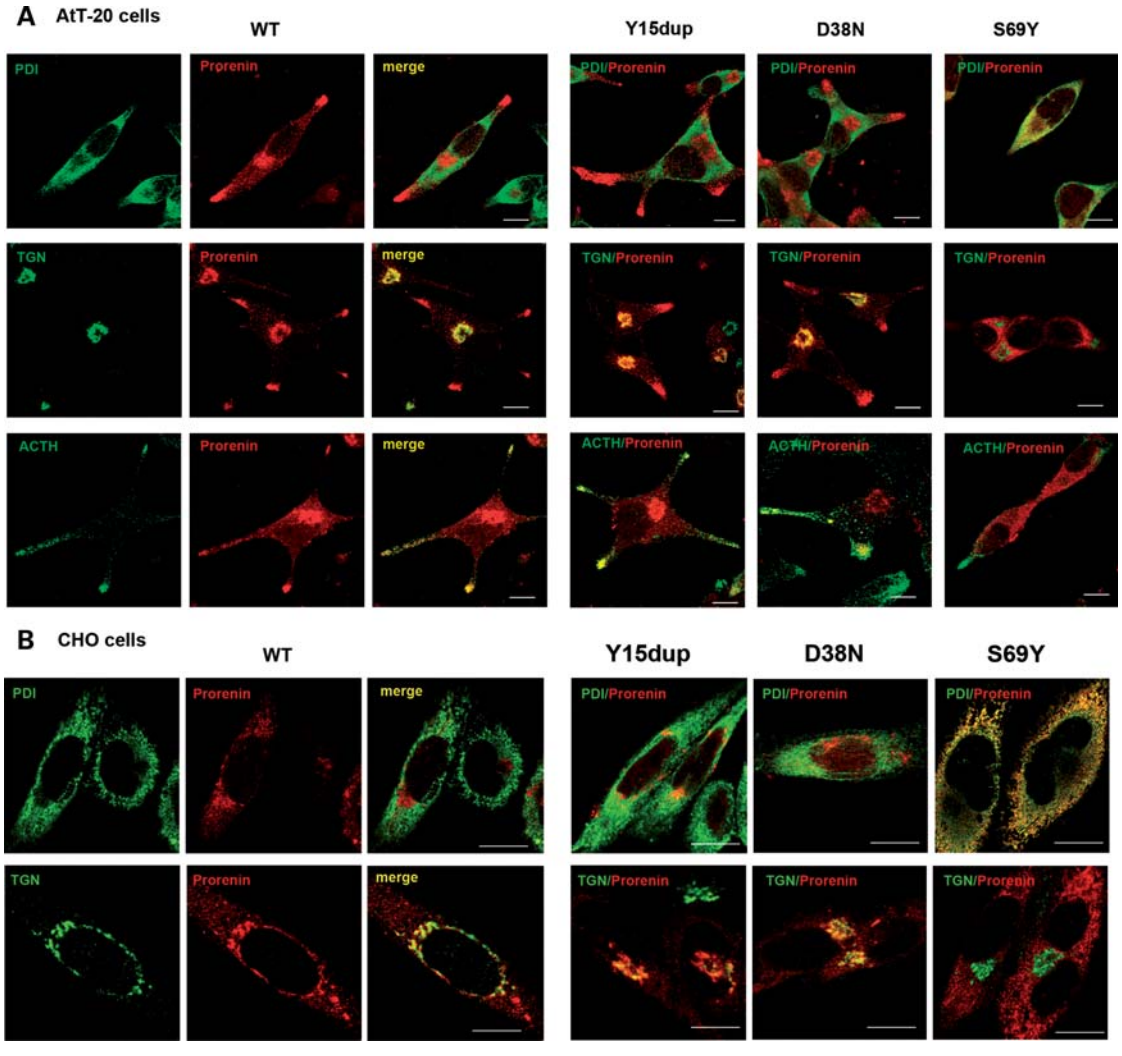

Figure 5. Co-localization of WT prorenin, Y15dup, D38N and S69Y mutants with markers of different intracellular compartments in AtT-20 and CHO cells. (**A**) AtT-20 cells stably transfected with WT, Y15dup, D38N and S69Y were fixed and co-stained with antibodies against prorenin/renin (R12 or 1A12) shown in red, ER-resident PDI (upper panels), Golgi resident TGN38 (middle panels), ACTH representative of secretory granules (lower panels), all shown in green. Only merge images are shown for all the mutants. Bar represents 10 μm. (**B**) CHO cells stably transfected with WT and Y15dup, D38N and S69Y were fixed and co-stained with antibodies against prorenin/renin (R12 or 1A12) shown in red, ER-resident PDI (upper panels), Golgi resident TGN38 (lower panels), all shown in green. Only merge images are shown for all the mutants. Bar represents 10 μm.

immunoassay and were also enzymatically inactive. These three mutations have more or less severe effects on the biosynthesis and the production of the corresponding proteins. Since these mutations produced abnormal prorenin or renin secretion, we investigated their subcellular localization. The Y15dup and D38N mutations disturb the correct sorting of (pro)renin into vesicules that undergo regulated release of their cargo. In the case of Y15dup, intracellular prorenin and renin were detected, indicating a proper processing of prorenin. The release of Y15dup prorenin was similar to that of WT, whereas Y15dup renin was minimal even under forskolin treatment, indicating that renin is misdirected to a compartment that cannot undergo exocytosis. In the cell, the Y15dup distribution was similar to WT. We hypothesize that the tyrosine 15 duplication likely disturbs renin structure and impedes substrate recognition or correct folding of active renin.

Exchange of catalytically important aspartates to asparagine in aspartic proteinases inevitably leads to inactive enzymes (22–25) with reduced stability (26,27). As expected, the D38N mutant was inactive and the loss of enzymatic activity was accompanied by changes in stability and rate of transport. The D38N mutant appears to release both prorenin and renin. Renin was not detected intracellularly, which suggests either its very rapid secretion or an intracellular degradation. Improper folding of D38N is also suggested by the presence in the Golgi complex of prorenin in an open configuration.

Unlike the Y15dup and D38N mutants, S69Y prorenin was fully retained within AtT-20 and CHO cells. S69Y prorenin was trapped in the ER, as shown by confocal microscopy in the two cell culture systems. Labeling with the antibody specific for the renin-active site showed that the profragment was in an open configuration not occupying the active site. It was difficult to rescue this mutant by lowering the

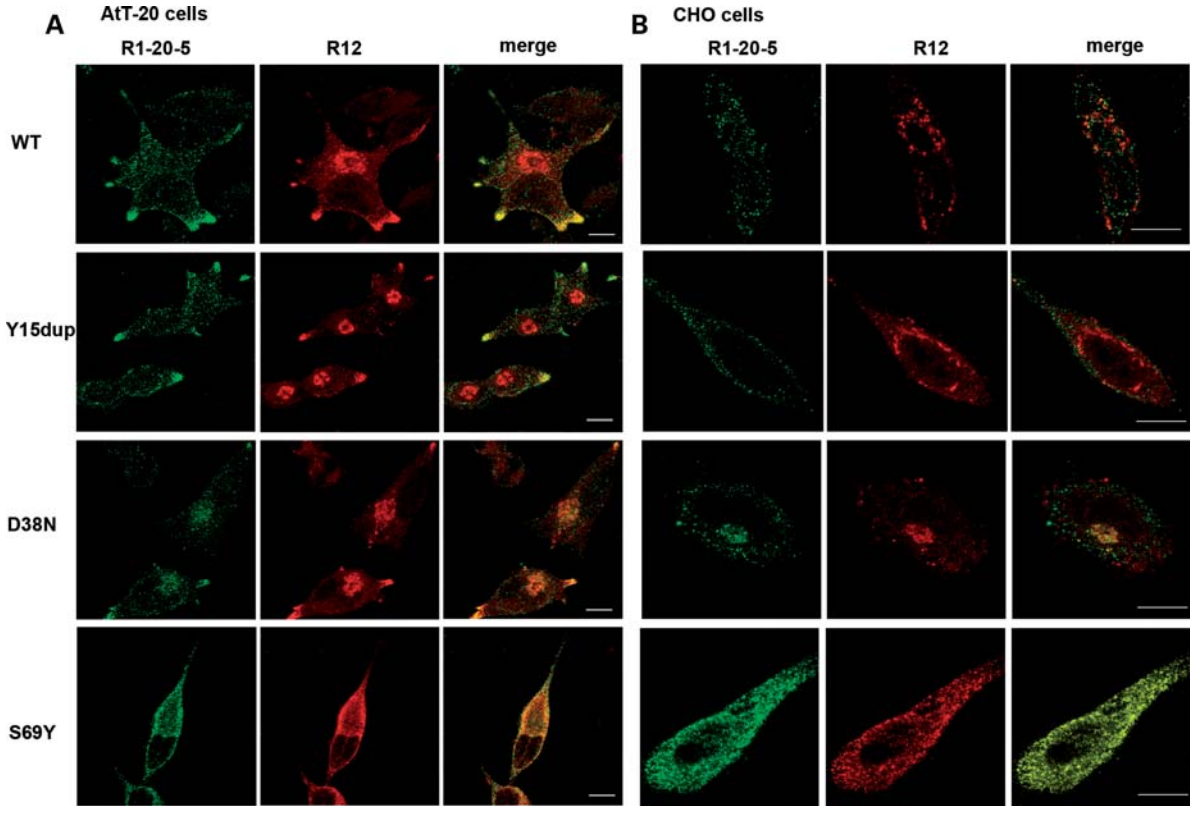

Figure 6. Co-localization of WT, Y15dup, D38N and S69Y prorenins and renins in AtT-20 and CHO cells. AtT-20 cells (**A**) and CHO cells (**B**) were fixed and co-stained with antibody against exposed active site of renin (R1-20-5) shown in green and antibody against prorenin and renin (R12) shown in red. Merge images are shown in the right columns. Bar represents 10 μm.

temperature of the culture and by inhibition of the proteasome with lactacystin.

There are some indications how structural determinants of prorenin and renin affect *in vitro* and *in vivo* protein trafficking and sorting. Glycosylation of renin is needed for correct trafficking (10,28–31). Deletion of 20 amino acids of the C-terminal part of renin leads to only prorenin production in cell culture (32). There are several data suggesting a role for the renin profragment in correct sorting: site-directed mutagenesis revealed the role of the prosegment and of the renin protein moiety in prorenin/renin trafficking (7,10). In absence of the 3D structure of human prorenin but by analogy with a molecular model built on pepsinogen (33–35), Deinum *et al*. (36) suggest that the profragment, which interacts in a very specific manner with the sequence of the mature protein, plays a role in the activation process. The proper attachment of the N-terminal part of the propeptide, $-F8^P$ to $-R15^P$, as the first of six strands in a highly conserved β-sheet, may stabilize the inactive form of human prorenin (6). The propeptide appears to interact with the mature protein also outside the active site (37). Another interesting observation is the discovery of a novel form of mRNA coding for active renin in rat and human brain. This renin that lacks part of the profragment is not secreted but present in the cytoplasm, where it is functionally active (38–41).

According to these data, we suggest the following hypothesis on prorenin/renin structure. In a model of two gastric aspartic proteinases, pepsinogen and progastricsin, proposed by Khan and James (35), Tyr9 residue, which is analogous to renin Tyr15, seems to play a crucial role in structural rearrangement of these zymogens during the activation pathway. There is a conserved TyrXAsp motif in renin (15–17), pepsin A (9–11), gastricsin (9–11), cathepsin D (10–12) and cathepsin E (20–22). In the structures of the pro-enzymes pepsinogen (3psg) and progastricsin (1htr, 1avf), there is an H-bond between the conserved tyrosine and the catalytic aspartate (D38 in mature renin) in the N-terminal domain, and also a conserved salt bridge between the Asp of this motif (15–17) and a conserved Arg in the SerXArg motif in the sequence of pepsinogen (11^P–13^P), progastricsin (11^P–13^P) and prorenin (18^P–20^P) (Fig. 2). This highly conserved salt bridge is assumed to be broken upon pH lowering, which subsequently leads to unraveling of the local tertiary structure and subsequent cleavage of the pro-part. Since Tyr15 as well as Asp17 in this TyrXAsp motif seems to have important structural tasks to fulfill and both residues are buried inside the protein, it seems reasonable to assume that a duplication of this crucial Tyr leads to a significant rearrangement of the local protein structure and eventually leads to a misfolding of the newly formed N-terminal part of mature renin or a non-recognition of the substrate.

The D38N mutant has exchanged one of the two catalytically required aspartate residues of the active site with an asparagine residue resulting in a completely inactive renin

mutant. It displays reduced stability due to the lack of the specially required versatility of H-bond donors and acceptors as well as the peculiar structural arrangement of the two catalytically important aspartates.

Ser 69 is located in a highly conserved region of the renin molecule, close to the beginning of a lengthy β-hairpin structure called 'flap' (Fig. 7A), which is required for proper enzymatic function. The flap consists of a hinge region, a β-hairpin structure and a highly flexible tip which serves as a cover of the active site that tightly interacts with either substrate or peptidomimetic inhibitor (42). In order to evaluate the relevance of S69 for structural integrity, we scrutinized an X-ray structure of human renin (3–5) and produced a model with an S69Y mutation (for details, see Materials and Methods). The structure preceding the flap in eukaryotic aspartic proteases is formed by a triad of hydrophilic small amino acids, starting with a very highly conserved serine (S69 in mature human renin) followed by a small amino acid (Ser, Ala, Gly) in the second position and ending with either serine or threonine. The highly conserved and buried S69 is involved in an extended hydrogen bond network (Fig. 7B), e.g. with the very highly conserved aspartate 95 (D95), another hallmark of eukaryotic aspartic proteases. In renin, this aspartate is also in hydrogen bond contact with the third amino acid (S71) of this triad. Serine 69 is involved in a further hydrogen bond with a neighboring backbone NH. Introduction of a large and voluminous tyrosine side chain instead of the slim serine in position 69 (sketched in Fig. 7C) has been modeled and found to destroy the delicate hydrogen bond network (for details, see Materials and Methods), thereby leading to a dramatic change of the local protein structure (data not shown) that is responsible for linking the long and flexible flap to the body of the protein. In the absence of 3D structure of human prorenin, we propose that the S69Y mutation might prevent correct positioning of the N-terminal part of renin and of its profragment, respectively. An accurately folded profragment and correct positioning of the flap in prorenin appear to be absolutely essential for renin to be eventually stable and functional. This finding most likely also applies to other eukaryotic aspartyl proteases since they display a high amino acid and structure conservation in this part of the respective structures.

In conclusion, this study documents for the first time abnormalities of human renin folding, trafficking and sorting which ultimately result into RTD, a fatal disease. The mutations involved are located in different domains of renin and are associated with distinct molecular and cellular mechanisms of renin inactivation and function. These findings allow some initial insights into the complex mechanisms associated with renin production, maturation, secretion and function.

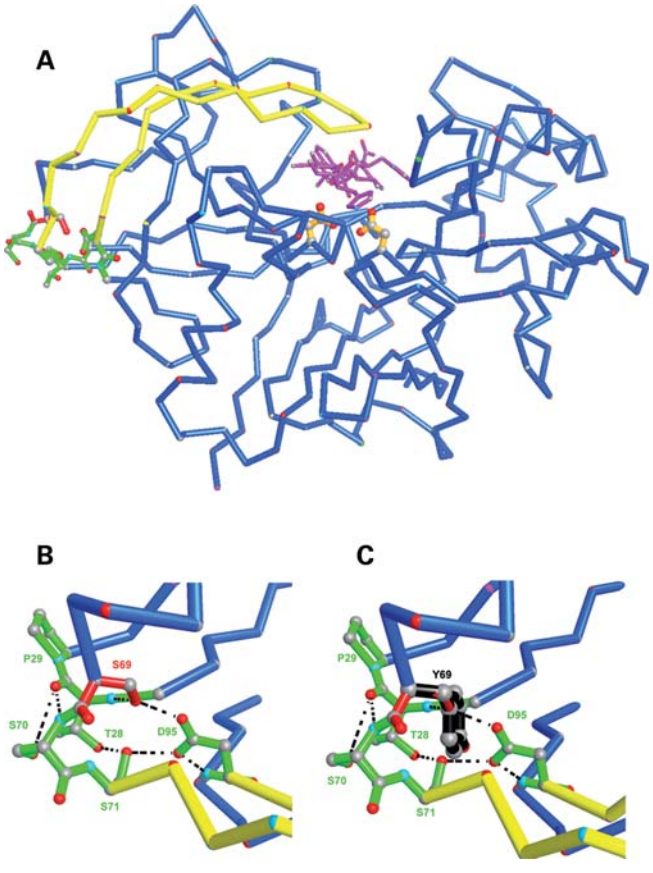

Figure 7. Structure of renin with S69Y modeled. (**A**) Renin (blue) is shown in C-alfa representation with the flap highlighted in yellow and the two catalytic aspartates (orange) at the bottom of the active site comprising a peptidomimetic inhibitor (magenta). (**B**) Selected amino acids involved in an extended hydrogen bond network (dotted black lines) are shown in green with serine 69 highlighted in red. (**C**) Sketch of a hypothetical situation where serine 69 (red) has been replaced by a voluminous tyrosine (black) which obviously does not fit in this protein arrangement and therefore will destroy large parts of the protein structure and the extended hydrogen bond network, respectively.

MATERIALS AND METHODS

Patients

Y15 dup mutation. After a first uncomplicated pregnancy, the second pregnancy was complicated by a complete ahydramnios detected at 30 weeks during the first consultation in Mayotte. Renal ultrasound revealed hyperechogenic kidneys with poor corticomedullary differentiation. The baby was delivered at 31 weeks. She had skull ossification defects and died at 3 days with persistent anuria, severe respiratory distress and limb deformations as consequences of ahydramnios. A homozygous duplication of Tyr15 was detected by gene sequencing according to the method described by Gribouval *et al.* (1).

D38N mutation. The mutation of an aspartyl residue of the active site into an Asn (Asp38Asn) has been previously mentioned (1).

S69Y mutation. The history of the first patient (patient I) has been reported previously by Zingg-Schenk *et al*. (8). The second patient (patient II), from a distinct family, comes from parents originating from Morocco who are first-degree related cousins. After a first uncomplicated pregnancy, the second pregnancy was complicated by oligohydramnios at 21 weeks' gestation and complete anamnios at 24 weeks. At that time, ultrasound examination showed normal-size hyperechogenic kidneys. The gestation was continued and the baby was

delivered at 32 weeks and died 1 h later. No clinical or post-mortem examination was permitted by the family. The third pregnancy was uneventful until 28 weeks of gestation when ultrasonography showed anamnios in a male fetus (patient II). Normal-size hyperechogenic kidneys were found by abdominal echography. Pregnancy was terminated at 29 weeks and kidney pathology was performed. A homozygous mutation of Ser 69 into Tyr was detected. Studies were done in accordance with French ethical committees' recommendations.

Immunohistochemistry

Immunostaining. Slides were deparaffinized in toluene, rehydrated in decreasing concentrations of ethanol and endogenous peroxidase inactivated with 3% H_2O_2 in PBS. Sections were prepared by incubation in 3% normal goat serum (NGS) for 30 min, followed by the R15 primary antibody specific for renin (43) diluted 1/500 in PBS + 1% NGS (1 h). Sections were then incubated for 30 min with biotinylated goat anti-rabbit IgG (1/500, BA-1000, VECTOR Laboratories). Antigen–antibody complexes formed on the section were detected with the ABC-peroxidase complex (PK-6100, Vectastain ABC Elite kit) and revealed with DAB$^+$ peroxidase substrate kit (K3468, DAKO). Control sections were incubated with a preimmune rabbit IgG and they showed no specific staining.

Plasmid constructs

The WT human preprorenin cDNA (32) was kindly provided by Dr F. Soubrier (Inserm U525) and amplified by PCR using the flanking primers 5′-AAGCTTAAGATGGATGG ATGGAGAAGGATGC-3′ (primer A) and 5′-CTTGGATCC TCAGCGGGCCAAGGCGAAGC-3′ (primer B), which contain restriction sites *Hin*dIII and *Bam*HI, respectively. The PCR product was then digested by *Hin*dIII/*Bam*HI (New England BioLabs) and subcloned into pcDNA3 plasmid (Invitrogen).

Site-directed mutagenesis of S135Y preprorenin (preprorenin numbering) or S69Y (renin numbering) was performed by two-step PCR. Two overlapping regions of preprorenin were amplified separately using primer A with the mutagenesis reverse primer S135/Y R 5′-CTTGTAGCTGGA**GTA** ATCCGAAGCATCG-3′ or the mutagenesis forward primer S135/Y F 5′-CGATGCTTCGGAT**TAC**TCCAGCTACAA G-3′ with primer B (the bases in boldface encode the new amino acid residue). The products of the two first amplifications (A-S135/Y R and S135/Y F-B) were used as templates for a second PCR with the primers A and B. The 1239 bp PCR product was then digested by *Hin*dIII/*Bam*HI restriction enzymes and subcloned into pcDNA 3. The plasmid constructs for Y81dup and D104N (preprorenin numbering) or Y15dup and D38N (renin numbering), respectively, were performed by Genosphere. Sequences of the constructs were verified by automated sequencing.

Expression of WT human prorenin, Y15dup, D38N and S69Y mutants in AtT-20 and CHO cells

Cell cultures. Established AtT-20 and CHO-K1 cells were obtained from ATCC. Cells were stably or transiently transfected using Lipofectamine 2000 (Invitrogen). Cells expressing the recombinant WT or the mutants were established by transfecting cells with either the cDNA of the WT preprorenin or with cDNA of the mutants in pcDNA3. Cells were transfected in 35 mm dishes and, 24 h later, maintained in Opti-MEM medium (Invitrogen) for 24 to 72 h. Media were harvested and cells scrapped in PBS for biochemical studies. For stable expression, transfected cells were selected for resistance to geneticin and clones screened by immunoblotting.

Protein extracts and immunoblots. Stably transfected cells were grown to confluency in complete medium, then depleted in serum-free medium Opti-MEM for 24 h and incubated without or with forskolin (10 μM). The conditioned media were then sampled, concentrated by centrifugation (ultrafree-4 biomax-10 kDa membrane, Millipore). Cells were harvested by scraping into PBS and resuspended in PBS with a protease inhibitor cocktail (bestatin 40 μg/ml, chymostatin 25 μg/ml, E64 5 μg/ml, phosphoramidon 200 μg/ml and EDTA 1 mM) (Roche Diagnostics), sonicated for 30 s and then centrifuged at 18 000*g* for 15 min at 4°C to remove the insoluble material for whole-cell lysates preparation. Prorenin in culture media and cell lysates were loaded onto 12.5% SDS–PAGE gels for western blotting analysis. Cell lysates were first immunotrapped with 3E8 mAb-coated tubes (Cisbio), to eliminate non-specific protein binding. The membrane was probed with the polyclonal R12 (43) antibody, revealed with the biotin–streptavidin system (GE) using NBT/BCIP as substrate.

Metabolic labeling and immunoprecipitation

Transfected cells were seeded in six-well plates. The cells were depleted in serum-free DME without cysteine and methionine and radiolabeled for 1.5 h with 50 μCi of [^{35}S]Cys/Met (PerkinElmer Life Sciences) in 750 μl of DME per well. For chase periods, cells were washed twice with PBS and incubated in 900 μl of Opti-MEM medium for chase period. After the chase period, media were harvested, adjusted to 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% (v/v) Triton X-100 and centrifuged at 2000*g* for 10 min at 4°C. To study the cellular fractions, cells were washed three times with PBS, scraped and solubilized with 300 μl of 20 mM Tris–HCl buffer, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% (v/v) Triton X-100 and the protease inhibitor cocktail. The resulting lysate was centrifuged at 18 000*g* for 15 min at 4°C to remove the insoluble material. Cell lysates (300 μl) and culture media (1 ml) were incubated overnight at 4°C with the selected antibody (R12 for renin) and protein A-Sepharose. The immune complex protein A was collected by centrifugation. Proteins were resolved by 12.5% SDS–PAGE, revealed by autoradiography and quantified with Quantity One software (BioRad). CST (Applied) was present or not during starvation and pulse-labeling at a final concentration of 1 mM; CNX was then immunoprecipitated (antibody from Sigma). For immunoprecipitates with Bip/GRP78 (Euromedex), cells were lysed in a buffer containing 50 U of Apyrase (Sigma). The proteasome inhibitor Clasto-lactacystin β-Lactone (Calbiochem) was added to the culture medium at a

final concentration of 10 μM at 37°C for 16 h throughout the incubation and the pulse-chase experiment.

PNGase F digestion. Radiolabeled immunoprecipitates were eluted from protein A-Sepharose as described and treated with PNGase F for 20 h at 37°C as recommended by the manufacturer (New England BioLabs).

Immunofluorescence microscopy

Stably transfected AtT-20 or CHO cells were seeded on 14 mm diameter coverslips. The cells were fixed with 4% paraformaldehyde plus PBS for 20 min and permeabilized by incubation in PBS, 1% Triton X-100 for 15 min. The cells were rinsed three times in PBS and saturated with 3% of NGS for 30 min and finally incubated for 1 h at room temperature with one of the following primary antibodies: R12 antiserum (1:5000 dilution); 1A12 monoclonal human renin antibody (44) (1:1000 dilution); R1–20-5 monoclonal human renin-active site (12) (1:2000 dilution) prior to staining with anti-PDI mAb (1:1000 dilution) (Stressgen) or rabbit polyclonal anti-TGN38 (1:1000 dilution in PBS) (a generous gift from S. Milgram, Chapel Hill, NC, USA) or anti-ACTH mAb (1:2000 dilution) (Dako), 1% NGS for 1 h at room temperature. The cells were then incubated for 1 h with the appropriate dye-labeled secondary antibodies (Molecular Probes): anti-rabbit or anti-mouse Alexa 488 or 555. Cells were examined with a Leica TCS SP II (Leica Microsystems, Heidelberg, Germany) confocal laser scanning microscope equipped with an argon/krypton laser and configured with a Leica DM IRBE inverted microscope. All the double-labeling experiments were analyzed in sequential scanning mode. Images (1024 × 1024 pixels) were obtained with a ×63 magnification oil-immersion objective. Each image corresponds to two or three cross-sections of the cell.

Renin immunoassay and enzymatic assay

Immunoreactive active renin was determined with a radioimmunometric assay kit (Cisbio) according to the manufacturer. Total renin concentration, which corresponds to both prorenin and active renin, was determined using the same kit after proteolytic activation of prorenin by trypsin. Proteolytic activation was performed in aliquots of media and cell lysates. Aliquots were incubated for 10 min at 4°C with 25 μl of a 50% suspension of trypsin immobilized on beaded sepharose (PIERCE) in a final concentration of 100 mM PBS, pH 7.4. Supernatants were collected by centrifugation.

For enzymatic activity, prorenin or renin was trapped with 3E8-coated tubes. After four washes, $5–10^{-7}$ M of human recombinant angiotensinogen was added at pH 7.5 in phosphate buffer. A kinetic analysis was performed successively on the same tube for each renin extract in order to obtain initial velocity by Ang I quantification. Finally, the specificity of the reaction was confirmed in the presence of pepstatin A (Sigma). Renin bound to the antibody of each tube was eluted and performed by western blotting to control the quantity. The amount of Ang I was determined by radioimmunoassay according to Lachurie *et al* (45). Human recombinant angiotensinogen was produced by expression in CHO cells and purified in our laboratory as described previously (46).

Modeling of renin mutants

Modeling was performed on a structure of human renin (PDB code: 3D91). All calculations were done on a Dell Precision 670 workstation using the program Moloc (47) (www.moloc.ch). Insertion of the tyrosine side chain instead of serine 69 could be performed straightforward applying a dedicated functionality in Moloc. Optimization of the resulting S69Y renin mutant structure led to a dramatic reordering of the protein structure in this area thereby completely destroying the local hydrogen bond network (data not shown).

SUPPLEMENTARY MATERIAL

Supplementary Material is available at *HMG* online.

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Conflict of Interest statement. None declared.

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