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Biosynthesis of renin: Multiplicity of active and intermediate forms

(renin processing/renin secretion/renin purification/mouse submandibular glands)

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ABSTRACT **Processing of renin involves sequential proteolytic cleavages of a preproform to the active mature forms. Preprorenin is rapidly internalized cotranslationally into the rough endoplasmic reticulum and hydrolyzed by signal peptidase to produce prorenin. In the Golgi, prorenin is converted (within 15 min) to a form of renin that is enzymatically active. Over the next 12 hr, a slow intracellular process removes a dipeptide near the carboxyl terminus, converting the one-chain renin into two chains joined by a single disulfide bond. This conversion occurs during formation, condensation, and packaging of renin granules. The resultant two-chain renin is approximately one-sixth as active as the one-chain form. The intact renin molecule is obligatory for enzymatic activity because heavy chain alone has little or no activity. Both one- and two-chain renins are secreted, but prorenin is not. Multiple isoelectric forms of prorenin, one-chain renin, and two-chain renin are also observed. This microheterogeneity probably results from minor differences in amino acid composition as a consequence of variations in cleavage positions during processing. Thus, these data suggest that renin synthesis and secretion is complex and may be subject to regulation at multiple steps. Furthermore, based on the results of this study, we also propose that renin can be secreted by two different pathways.**

The renin angiotensin aldosterone system plays a central role in the regulation of blood pressure and extracellular fluid volume. Although the kidney is the major source of plasma renin (EC 3.4.99.19), other tissues are also capable of renin synthesis and secretion (1). For example, the mouse submandibular gland (SMG) produces an enzyme identical to renal renin in catalytic, antigenic, and molecular properties (2). In some strains (3), this gland produces large quantities of the enzyme and, thus, is an ideal model system for elucidating the pathway of renin biosynthesis. Recently, the sequence of SMG renin and the cDNA to renin mRNA have been reported (4, 5). Renin mRNA is 1,600 nucleotides long and encodes a protein of M_r 45,000. The mature enzyme is M_r 36,500 and consists of a heavy chain of 288 amino acids (M_r 31,036) and a light chain and 48 amino acids (M_r 5,458) held together by a single disulfide bridge.

Although a model for renin processing has been postulated (5), the entire biosynthetic pathway has not been completely elucidated. To date, no studies have directly examined whether the M_r 45,000 translational product of renin mRNA is converted to an intracellular precursor [such as Parathormone (6)] or directly to the final active product [such as prolactin (7)]. Additional questions include the following ones. Does processing involve several biosynthetic intermediates? What is the temporal sequence of the proteolytic cleavages—i.e., which occurs first, the carboxyl-terminal or the amino-terminal cleavage? Is the cleavage of the one-chain protein or two chains a specific biosynthetic process or is it an artifact due to the numerous steps necessary for purification? Is the one-chain renin biologically active or is processing to the two-chain renin obligatory for activity? Is the heavy chain alone active? Is there heterogeneity in processing? What are the storage and secretory forms of renin? Finally, determination of the kinetics of the various processing steps is important for the understanding of regulation and secretion of renin. This study was undertaken to examine the above issues by using mouse SMG renin as a model system.

MATERIALS AND METHODS

Outbred Swiss adult male or female Crl: CD-1(ICR) BR mice were studied. Poly(A)$^+$ mRNA isolated by guanidine hydrochloride extraction was translated in the presence (6) or absence of dog pancreatic microsomes by using rabbit reticulocyte lysates (New England Nuclear) (8). *In vitro* pulse-labeling experiments were performed on glands from female mice stimulated with testosterone for 5 days (3, 8). Minced glands were incubated at 37°C for various times in methionine-free Dulbecco's modified Eagle's medium under 95% O_2/5% CO_2 to which 250 μCi (1 Ci = 37 GBq) of [^{35}S]methionine (>1,000 Ci/mmol; New England Nuclear) was added. The tissue and media were separated by centrifugation and processed immediately at −70°C. To confirm the findings of the *in vitro* experiments, *in vivo* pulse labeling was performed in an intact anesthesized testosterone-treated female mouse to which 1 mCi of [^{35}S]methionine was injected into a lobe of a SMG. Eight hours later, the mouse was sacrificed and the excised gland was frozen immediately. [^{35}S]Methionine-labeled renin and renin precursors in the extracts of tissues from the above experiments were immunoselected with renin antiserum (8 μl/100 μl of extract), the immunocomplexes were purified by absorption with heat-killed *Staphylococcus aureus* and analyzed by 11% NaDodSO$_4$/polyacrylamide gel electrophoresis (9), followed by fluorography and densitometry. The pIs of the various renins were determined by two-dimensional gel electrophoresis (10). Peptide maps of the purified renins and the *in vitro* radiolabeled renin and renin precursors were compared electrophoretically after limited digestion with V-8 protease (11).

To examine the subcellular localization of precursor and mature renins, enriched Golgi (12) and renin granules (13) were isolated by subcellular fractionation with differential centrifugation. Isolated fractions were lysed in 2% NaDodSO$_4$ and analyzed on NaDodSO$_4$/polyacrylamide gel electrophoresis, followed by electrophoretic transfer of proteins to nitrocellulose sheets (14). Renin was immunochemically detected by using renin antiserum and horseradish peroxidase conjugated to goat anti-rabbit IgG (15).

Enzymatic activity of the biosynthesized radiolabeled renin, its precursors, and intermediates was assessed by their inter-

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Abbreviation: SMG, submandibular gland.

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action with the active-site ligand pepstatin conjugated to aminohexyl-Sepharose (16).

Renin was purified from male outbred Swiss CD-1 mice by the procedure of Cohen *et al.* (2), modified to include the protease inhibitors (0.25 mM EDTA, 0.25 mM sodium tetrathionate, and 0.1 mM phenylmethylsulfonyl fluoride) in all buffers. The purified proteins were analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and visualized by staining with Coomassie blue or silver nitrate. Isolation of the heavy chain from the two-chain renin was achieved by incubation of the latter in 0.1 M dithiothreitol for 1 hr at room temperature in order to dissociate heavy from light chain. Light chain and dithiothreitol were removed by dialysis against two changes of 0.01 M sodium phosphate buffer (pH 7.4) at 4°C overnight.

Renin activity was assayed as angiotensin I generation at pH 7.4 (4). Trypsin activation (final concentrations, 1×10^{-7} to 5 mg/ml) was performed at 25°C for 30 min. Carbohydrate analysis was performed by the gas chromatographic method of Rheinhold (17). Protein concentration of purified renin was determined by UV absorbance at 280 nm.

RESULTS

Among the cell-free translational products of male SMG mRNA is a M_r 47,000 ± 2,000 protein that has been identified as a renin precursor (8). This protein is immunoprecipitated by renin-specific antiserum (Fig. 1*A*), and its specific precipitation is blocked by prior treatment of the antiserum with pure renin (data not shown). The "prepro" nature of this protein was demonstrated by cell-free translation of renin mRNA in the presence of dog pancreatic microsomes that contain signal peptidases capable of cotranslationally hydrolyzing the presequence from nascent polypeptide chains (7). Microsomal-enclosed renin, presumably prorenin, has an apparent M_r of 44,000 ± 2,000 (Fig. 1*A*), which is 3,000 less than the primary translational product, strongly suggesting the loss of a signal peptide.

Further evidence for the existence of prorenin was obtained in biosynthetic pulse-labeling studies of minced SMG, which demonstrated the rapid (within 15 min) intracellular synthesis of the immunoreactive ^{35}S-labeled protein of M_r 44,000 ± 2,000 (Fig. 1*A*). This prorenin was rapidly converted (within 15 min) to a M_r 38,000 ± 2,000 form of renin as shown by $NaDodSO_4$/polyacrylamide gel electrophoresis. Regardless of whether this electrophoresis was performed under reducing or nonreducing conditions, the molecular weight of this renin remained unchanged, demonstrating that it corresponds to a one-chain renin that kinetically precedes the removal of the Arg-Arg dipeptide at positions 352–353 (4, 5). The processing of prorenin to single-chain renin was rapid (Fig. 1*B*). After 30 min of this initial labeling, 50% of the newly synthesized renin was in this one-chain form. At 3 hr, the one-chain form constituted 75% of total radiolabeled renin, and a significant fraction of this renin had been secreted into the medium. By 6 hr of labeling, a small quantity of a new form of renin, representing 15% of total labeled intracellular renins, appeared in the tissue. This renin has an apparent M_r of 34,000 ± 2,000 on reducing and 38,000 ± 2,000 on nonreducing $NaDodSO_4$/polyacrylamide gel electrophoresis. Based on the differential electrophoretic behavior, this protein probably corresponds to the two-chain renin described by Misono *et al.* (4). The ratio of percentages of the prorenin to one-chain renin to two-chain renin at this time was approximately 15:70:15 based on densitometry of the fluorogram. After another 12 hr of incubation in the presence of excess unlabeled methionine, 90% of the ^{35}S-labeled renin was in the two-chain form, suggesting intracellular processing to this final form of renin. However, small amounts of radiolabeled prorenin and one-chain renins were still present in the tissue. Analysis of the media of the pulse-labeling experiments at this time revealed the presence of both the one- and two-chain renins (Fig. 1*B*). In contrast, at no time in our study was prorenin detected in the media. The latter finding suggests that prorenin is not secreted by the gland.

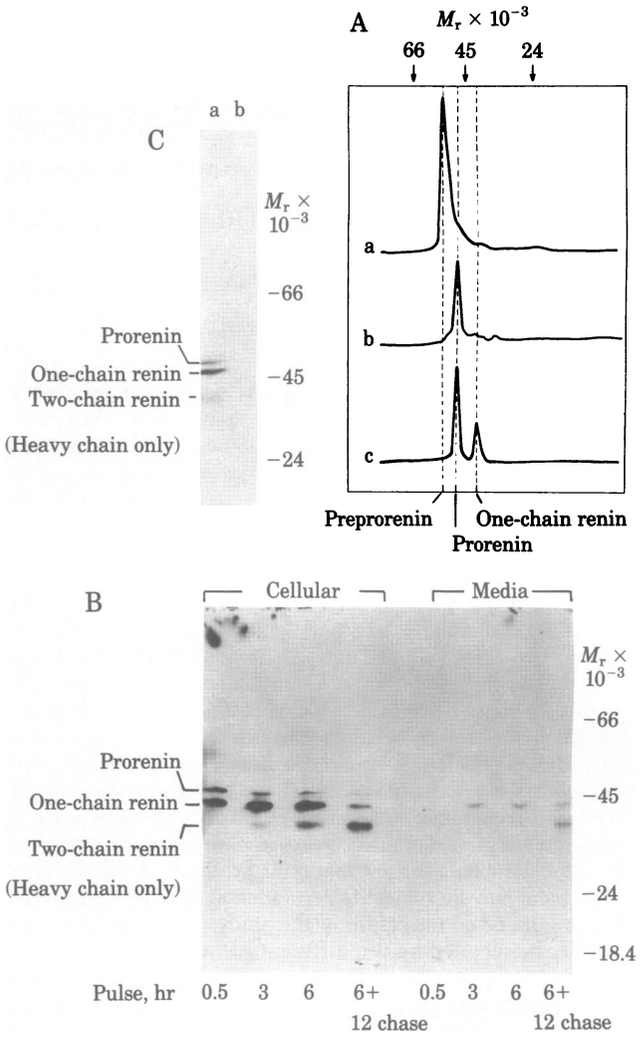

FIG. 1. (*A*) Synthesis of prorenin. [^{35}S]Methionine-labeled cell-free translational products of male glandular mRNA were synthesized in the presence of dog pancreatic microsomes. After digestion of extramicrosomal proteins with trypsin (7), the intramicrosomal proteins were immunoselected with renin antiserum and analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis under reducing conditions, followed by fluorography and densitometry (trace b). Note the decrease in molecular weight as compared to immunoselected translation products synthesized without microsomes (trace a) and the similarity in molecular weight to the *in vitro* biosynthesized prorenin (trace c). The arrows indicate the position of the molecular weight standards, and the positions of the various forms of renin are indicated at the bottom. (*B*) *In vitro* synthesis of renin. Fluorographs of immunoprecipitates from mouse SMG labeled with [^{35}S]methionine for the indicated times (lanes labeled cellular). $NaDodSO_4$/polyacrylamide gel electrophoresis was performed under reducing conditions. Similar analysis of the media from each of the time points is shown in lanes labeled media. (*C*) *In vivo* synthesis of renin. [^{35}S]Methionine (1 mCi) was directly injected into a gland of an intact mouse; 8 hr after injection, the gland was excised, homogenized, and analyzed with renin antiserum (lane a) or control serum (lane b).

The slow processing of the one-chain to the two-chain form was the result of specific proteolysis and not due to nonspecific breakdown after cell death and lysis. Indeed, incorporation of radioactivity into protein by the tissue was linear during the 6 hr of tissue incubation (data not shown). Furthermore, the results of the *in vivo* labeling experiment confirmed the *in vitro*

labeling data. Eight hours after injection of [^{35}S]methionine into an intact mouse, the gland was removed and immediately processed without incubation. The ratio of glandular percentages of prorenin to one-chain renin to two-chain renin was 10:70:20 (Fig. 1*C*), similar to percentages observed in the *in vitro* incubated tissues (Fig. 1*B*), thus excluding cell death and nonspecific proteolysis as a possibility. This postulate is further supported by the demonstration that isolated intact renin granules contain predominantly the two-chain form (see below).

The presence of renins in the media is due to specific secretion and not to cell leakage. At no time was prorenin detected in the media, demonstrating cell integrity and indicating that release of renin into the media is a selective process. Indeed, vincristine greatly inhibited both the secretion of renin activity and radiolabeled proteins (data not shown), indicating that the renins in the media represent bona fide secretory products. The presence of the two-chain renin in the medium is not due to extracellular proteolysis of the single-chain polypeptide after prolonged incubation because the *in vivo* labeled gland (containing 20% two-chain renin as described above) released the two-chain renin into the medium within minutes of *in vitro* incubation of the tissue.

To assess the enzymatic activities of the prorenin, one-chain renin, and two-chain renin, extracts of radiolabeled gland were subjected to pepstatin affinity chromatography, which binds active renin but not inactive renin (16). Therefore, it is highly likely that binding to the affinity column indicates the active state of the enzyme. Our results demonstrated that both the biosynthesized one-chain renin and the two-chain renin were enzymatically active because they were retained on the column. However, prorenin did not bind.

To further examine the enzymatic activities of the one-chain and two-chain renins, both forms of enzyme were purified from mouse SMG by a modification of the procedure of Cohen *et al.* (2). These renins were separated by carboxymethylcellulose into two separate major peaks (Fig. 2*A*). Both peaks contained pure renin because nonreducing NaDodSO$_4$/polyacrylamide gel electrophoresis demonstrated a single band of protein in each peak. Peak I consisted predominantly (95%) of the two-chain renin, whereas peak II consisted of both the one-chain (20%) and the two-chain renin (80%) as analyzed by reducing NaDodSO$_4$/polyacrylamide gel electrophoresis (Fig. 2*B*). The specific activity of peak I is 2.52×10^6 ng of angiotensin I released per hr/mg of protein and is half that of peak II (4.7×10^6 ng of angiotensin I released per hr/mg of protein). Based on the ratios of the two renins in each peak and the relative specific activities of each peak, we estimate that the one-chain renin has a 6-fold higher specific activity than the two-chain form. However, purified heavy chain, confirmed by NaDodSO$_4$/polyacrylamide gel electrophoresis, retained less than 1% of the activity of the native two-chain molecule, suggesting that the light chain is essential for enzymatic activity.

Heterogeneity in isoelectric points was observed in various forms of renins (Fig. 3). Each renin appeared to have four or five isoelectric points. The pIs of biosynthesized radiolabeled one-chain renin were similar to those of the purified one-chain renin (pI 5.6–6.4) and were more basic than the biosynthesized or purified heavy chain (pI 5.25–5.7). Prorenin has four isoelectric forms with pIs similar to those of one-chain renin. Preprorenin, on the other hand, showed only one isoelectric form with a pI at 5.3. The microheterogeneity in pIs is not a result of glycosylation of SMG because carbohydrate analysis of pure mouse SMG renin failed to detect significant carbohydrate moieties.

The subcellular localization of prorenin, one-chain renin, and two-chain renin was studied with subcellular fractions and electrophoretic blotting techniques. Enriched Golgi contained predominantly the one-chain renin (Fig. 2*C*). Trace quantities of prorenin and two-chain renins also were detected. On the other hand, mature granules contained primarily the two-chain form (80–90%) and, to a lesser extent, the one-chain renin (10–20%).

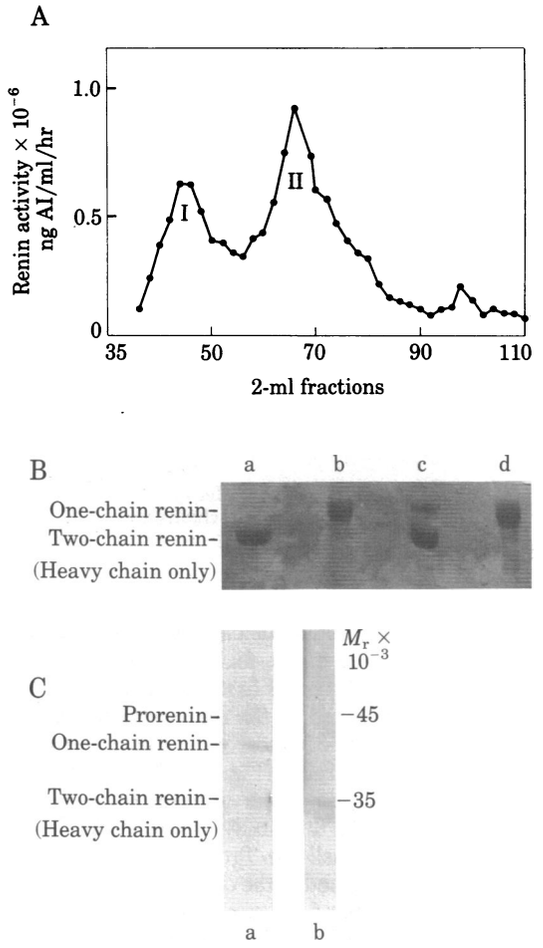

FIG. 2. (*A*) Renin purification on carboxymethylcellulose. Semipurified renin [Cohen *et al.* (2), step 5] was dialyzed against 0.05 M sodium acetate (pH 5.5) and applied to a 10-ml column of CM-52 cellulose equilibrated with the same buffer and eluted with a linear concentration gradient of 0–0.15 M NaCl. Two main peaks of renin activity are eluted (peaks I and II) with specific activities, respectively, of 2.5×10^6 and 4.7×10^6 ng of angiotensin I released per hr/mg of protein. (*B*) Analysis of purified renins. The two major peaks from *A* were analyzed on NaDodSO$_4$/polyacrylamide gel electrophoresis under reducing or nonreducing conditions. Lanes: a and b, peak I renins analyzed with or without 2-mercaptoethanol, respectively; c and d, peak II renin analyzed with or without 2-mercaptoethanol, respectively. The proteins were located by staining with Coomassie blue. (*C*) Subcellular localization of the various forms of renin. Enriched Golgi (lane a) and granules (lane b) were isolated, and analyzed by reducing NaDodSO$_4$/polyacrylamide gel electrophoresis, followed by electrophoretic transfer to nitrocellulose sheets, which were then reacted with renin antiserum (1:250 dilution) followed by goat anti-rabbit horseradish peroxidase (1:2,000) and then were stained with 4-chloronaphthol.

Finally, the identities of the biosynthesized renins were compared to those purified from the gland by using Cleveland analysis. Peptide maps of the radiolabeled biosynthesized renins were identical to the purified renins, indicating structural homology (Fig. 4).

DISCUSSION

This study characterizes fully the pathway of renin biosynthesis using the mouse SMG as a model. The primary translational product of renin mRNA is preprorenin, which is cotranslationally processed to prorenin. As suggested by previous structural

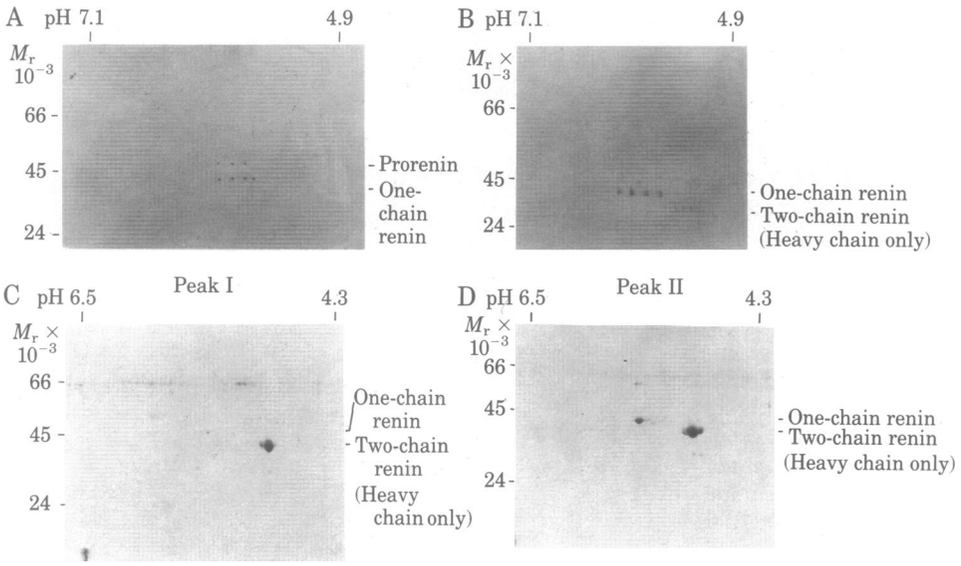

FIG. 3. (*A*) Two-dimensional patterns of the various forms of renin. Labeled prorenin and one-chain renin were analyzed by equilibrium isoelectric focusing (10), followed by reducing $NaDodSO_4$/polyacrylamide gel electrophoresis and fluorography. (*B*) Similar analysis was performed on labeled one-chain and two-chain renins. (*C* and *D*) Results of analysis of peak I and peak II renins, respectively, by two-dimensional gel electrophoresis as described and stained with Coomassie blue. The pH gradient for each gel is indicated on the top.

studies of renin, subsequent intracellular processing of renin involves several other proteolytic cleavages. Our data indicate that the cleavage of the amino-terminal "pro" sequence between Arg-63 and Ser-64 occurs first, yielding the single-chain renin. This is then followed by the removal of the Arg-Arg dipeptide at positions 352–353 near the carboxyl terminus, resulting in the two-chain renin (Fig. 5). The kinetics of intracellular processing of renin precursor to the two-chain enzyme have been examined in this study. Preprorenin has an extremely short biological half-life. Prorenin appears within minutes and is rapidly processed to the one-chain renin. This cleavage betwen Arg-63 and Ser-64 occurs quickly in the Golgi, within minutes of prorenin synthesis. The removal of the Arg-Arg dipeptide at 352–353, on the other hand, is very slow. Two-chain renin only appeared after 3–6 hr of continuous labeling, and this slow intracellular processing appeared to require 12–18 hr for completion. This processing occurs during the formation, condensing, and packaging of mature storage granules. Indeed, mature renin granules consist predominantly of the two-chain renin, with the one-chain renin contributing only 10–20% of total renin in the storage granules.

Earlier reports contend that the smaller molecular forms of purified renal renins result from artifactual breakdown of native molecule during isolation (18, 19), suggesting that the two polypeptide chains of renin reported by Misono *et al.* (4) and

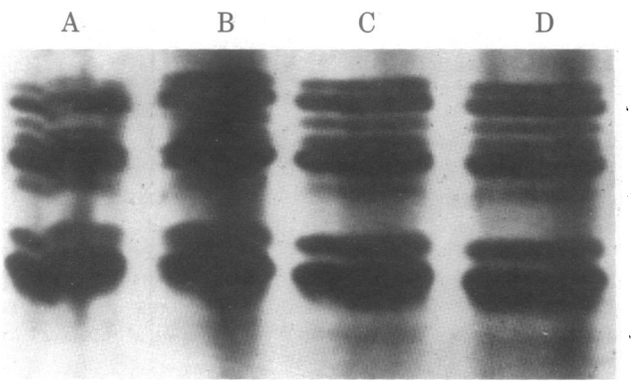

FIG. 4. Peptide maps of the various forms of renin. Immunoselected prorenin (lane A), immunoselected one-chain renin (lane b), purified one-chain renin (lane c), or purified heavy chain of renin (lane d) were digested with V-8 protease in the stacking gel. Identical patterns were observed for all of these proteins as analyzed by $NaDodSO_4$/polyacrylamide gel electrophoresis and staining with silver nitrate.

observed in our *in vitro* experiments represent *in vitro* artifacts of purification or prolonged tissue incubation. This possibility was excluded by the results of the *in situ* biosynthesis of renin in an intact mouse, which confirmed the *in vitro* results. Further evidence for the existence of a native two-chain renin is provided by the analysis of renin storage granules in our study. Rapid isolation of intact renin granules from fresh tissue yielded predominantly the two-chain enzyme.

Both the biosynthetic one-chain and the two-chain renins are enzymatically active and prorenin is not. A surprising finding of this study is that the one-chain renin has a greater specific activity than the two-chain form. Our data also show that light chain plays a central role in maintaining renin enzymatic activity.

In the light of the recent findings suggesting that mice of the high-renin strain carry only two genes for renin (20), it was surprising to find that prorenin, one-chain renin, and two-chain renin all displayed multiple isoelectric points. This heterogeneity was not due to glycosylation, as direct carbohydrate analysis of purified SMG renin failed to detect carbohydrate moieties. However, it may be due to differences in amino acid compositions as a result of minor variations in the cleavage positions. The latter hypothesis is supported by recent data that the amino terminus of the heavy chain showed sequence variability (4). In addition to commencing with Ser-64, small populations of the heavy chain began with residues Ser-65 or Thr-67. Alternatively, heterogeneity may arise from other post-translational modifications such as methylation, acetylation, or phosphorylation. Finally, because outbred strains of mice were used in this study, germ-line polymorphism resulting in heterogeneity of renins is another possibility, although this is unlikely because only one form of preprorenin was observed in this study.

An interesting finding is that both the one-chain and two-chain renins are secreted. On the other hand, neither radiolabeled prorenin nor trypsin-activatable renin activity can be detected in the incubation medium. The biologic relevance of two secreted forms of active renin with different enzymatic activities and multiple isoelectric forms is uncertain. These multiple forms may represent multiple intracellular pools of renin. As in other endocrine systems (21, 22), there may be two pathways of renin secretion. For example, the "earlier" form (one-chain renin) may be secreted in preference to the storage form (two-chain renin) under certain circumstances. As to the issue of multiple isoelectric forms, recent data on renal renin suggest that multiple isoelectric forms may represent different intracellular pools,

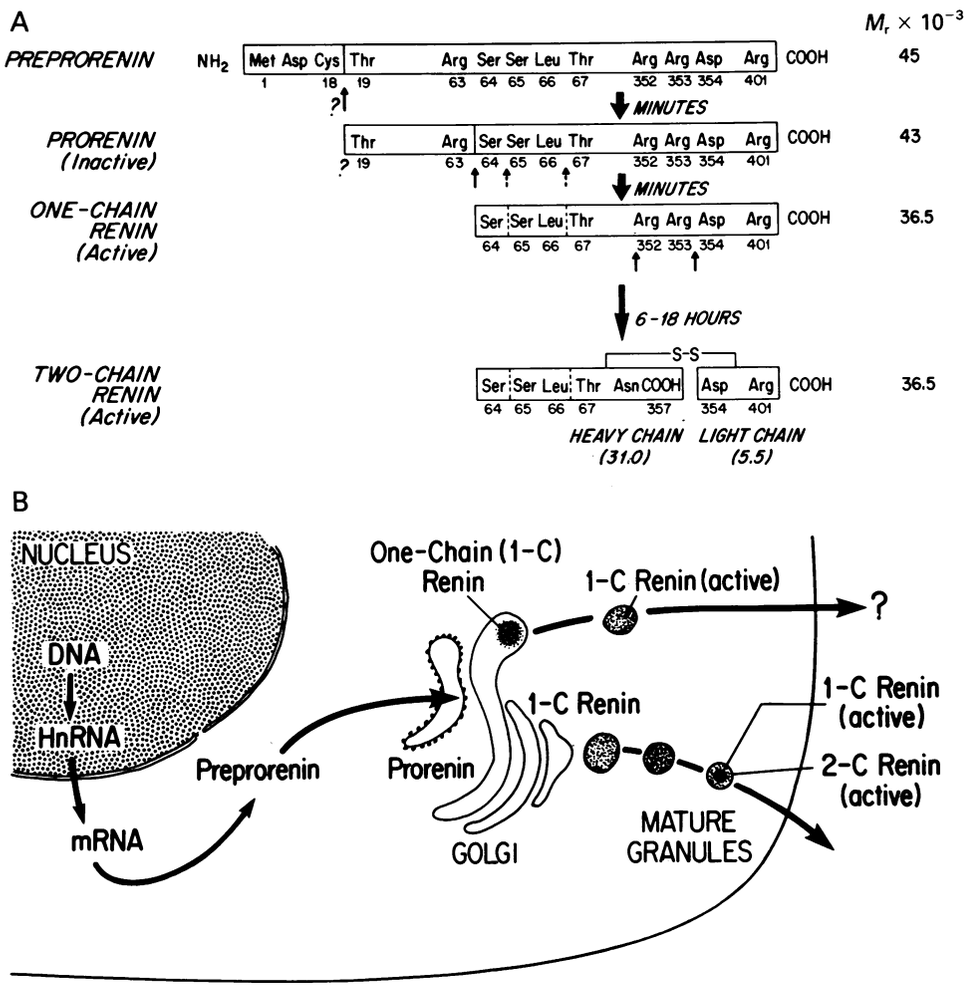

FIG. 5. Models of renin biosynthesis and secretion. (*A*) Proposed sites (↑) and alternative sites (↟) of processing of renin (5) with the relative kinetics of each step shown, based on data from this report and from other studies (4). The molecular weights were deduced from the amino acid sequence (4). The question mark at the Cys-Thr cleavage site at positions 18–19 indicates the proposed but unconfirmed cleavage location of preprorenin to prorenin (5). (*B*) Proposed intracellular localization of the various forms of renin during biosynthesis and secretion.

which can be released differentially in response to various stimuli (23).

Our data suggest that renin biosynthesis and secretion is complex and may be controlled at multiple points. The regulation of the kinetics of conversion of prorenin to one-chain and two-chain renins needs further elucidation. The potential existence of multiple secretory pools of renin and multiple secretory pathways may have important physiologic implications in the control of a system which plays a major role in cardiovascular homeostasis.

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