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The Molecular Biology of Human Renin and Its Gene

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The molecular biology of renin, prorenin, and the renin gene have been studied. A tissue-specific pattern of expression was found in rat and human tissues. In the human placenta, the transfected and endogenous renin promoters are active, and renin mRNA levels and transfected promoter activity are increased by a calcium ionophore plus cAMP. Cultured pituitary AtT-20 cells transfected with a preprorenin expression vector mimick renal renin release by converting prorenin to renin and releasing renin in response to 8Br-cAMP. Studies with mutant renin genes suggest that the body of renin directs renin to the regulated secretory pathway, and renin glycosylation affects its trafficking. Chinese hamster ovary cells were used to produce recombinant prorenin. Infused prorenin was not converted to renin in monkeys. Renin crystals were used to determine its three-dimensional structure. Renin resembles other aspartyl proteases in the active site and core, but it differs in other regions that probably explain renin's unique substrate specificity. Based on structural and mutational analysis, a model for human prorenin was built that suggests lysine -2 of the prosegment interacts with active site aspartate residues, and that the prosegment inactivation of renin is stabilized by binding of an amino terminal β strand into a groove on renin.

INTRODUCTION

The renin angiotensin system is involved extensively in regulating blood pressure, fluid balance, and other cardiovascular and renal functions [1,2]. It is critical for the body's responses to fluid loss and shock, and abnormalities in it are implicated in the pathological derangements of hypertension, heart failure, and other disorders. Renin is rate-limiting for the production of angiotensin II, the major active component of the system [1,2]. In the circulation and possibly at other local sites, renin cleaves angiotensinogen to release the decapeptide angiotensin I [1,2] that is converted to angiotensin II by converting enzyme. Since converting enzyme inhibitors are widely used in treating hypertension and heart failure [3], renin is also a target for blockade by pharmaceuticals [4,5]. The effects of renin and converting enzyme inhibitors could even differ, since converting enzyme acts on substrates, such as bradykinin [1,3], in addition to angiotensin I, whereas renin acts only on one known substrate in spite of its homology with other aspartyl proteases, such as pepsin that have other substrates

Abbreviations: CAT: chloramphenicol acetyltransferase CHO: Chinese hamster ovary

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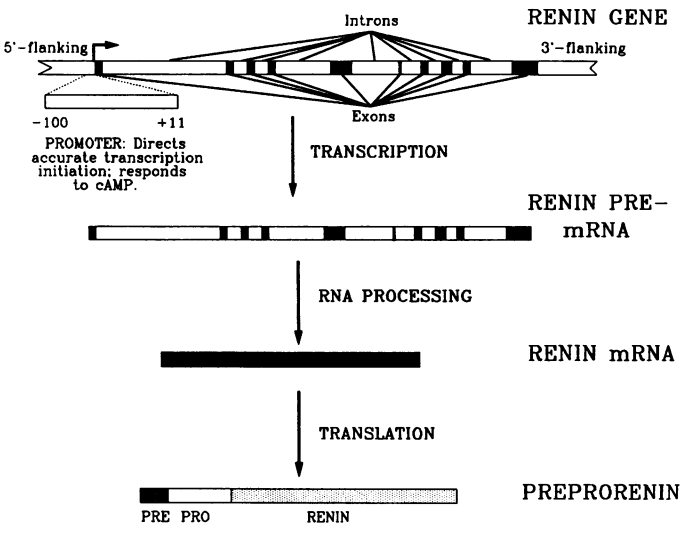

FIG. 1. Structure and expression of the human renin gene. Shown schematically are the various elements of the gene, including promoter elements, with the location of the element that responds to cAMP, introns (*open rectangles*), exons (*solid rectangles*), and flanking DNA (*open rectangles*). The introns and exons of the pre-mRNA and mRNA are also shown.

[4–11]. Knowledge of the structure of renin could facilitate the design of such inhibitors.

The major source of circulating renin in man is the renal juxtaglomerular cells, in which renin mRNA is translated into preprorenin that is processed to prorenin upon its insertion into the rough endoplasmic reticulum [1,2,12–15]; prorenin is catalytically inactive [12,16]. Some prorenin is released [17,18], but much of it is converted in the juxtaglomerular cells to active renin by proteolytic removal of the 43 amino acid prosegment [10,11,16–18]. This renin can be stored and released in response to such provocative stimuli as beta-adrenergic activation [18,19]. Elements of the renin angiotensin system are also expressed in extrarenal tissues, and there is growing suspicion that local renin angiotensin systems play important roles [12,20–26]. At external sites, prorenin rather than renin is predominately released. Thus, knowledge of how the expression of the renin gene is controlled, renin release is regulated, and conversion of prorenin to renin occurs is relevant to understanding the role of renin in physiological and pathological states. To address these issues, we have been examining aspects of renin and its gene.

CLONING AND STRUCTURAL ANALYSIS OF THE HUMAN RENIN GENE

To obtain human renin gene sequences, we earlier cloned the human renin chromosomal gene [10], using a mouse cDNA probe [27], and later cloned a human renin cDNA [28]. The chromosomal gene was sequenced in the 5'- and 3'-flanking DNA, the exons, and portions of the introns. The gene spans over 11 kb of DNA, contains ten exons separated by nine introns, and there is repetitive DNA in the 5'- and 3'-flanking regions (Fig. 1). The gene shows organization similar to that encoding the related pepsinogen [8].

TRANSCRIPTIONAL CONTROL OF RENIN GENE EXPRESSION

Transcriptional control of renin gene expression involves factors that direct its tissue-specific pattern of expression and that regulate the level of expression in

response to regulatory signals. The tissue-specific expression of the gene in the rat was examined by hybridization histochemistry and immunocytochemistry [29]. The gene is most actively expressed in the renal juxtaglomerular cells, but there are lower levels of expression in the adrenal glomerulosa, testicular leydig cells, ovary, and anterior and intermediate pituitary. In the human, expression was detected in placental cells [30,31]. (The specific cell type has not yet been rigorously established.) These results suggest a highly tissue-specific pattern of expression. A transfected gene containing the renin promoter and chloramphenicol acetyltransferase (CAT) coding sequences (renin-CAT) was expressed by the placental cells, although it is not clear that this promoter is sufficient for directing tissue-specific expression ([31]; Fig. 1).

Since renal renin release is regulated by such agents as β-adrenergic agonists and angiotensin II that act through second messengers such as cAMP and calcium ion [1], we examined whether some of these second messengers could affect either renin mRNA levels or promoter activity. In the placental cells, a calcium ionophore increased endogenous renin mRNA levels, and the increase was greater with the ionophore plus forskolin that activates adenylate cyclase [31]. The combination of forskolin plus a phorbol ester that activates C-kinase also increased renin mRNA levels. Thus, these messengers could affect renin production in part by regulating renin mRNA levels. These influences could be due to effects on the renin promoter, since expression of the renin-CAT construct, when transfected with calcium phosphate precipitation (which increases calcium ion uptake), was increased by forskolin (Fig. 1). The effect was observed with constructs containing as few as 100 bp of the proximal renin 5′-flanking DNA.

POST-TRANSCRIPTIONAL EVENTS IN RENIN GENE EXPRESSION

Since cultured human juxtaglomerular cells that produce prorenin and process it to renin are not available, we have developed a model system to study regulated release of renin by transfecting cultured cells with a gene that expresses human renin gene coding sequences [32]. Thus genes containing the human metallothionein or Rous sarcoma virus promoters that drive expression of preprorenin coding sequences have been transfected into cultured pituitary AtT-20 or Chinese hamster ovary (CHO) cells [32]. AtT-20 cells process other preproteins such as proopiomelanocortin or proinsulin, and release of the mature hormone is controlled in these cells by secretagogues [33]. In contrast, CHO cells do not process prohormones such as these, and the secretion of the proteins is not affected by secretagogues [33].

It was of particular interest to determine whether prorenin was converted to renin in these cells, because the only known sources of plasma renin in humans and rats are the kidneys [1,12,14,15]. Most other tissues that have been found to express the renin gene apparently release only prorenin [12,14,20–26,29–31]. These data could imply that the prorenin processing enzyme has a highly restricted tissue distribution; however, the prorenin synthesized by the transfected AtT-20 cells was cleaved to active renin at the natural cleavage site (documented by amino terminal sequencing), and the release of renin was regulated by the same stimulus (cAMP; in this case, 8Br-cAMP) that promotes the release of renal renin [32]. Similar results were obtained by Pratt et al. [34]. Thus, prorenin processing enzymes are not restricted to the kidney, and certain aspects of regulated renin release can be reconstituted in the AtT-20 cells, which can therefore serve as a useful model.

ROLE OF GLYCOSYLATION IN PRORENIN AND RENIN RELEASE

Renin is known to be glycosylated [28,36,37]. In the rat, several different forms of glycosylated renin have been identified, and these have been proposed to vary in their clearance from the circulation [37]. The presence of sugar moieties on other proteins has also been shown to affect their intracellular trafficking [36]. This presence can promote the binding of the proteins to the mannose-6-phosphate receptor on the lysosome, with consequent uptake into the lysosome and degradation [36]. In our studies with recombinant DNA-produced prorenin, treatment of prorenin with endoglycosidase F that removes much of the carbohydrate did not activate prorenin, suggesting that the sugar moieties were not involved in activating prorenin [28,35]. Faust et al. presented evidence, using human prorenin expressed in Xenopus oocytes, that a significant amount of the prorenin produced is targeted to the lysosome where it is degraded [36].

To explore more rigorously the role of glycosylation in prorenin and renin trafficking in the cell and activation, we prepared a mutated prorenin coding vector in which one or both of the only two glycosylation sites of renin (asparagine residues at positions 5 and 75) were changed to serine residues [38]. The mutant deglycosylated prorenins were then expressed in CHO and AtT-20 cells. No differences between native and mutant deglycosylated prorenin secretion or activity were observed with CHO cells. In contrast, a higher proportion of the total prorenin and renin produced in the AtT-20 cells was active renin. Thus, the sugar moieties do not appear to contribute to the inactivation or activation of prorenin, but they may affect the intracellular trafficking of prorenin and possibly the intracellular processing of prorenin. If the sugar moieties do target prorenin to the lysosome, then the AtT-20 cell data could imply that the absence of the sugar moieties could target more of the prorenin to the regulated secretory granules, with consequent processing to renin and release in response to secretagogue stimulation.

ROLE OF THE SIGNAL PEPTIDE AND PROSEGMENT SEQUENCES IN RENIN TRAFFICKING

To determine the role of the signal peptide and prosegment sequences in the intracellular trafficking of renin (Fig. 2), we prepared two mutant renin genes [38]. One of these contained a deleted prosegment and the other contained the signal peptide sequences of the mouse IgM immunoglobulin, which is not targeted to the regulated secretory pathway. These genes were transfected into AtT-20 cells. The prosegment-deleted gene expressed active renin whose release was increased by secretagogue stimulation. The gene containing the immunoglobulin leader sequence expressed both prorenin and renin, and renin release was increased by secretagogue treatment. These results suggest that the signals directing renin to the regulated secretory pathway are not contained within the signal peptide or prosegment sequences and therefore must be contained within the body of renin.

RECOMBINANT PRODUCTION OF PRORENIN AND RENIN

Renin and prorenin circulate at very low levels, and only small quantities of these proteins are available from natural sources for purification. To obtain prorenin and renin for purification, we developed CHO cell lines that express larger quantities of human preprorenin [28]. The cells were co-transfected with two vectors. One of these

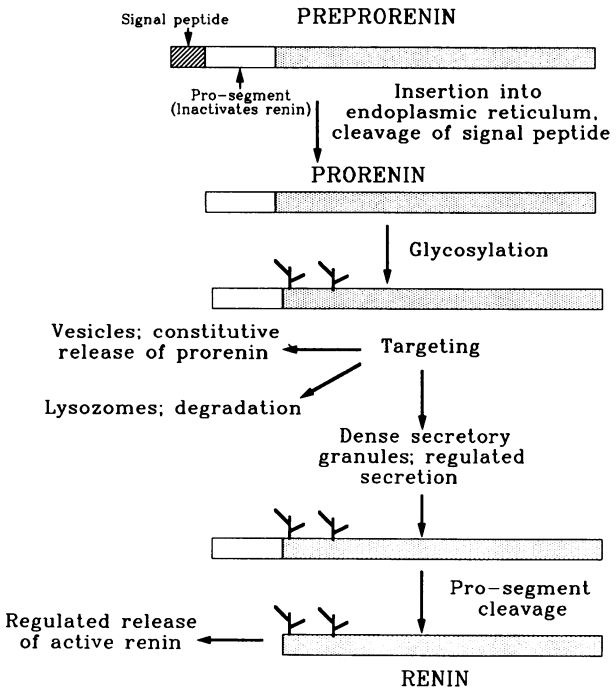

FIG. 2. Post-translational events in prorenin and renin processing, subcellular targeting, and release.

contained the metallothionein promoter that is highly active in these cells, human preprorenin coding sequences, and a 3'-terminal region from the human growth hormone gene. The other vector contained the entire metallothionein gene. Metallothionein protects cells from being killed by cadmium ions. Thus, by growing cells in progressively increasing cadmium ion concentrations, cell lines can be selected in which the metallothionein gene is amplified. Because the preprorenin and metallothionein vectors are usually integrated into the DNA together, both genes will be amplified by the cadmium ion selection, and thus cadmium-resistant cells will produce higher levels of prorenin. In this way, lines that express relatively larger quantities of prorenin were obtained from the original transfected cells. Prorenin was subsequently isolated from these cells and was either purified as such or converted to renin by trypsin treatment and purified [7,29,35]. The properties of the purified prorenin and renin were identical to those of naturally occurring renin in all of a number of parameters studied, such as mobility on several gels, apparent carbohydrate content, inhibition by pepstatin that blocks renin activity, substrate affinity, and activation by trypsin [16,28,35].

ACTIVITY OF INFUSED PRORENIN

There is controversy as to whether prorenin can be converted to renin in the circulation. Whereas most investigators have concluded that there is no conversion of prorenin to renin in the circulation [1,12,14,15], it has been reported that the rat kidney releases a protease that cleaves renin in the circulation [39]. Others have proposed that prorenin might have *in vivo* activity, for example, through reversible activation or after its uptake by peripheral tissues [12,21,40]. To address issues such as

these, prorenin was infused into rhesus monkeys over a 40-minute period [41]. The infusion resulted in three- to fourfold elevations of prorenin levels. There was, however, no increase in plasma renin levels and no response to several potential sequelae of renin actions to generate angiotensin II production, such as changes in blood pressure, aldosterone release, or urinary sodium or potassium secretion. These studies therefore suggest that circulating prorenin is not converted to renin and is generally inactive in terms of classical renin activity.

X-RAY CRYSTALLOGRAPHIC STRUCTURE OF HUMAN RENIN

The availability of prorenin and renin produced by recombinant DNA methods provided a stimulus to determine the three-dimensional structure of human renin. To do this, the recombinant DNA-produced and purified renin from the CHO cells was first crystallized [7]. By obtaining highly purified renin and following about 1,500 crystallization trials, crystals acceptable for X-ray diffraction experiments were obtained [7]. Solution of the structure was facilitated by the availability of coordinates of the closely related porcine pepsinogen [6]. Based on these coordinates and the amino acid sequence homology between renin and pepsinogen, a model for human renin was constructed. The placement of this model in the renin crystallographic cell was carried out by the molecular replacement technique. The electron density maps obtained from the X-ray diffraction data and molecular replacement phases revealed that the model was correct for those regions of renin expected to be most similar to pepsin, for example, in the active site and the hydrophobic core of the molecule. The agreement was much worse for regions where the sequence homology is weak, however; i.e., those regions where renin is unique. These regions are probably of the greatest interest in explaining the unique substrate specificity of renin and for designing inhibitors of renin. These results also point to limitations of current computer modeling and other methods to define tertiary structures. To improve the structure of renin, molecular dynamics refinement techniques that included the crystallographic data as a restraint were used to resolve the differences between the model and the data. The final stages of refinement were carried out at 2.5-angstrom resolution by using conventional restrained parameters least-squares algorithms. The current model (Fig. 3) still contains poorly defined surface loops, whereas the interior of the molecule is well defined.

MODEL FOR PRORENIN AND MECHANISMS OF PROSEGMENT ACTIVATION

Prorenin has very weak renin catalytic activity [12,16,35]. This low level of activity is due to inhibition of the active site of the enzyme by the prosegment [6,12,42]. Pepsinogen is analogous to renin in this respect, and for this zymogen the prosegment folds into a cleft in the protein that harbors the two aspartate residues of the active site. The amino terminal segment forms part of a β-pleated sheet located in the back of the molecule; it then forms two α helices that fit into a hydrophobic pocket near the aspartate residues of the active site [6]. When the prosegment of pepsinogen is removed, the amino terminus of the mature pepsin molecule undergoes an enormous conformational change in which the amino terminal amino acids move into the same β-sheet structure previously occupied by the prosegment [6]. Inspection of the structure of renin [7] suggests that its amino terminal amino acids occupy an analogous position relative to the renin molecule. Thus, it is likely that a similar

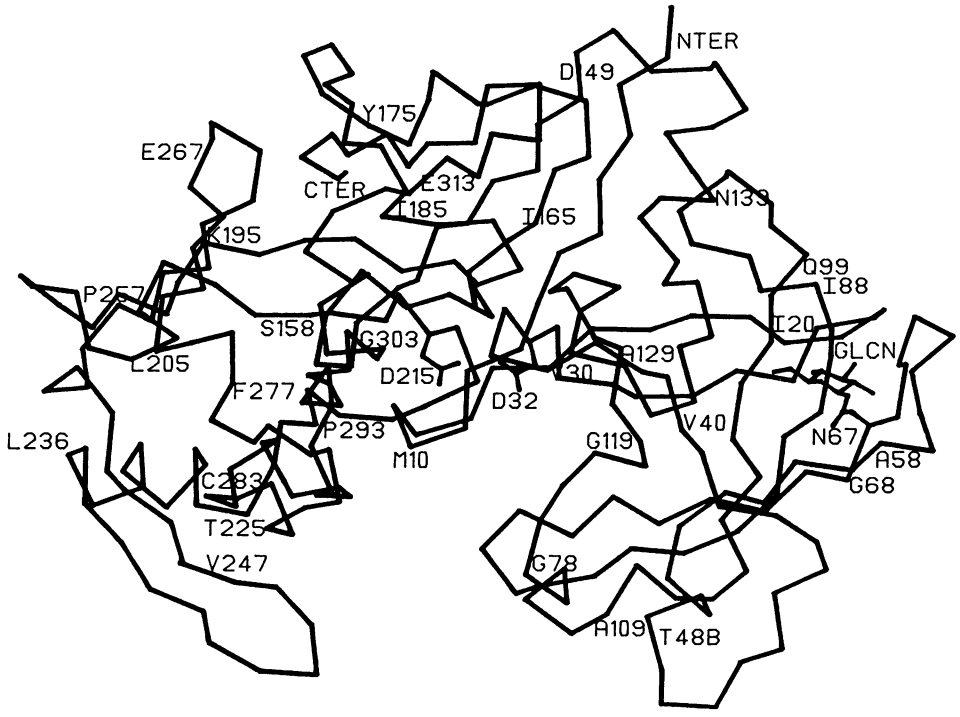

FIG. 3. Structure of human renin. Data from Sielecki et al. [7]. Shown is the alpha-carbon tracing of the molecule. The overall croissant-like shape of the molecule with a cleft is apparent. In the interior of the cleft are the active site aspartate residues (**D32** and **D215**) whose side chains are also portrayed. The amino terminus (**NTER**) that is folded around the back of the molecule fits into a pocket proposed to be occupied by the amino terminus of prorenin. The carboxyl terminus (**CTER**) and the single monosaccharide (**GLCN**) bonded to Asn 67 (**N67**) that remained on the glycosidase-treated renin used for the structure analysis are also indicated. The numbering system used here is based on that of porcine pepsin. Thus, Asn 67 is Asn 75 of renin, Asp 32 is Asp 38 of renin, and Asp 215 is Asp 226 in renin.

situation exists with prorenin. Based on a comparison of the structures of renin and pepsinogen, it appears the lysine at -2 of the prorenin prosegment is the amino acid that forms ionic interactions with the active site aspartate residues and that a β strand of more amino terminal amino acids of the prosegment of prorenin fits into the β sheet on the back surface of the molecule in a manner analogous to that found in pepsinogen.

To assess the importance of these interactions, we prepared two mutant renin genes. In one of these, the lysine at -2 was converted to an alanine. This gene, when transfected into CHO cells, expressed only prorenin, suggesting that the putative interaction of the lysine at the active site is not critical for prosegment inactivation. In contrast, when an arginine at prosegment residue -32 in the proposed β sheet was changed to an aspartic acid residue that would be expected to create a charge repulsion effect on the interaction of the β sheet with the body of the molecule, the CHO cells released active prorenin. These results suggest that the β-sheet interactions with the body of the molecule are important for keeping the prosegment amino acids fitted into the active site, where they presumably block the catalytic activity of the enzyme by preventing the substrate (angiotensinogen) from reaching the active site.

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