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Prorenin processing by cathepsin B in vitro and in transfected cells

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Abstract Renin, which catalyzes the initial proteolytic cleavage reaction in the production of angiotensins, is first synthesized as a zymogen, prorenin, and requires the proteolytic removal of an amino-terminal prosegment for activation in vivo. The lysosomal hydrolase cathepsin B has been proposed as a prorenin processing enzyme based on reports of its co-localization with renin in the secretory granules of certain tissues and its ability to activate prorenin in vitro. In the current study, scanning mutagenesis was used to identify the amino acids which determine the site selectivity of prorenin cleavage by human cathepsin B in vitro. Co-expression assays in AtT-20 cells were also used to test for the ability of cathepsin B to cleave human prorenin within cells. Our results suggest that a basic lysine residue at the −2 position from the cleavage site is required for cathepsin B cleavage of prorenin in vitro and that the structure of prorenin itself may account for the selection of the proper cleavage site. In addition, although cathepsin B appears to be correctly sorted to lysosomes, the enzyme exhibits prorenin processing activity in transfected AtT-20 cells, raising the question of the cellular localization in which the processing event occurs.

Key words: Prorenin; Renin; Cathepsin B; Protein processing

1. Introduction

The aspartyl protease renin catalyzes the rate-limiting step in the generation of angiotensins by cleaving circulating angiotensinogen in the renin angiotensin system (RAS). Although the main source of plasma active renin is the kidney [1], many other tissues express the renin gene [2–5]. Some of these tissues have been shown to express the other components of the RAS suggesting the existence of locally active tissue RASs. Like many proteases, renin is first synthesized as a larger inactive precursor, prorenin. In humans, processing of prorenin to active renin involves the proteolytic cleavage of a 43 amino acid prosegment following a pair of basic amino acids (Lys-Arg) and occurs specifically in secretory granules [6]. Indeed, cultured cells devoid of these organelles are unable to process prorenin in transfection assays [7]. In vivo, it is possible that multiple prorenin processing enzymes (PPE) exist and are active in different renin-producing tissues [8,9].

Cathepsin B was first proposed as a renal PPE on the basis of its ability to cleave prorenin in vitro [10] and more recently, on its capability to process prorenin co-transfected with cathepsin B in GH_4C1 cells [11]. Immunohistochemistry studies have also indicated that renin and cathepsin B are co-localized in granules of different tissues: epithelial cells of the renal proximal tubule [12], human pituitary lactotrophs [13], mouse adrenocortical cells [14] and submandibular gland [15], rat anterior pituitary gonadotrophs [16] and juxtaglomerular cells [17]. Cathepsin B is most commonly known as a lysosomal cysteine protease of the papain superfamily that possesses endopeptidase as well as exopeptidase activities with complex pH dependencies [18]. Its endopeptidase activity was reported to show broad specificity with a preference for basic (particularly Arg) and aromatic (particularly Phe) residues at the −2 position from the cleavage site [19]. However, these data have mostly been established using synthetic peptide substrates and little evidence exists regarding the amino acids important for the selection of the proper cleavage site by cathepsin B with protein substrates like prorenin.

Cathepsin B is synthesized as a glycosylated zymogen of 39 kDa that is converted to a 33 kDa active form by the proteolytic removal of the prosegment. The activation of procathepsin B has been proposed to result from an intramolecular autocatalytic event that occurs primarily within the lysosome in the pH range of 4–6 [20]. Additional processing of active cathepsin B involves an internal cleavage that generates the active two-chain form containing a heavy chain of 27 kDa and a light chain of 5 kDa, linked by a disulfide bridge [21]. In order to act as a PPE, cathepsin B would need to be capable of sufficient autoactivation within the secretory pathway to be active on the prorenin in the secretory granules.

In the current study we have sought to identify the protein sequences which dictate the cleavage specificity of human cathepsin B on prorenin and to test whether cathepsin B is capable of cleaving human prorenin when co-expressed in cells containing secretory granules.

2. Materials and methods

2.1. Recombinant plasmid construction

Construction of the expression vectors for native human prorenin and for prorenins generated by site-directed mutagenesis around the Lys-Arg cleavage site have been described previously [22]. The expression vector for human procathepsin B was generated by overlap extension polymerase chain reaction (PCR) [23] of the human cathepsin B cDNA ([24]; a gift from Dr. D. Steiner) using the following oligonucleotides: forward: 5′-CGCGATATCGAGTAGTGGATCTAGG-3′; reverse: 5′-GCGGGATCCCAGGACTGGCACGAC-3′. The amplified fragment was digested with the restriction enzymes *Eco*RV and *Bam*HI (recognition sites underlined in the amplifying oligonucleotides) and was inserted into the RSV globin expression vector [25]. The entire coding sequence of cathepsin B was verified by DNA sequencing.

2.2. Cell culture

GH_4C1 and AtT-20 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 0.1% SerXtend (Irvine Scientific, Santa Ana, CA) and 10 μg/ml gentamicin.

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2.3. Assay of prorenin processing by cathepsin B in vitro

GH_4C1 cells were plated at a concentration of 10^6 cells per 35 mm well and transfected 24 h later with 20 μg of an expression vector for native prorenin or a mutated prorenin using the lipofectin reagent method (Gibco-BRL). After 48 h of incubation, the transfected cells were transferred to 25 mm wells; 24 h later the transfected cells were incubated 2 h with methionine-free DMEM and labeled overnight with 300 μCi of [^{35}S]methionine per well in serum-free medium. Culture supernatants containing labeled native prorenin or mutated prorenins constitutively secreted by the transfected cells, were collected and their pH was lowered by the addition of MES buffer (pH 6.0) to a final concentration of 100 mM MES with 1 mM EDTA, 5 mM dithiothreitol and 0.001% BSA. Buffered supernatants were incubated with or without 0.4 units of cathepsin B purified from human liver (Calbiochem) at 37°C for up to 6 h. The supernatants were subsequently immunoprecipitated with an anti-human renin antibody and analyzed by SDS-PAGE followed by fluorography as previously described [25]. The relative intensities of the bands corresponding to prorenin and renin were evaluated by scanning densitometry of autoradiograms using an IS-1000 Digital Imaging System (Alpha Innotech Corporation).

2.4. Transient expression in AtT-20 cells

Purified expression vectors for native or mutated prorenin and for cathepsin B or a control plasmid (50 μg total DNA) were mixed with 10^7 AtT-20 cells and electroporated with a single pulse of 300 V/4 mm, 1000 μF. Electroporated cells were then transferred to 25 cm^2 flasks; the next day, the cells were transferred to 12 well plates at a concentration of 5×10^5 cells per well. Twenty-four hours later, cells were incubated for 2 h with methionine-free DMEM containing 10% dialysed FBS, followed by a 5 h labeling period using 300 μCi of [^{35}S]methionine per well. Tunicamycin treated cells were incubated 3 h with methionine-free DMEM with or without tunicamycin at a dose of 15 μg/ml, labeled for 2 h and chased overnight in complete DMEM with or without tunicamycin.

2.5. Detection of renin and prorenin

Supernatants from labeled AtT-20 cells were immunoprecipitated using an anti-human renin antibody as described above; in parallel, secreted prorenin and renin were dosed using an angiotensin I generation assay followed by an angiotensin I radioimmunoassay described previously [22]. Briefly, supernatants were incubated with an excess angiotensinogen either directly (active renin content), or following an incubation with trypsin (total renin content=prorenin+renin). The percentage of active renin was calculated as (active renin content/total renin content)×100.

2.6. Immunoprecipitation of human cathepsin B

Culture supernatants from labeled AtT-20 cells were collected and the cells were lysed in 10 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 2% NP-40. Cell lysates and supernatants were immunoprecipitated with an anti-human cathepsin B antibody and analyzed by SDS-PAGE followed by fluorography as previously described [25].

3. Results

Determinants of cathepsin B cleavage of prorenin were evaluated using mutated prorenins, shown in Fig. 1, generated by alanine scanning mutagenesis in the region of the reported cleavage site [26]. The vectors expressing the native or mutated prorenins were transfected in GH_4C1 cells. These cells lack the capacity to process prorenin and constitutively secrete the unprocessed protein in the medium [7,27]. Following transfection, the cells were labeled overnight using [^{35}S]methionine. The next day, supernatants were collected and then incubated with purified human liver cathepsin B for up to 6 h at 37°C in a pH 6 buffer [26]. The generation of active renin was quantitated by scanning densitometry of autoradiograms. Digestion of native (non-mutated) human prorenin with cathepsin B under these conditions leads to a slow generation of active renin as confirmed by electrophoretic migration (Fig. 2) and renin activity assay (data not shown). In the time course of the digestion, no other degradation products appeared and no cleavage occurred in the absence of added cathepsin B (Fig. 2, −Cathepsin B). These results confirm that under the conditions used, human cathepsin B displays a high degree of cleavage site selectivity on human prorenin [10].

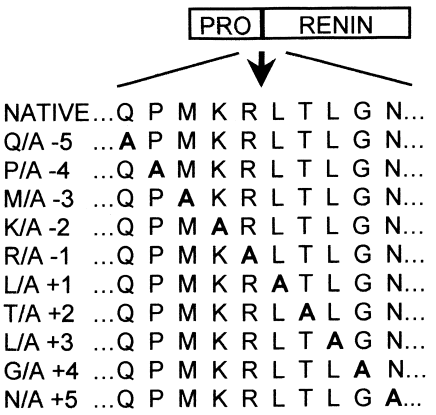

Fig. 1. Schematic representation of the alanine scanning mutations of residues around the cleavage site in human prorenin. Amino acids are represented by their one-letter code. Arrow indicates native processing site in human prorenin. Numbering is relative to amino acid 1 of active renin.

Fig. 3 shows the time course of cathepsin B cleavage of prorenins mutated at single amino acids in the region of the reported cleavage site. As with native prorenin, cathepsin B processed all of the mutated prorenins slowly and without showing any signs of non-specific degradation (data not shown). The overall effect of mutating residues to alanine on the amino-terminal side of the reported cleavage site was to diminish the processing efficiency by cathepsin B (Fig. 3, N-term.; dashed lines). Mutations on the carboxy-terminal side of the cleavage site (Fig. 3, C-term.; dashed lines) resulted in cleavage efficiencies that were equal to or greater than those obtained with native prorenin. The most marked effect on cleavage efficiency was observed with the mutation of the lysine at position −2 (K/A−2; numbering relative to the reported cathepsin B cleavage site) which virtually abolished cleavage by cathepsin B. The dependence of prorenin cleavage on the presence of the lysine residue was also observed using crystallographic grade recombinant human cathepsin B purified from yeast (a gift from Dr. John S. Mort) that efficiently processed native prorenin but showed no activity towards the

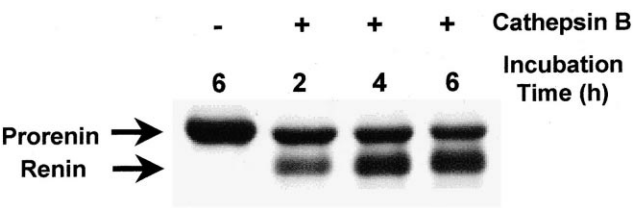

Fig. 2. In vitro cleavage of human prorenin by human cathepsin B. Tissue culture supernatant containing native (non-mutated) recombinant human prorenin was incubated with purified human cathepsin B for the indicated times and was immunoprecipitated with anti-renin/prorenin antibody. The expected migration for prorenin and renin was determined by co-electrophoresis of size markers.

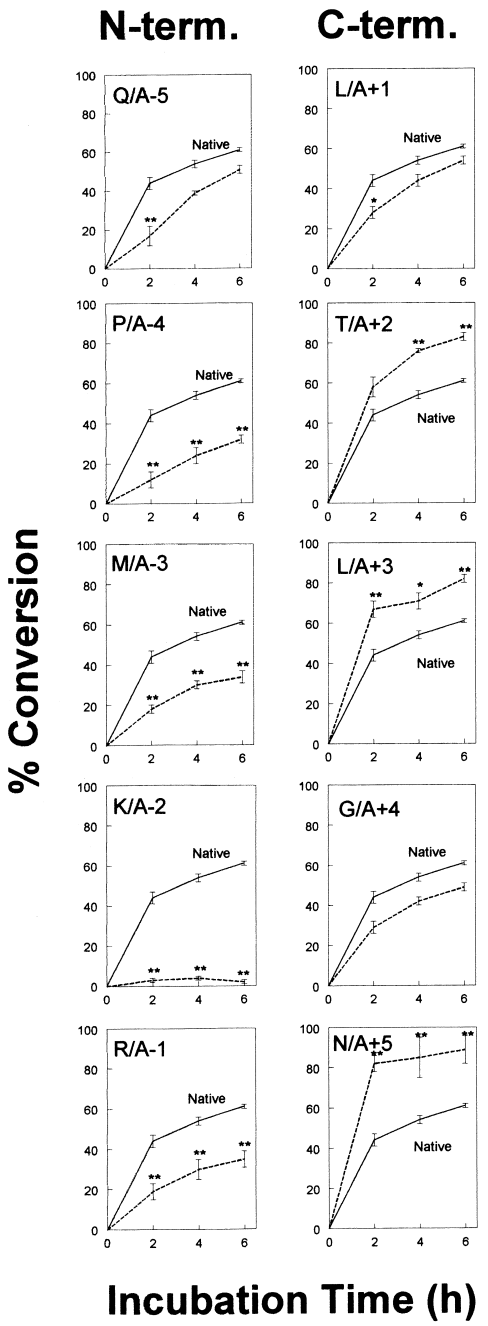

Fig. 3. Time course of activation of mutated prorenins by cathepsin B. Culture supernatants containing recombinant human mutated prorenins were subjected to limited digestion by human cathepsin B as shown in Fig. 1. Scanning densitometry was used to determine the percentage conversion to active renin. Solid lines and dashed lines represent the average time course of activation of native and the corresponding mutated prorenins, respectively. Nomenclature represents the mutation induced (e.g., K/A means a Lys is replaced with an Ala) and the position of the mutation with reference to the reported cleavage site for cathepsin B [26]. $*P<0.05$; $**P<0.01$ as compared to native prorenin using Dunn's multiple comparisons test. Data are derived from 3–4 independent assays.

K/A−2 mutant (data not shown), confirming that the cleavage of prorenin was not due to a contamination of the commercial preparation of cathepsin B.

To test if the processing activity observed in vitro could also take place in cultured cells, AtT-20 cells were used for co-transfection assays of prorenin and cathepsin B, since these cells represent an appropriate model to study prorenin processing [28]. AtT-20 cells contain an endogenous protease capable of activating native human prorenin ([28] and Fig. 4A), but this enzyme is incapable of cleaving prorenin mutated at the −1 position from the cleavage site ([22] and Fig. 4A). Since this mutated prorenin is processed by cathepsin B in vitro (Fig. 3, R/A−1), AtT-20 cells were co-transfected with the expression vectors for the mutated R/A−1 prorenin and human cathepsin B. Whereas expression of the R/A−1 mutant prorenin alone leads to the secretion of the unprocessed form only (Fig. 4A), co-transfection of cathepsin B and R/A−1 prorenin results in the secretion of the lower molecular weight form corresponding to active renin (Fig. 4A). It is noteworthy that the endogenous cathepsin B in AtT-20 cells, which is of murine origin, is incapable of processing the R/A−1 mutant prorenin, possibly indicating that murine and human cathepsins B have a different substrate specificity. The activity of the secreted R/A−1 renin was assessed using an angiotensin I generation assay which indicated that AtT-20 cells secreted 33% active renin when co-transfected with the R/

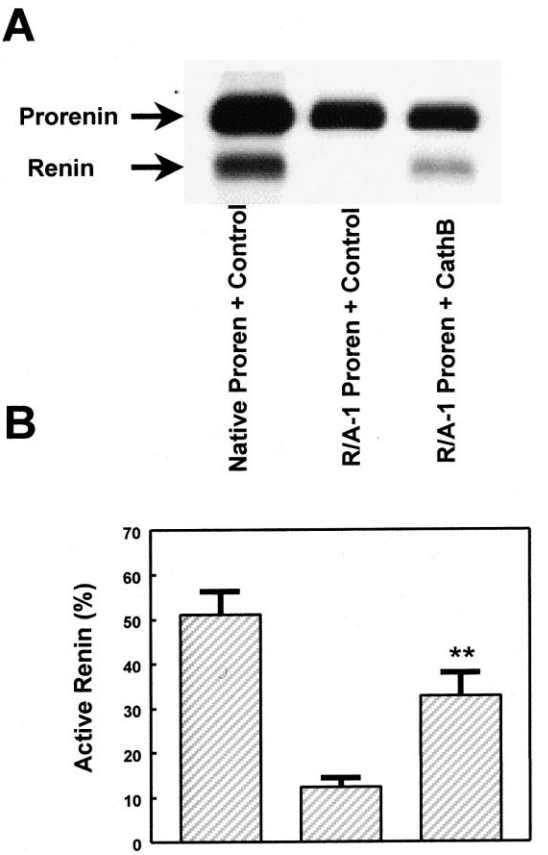

Fig. 4. A: Processing of human prorenin by human cathepsin B in AtT-20 cells. AtT-20 cells were electroporated with expression vectors for the indicated proteins. Following labeling of the cells, supernatants were immunoprecipitated with anti-renin/prorenin antibody. Protein bands corresponding to prorenin and renin are marked by arrows. Representative data are shown from one of six such experiments. B: Secretion of active renin by AtT-20 cells co-transfected with human prorenin and human cathepsin B. Prorenin and renin secreted in the supernatants of AtT-20 cells electroporated with expression vectors for the indicated proteins were dosed using an angiotensin I generation assay. $**P<0.01$ as compared to R/A−1 Proren+Control using Student's *t*-test.

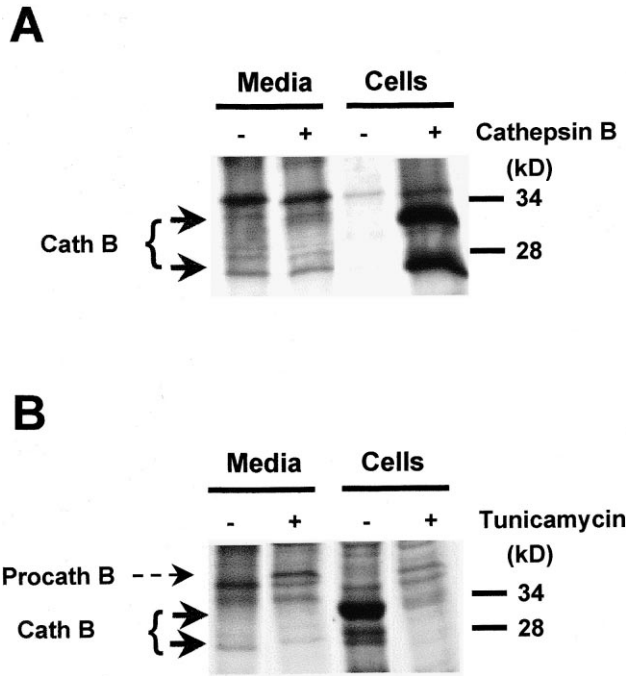

Fig. 5. A: Expression of human cathepsin B in transfected AtT-20 cells. AtT-20 cells were electroporated with an expression vector for human cathepsin B (+) or a control plasmid (−). Following pulse labeling of the cells for 5 h, cathepsin B was immunoprecipitated from supernatants (Media) and cell lysates (Cells) using an antibody specific for human cathepsin B. B: Sorting of human cathepsin B in AtT-20 cells. AtT-20 cells transfected with cathepsin B were pulse-labeled for 2 h and chased overnight, in the absence (−) or presence (+) of tumicamycin. Cathepsin B was immunoprecipitated as described above. Active forms of cathepsin B are indicated by arrows and the precursor form by a dashed arrow. Numbers at the left of the autoradiogram represent the apparent molecular weights (in kDa) of co-electrophoresed size markers. The data shown are from a representative experiment (of three).

A−1 mutant prorenin and cathepsin B (Fig. 4B). Thus cathepsin B exhibits prorenin processing activity both in vitro and in transfected AtT-20 cells.

Since procathepsin B is normally sorted to lysosomes while prorenin enters the regulated secretory pathway, the capacity of cathepsin B to process prorenin raises the question of the organelle localization in which the processing event occurs. As shown in Fig. 5A, AtT-20 cells transfected with human cathepsin B expressed abundant amounts of the enzyme in the active form as demonstrated by the presence of the 33 kDa single chain form and the 27 kDa subunit of the active enzyme. Labeled cathepsin B also appeared to be retained in the cell since no significant accumulation of the enzyme could be observed in the supernatant even after a 5 h secretion period (Fig. 5A). As lysosomal targeting of cathepsin B is mediated by modified mannose residues in the glycoprotein, cells were treated with tunicamycin, an inhibitor of glycosylation, to confirm that such a treatment would prevent human cathepsin B export to the lysosomes and therefore determine if the expressed protein was correctly sorted. The results demonstrate that tunicamycin treatment leads to a dramatic decrease of the active forms of human cathepsin B within the cell and the appearance of a higher molecular weight form in both the cells and the supernatant (Fig. 5B). This slower migrating form possibly corresponds to unglycosylated procathepsin B expected to be around 35 kDa. The levels of cathepsin B that could be immunoprecipitated both intracellular and extracellular were also markedly decreased and could reflect degradation of cathepsin B retained in the secretory pathway. In addition, when AtT-20 cells co-expressing the R/A−1 prorenin and human cathepsin B were treated with tunicamycin, only prorenin was found to be secreted in the medium (data not shown). Thus, human cathepsin B expressed in AtT-20 undergoes intracellular sorting in a glycosylation-dependent manner consistent with its sorting to the lysosomal compartment.

4. Discussion

Our current results support the previously reported role of cathepsin B in site-selective cleavage of human prorenin in vitro [26] and demonstrate that this cleavage is critically dependent on the lysine at position 42 in the prorenin prosegment. Based on the proposed mechanism of cathepsin B action on synthetic substrates [19], the resulting scissile bond would follow Arg-43, consistent with the reported amino-terminus of the renin produced after cathepsin B digestion of human prorenin [26]. Cathepsin B cleavage of substrates containing Arg at the −2 position (relative to the cleavage site) depends on a group with pK_a of 5.1 (suggested to be Glu-245) that has been proposed to interact with the Arg side chain and stabilize the transition state [19]. Similarly, crystal structure modeling predicts Glu-245 could also achieve efficient electrostatic interaction with Lys at the −2 position from the cleavage site [29]. Although human prorenin contains several other basic residues which could serve as potential cathepsin B cleavage sites [30] its preferential cleavage at position 43 in the prosegment reflects that of numerous other proteases including trypsin, thermolysin, kallikrein, plasmin and pepsin [31,32]. This site selectivity is thus quite likely to be a result of a combination both of site preference by cathepsin B and of the masking of additional cleavage sites due to the conformation of prorenin.

Comparison of the known protein sequences for prorenin indicates that the critical lysine in human prorenin cleavage by cathepsin B is conserved between species in the homologous proteins. In addition, this lysine is found at the −2 position from the cleavage site of mouse submaxillary prorenin [33,34]. However, N-terminal sequencing of rat renal renin has indicated cleavage at a site further C-terminal relative to the conserved lysine [35,36]. Thus it is still uncertain if the identity of the renal PPE is conserved between species.

Procathepsin B activation has been shown to occur preferentially in an acidic pH range of 4–6 and may be the result of an intramolecular autoactivation mechanism [20]. Denaturation of active cathepsin B increases exponentially with increasing pH and the enzyme is irreversibly inactivated at pH 7.0 [37]. Tumor-derived endocrine cell lines and particularly AtT-20 cells are known to exhibit a high rate of basal unstimulated release of granule content [38]. However, when AtT-20 cells were transfected with human cathepsin B, the mature enzyme could not be detected in the supernatant making it unlikely that the expressed protein was routed to the secretory pathway to be co-secreted with renin. Furthermore, retention of cathepsin B in the cells was dramatically decreased when glycosylation of the protein was inhibited indicating normal trafficking of cathepsin B to the lysosomes. These results raise the question of how cathepsin B could cleave prorenin if the two proteins are not co-resident within the cells.

In pancreatic β-cells, proinsulin and procathepsin B have been shown to be transiently associated in the immature secretory granules and to segregate to their respective cellular destinations as the secretory granule matures [39]. As the environment progressively acidifies in the *trans*-Golgi and in budding immature granules [40], it is possible that some degree of cathepsin B activation occurs before it is sorted to the lysosomes. In the context of an overexpression system such as transfected AtT-20 cells, this might generate sufficient active cathepsin B to process prorenin. It is unclear, however, if such a process is responsible for prorenin processing in renin-producing tissues. The granules of renal juxtaglomerular cells have been reported to contain numerous lysosomal enzymes and autophagic particles [17]. Prolonged storage of prorenin in these granules in the presence of an active form of cathepsin B could overcome the limitation of the apparently slow kinetics of cathepsin B action on prorenin seen in vitro [26].

Immunohistochemical studies have shown renin and cathepsin B co-staining in subcellular compartments of numerous tissues. In some cases, this possibly represents cathepsin B colocalization with renin in late endosomes or lysosomes which have taken up prorenin from the extracellular space. For example, the cortical proximal tubules of the kidney contain significant amounts of immunoreactive renin [12], although they appear to synthesize very little renin mRNA [41]. Immunoelectron microscopy of proximal tubular cells suggests that this renin is in lysosomes, consistent with its absorption from the lumen of the collecting duct [12]. Indeed, studies of monkeys injected with purified labeled human prorenin demonstrate that both the kidney and liver are involved in clearing the protein from the circulation and that the initial step in its degradation in the lysosomes involves an apparent removal of the prosegment [42]. While such a process would not be likely to play a major role in the generation of active renin for secretion, its importance in the generation of angiotensins within tissues or cells [43] remains an interesting topic for further study.

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