Twists and turns in the search for the elusive renin processing enzyme. Editorial

Focus on “Cathepsin B is not the processing enzyme for mouse prorenin”

KENNETH W. GROSS¹, R. ARIEL GOMEZ² AND CURT D. SIGMUND³

¹Molecular and Cellular Biology, Roswell Park Cancer Institute, Buffalo, New York 14263

²University of Virginia School of Medicine, Charlottesville, Virginia

³University of Iowa, Iowa City, Iowa

Running Head: Twists and turns in prorenin processing

Corresponding author:

Molecular and Cellular Biology, Elm & Carlton Streets, Buffalo, NY 14263

Phone: 716-845-4572 Fax: 716-845-5908

E-mail: kenneth.gross@roswellpark.org

Copyright © 2010 by the American Physiological Society.
Having worked for many years on relatively basic questions germane to the venerable renin-angiotensin system (RAS), we were musing with some fascination recently on the continuing evolution of our understanding of the system. In particular, we were taking note of the considerable amount of dogma, with varying levels of verification, that’s associated with the system, as well as, the almost continuous infusion of remarkable new twists and turns that seem to mark the passing of time. We were also struck by the existence of a surprising number of unresolved core issues.

This issue of AJP; Regulatory contains a contribution by Reudelhuber’s laboratory (Mercure, et al, 2010) that has direct bearing on one of these core issues-a central question that has eluded a completely satisfactory explanation for some time, namely, the identity of the prorenin processing enzyme (PPE) that generates active renin in the juxtaglomerular cell of kidney. This is not a trivial question for, while there may be local activation of renin at various tissue specific sites, the experimental evidence suggests that the preponderant source of systemically circulating active renin is the kidney. As the authors correctly point out, unequivocal identification of the PPE might thus provide a new pharmaceutical target to inhibit this critical rate-limiting step of the RAS, thus providing a potential novel therapy for hypertension and cardiovascular disease.

The article is of significance not for its positive identification of a novel PPE but rather for its rigorous exclusion of a longtime favorite candidate for the PPE, Cathepsin B, at least in the specific case of mice. Satisfactory resolution of the issue has been complicated by a number of factors including the fact that multiple enzymes appear to be able to generate “active” renin in vitro, different N-terminal sequences have been identified for the presumptive mature renal renin of human, mouse and rat origin and the in vitro cell systems in hand are for the most part non-optimal or non-representative of the renal site in question.
It was known for some time that a number of enzymes exhibited the capability of processing prorenin to active renin in vitro, e.g. cathepsins B, D, G, tissue kallikrein, convertases, trypsin, mouse submandibular gland prorenin converting enzyme, plasmin, pepsin, and others (2, 6, 10, 14, 17, 18). However, there were issues of proteolysis causing degradation or issues regarding the colocalization of the enzymes with renin in vivo which lead to uncertainty about their roles. Cathepsin B has enjoyed preferential, if not quite dogmatic status, as the PPE candidate of choice for a number of reasons. It was noted early on by Taugner that the secretory pathway in the renal JG cell of rats appeared to involve granules which had the characteristics of modified lysosomes (24). Not surprisingly Cathepsin B, along with a number of other lysosomal enzyme candidates, were shown to exhibit cellular and organelle co-localization with prorenin.

A series of straightforward studies undertaken by Hsueh and colleagues were particularly persuasive. They purified human active renin and undertook amino terminal sequencing (4). The results indicated that prorenin appeared to be converted to renin through cleavage at the carboxyl end of a Lys-Arg dibasic amino acid doublet (residues 65-66 of preprorenin). Using a recombinant human prorenin as a substrate they then went on to purify an enzymatic activity associated with a thiol protease from human kidney that accurately processed prorenin to renin in vitro, suggesting that a cysteine protease was the authentic renal PPE(23). Subsequent studies revealed that Cathepsin B, of both renal and liver origin, correctly corresponded with enzymatic activity, and that Cathepsin B, hydrolyzed the 43 amino acid prosegment of prorenin without further degrading renin (25). Importantly, Cathepsin B exhibited co-localization with renin in JG cell secretory granules. Confirmation that Cathepsin B was uniquely the JG PPE could have been demonstrated by showing that Cathepsin B inhibitors prevented prorenin processing in vivo, or in cultured JG cells. Whether the inhibition studies were performed is unclear.

Baxter and colleagues (18) showed that co-transfection of Cathepsin B and human preprorenin expression vectors into secretory granule-containing rat GH4C1 cells resulted in enhanced generation of secratable
active renin relative to the preprorenin vector alone. This suggests that Cathepsin B could localize to the appropriate cellular compartment to effect correct processing in an intact cell. The ratios of active renin to prorenin secreted however were not particularly high and they thus carefully pointed out that while these results were consistent with such a role for Cathepsin B they did not constitute proof that Cathepsin B was the bonafide PPE.

Reudelhuber’s laboratory used scanning mutagenesis to identify the amino acids determining site selectivity of human prorenin cleavage by human cathepsin B in vitro (9). Their results suggested that the basic residue, lysine, at -2 (relative to the cleavage site) was required for cleavage in vitro, consistent with predictions of Cathepsin B mechanism from activity on synthetic substrates.

All of the above data are consistent with Cathepsin B comprising the PPE, but a number of less satisfying observations have also been reported. In particular, amino terminal sequencing of active renins from other species revealed that the N terminus of active renin does not appear to be necessarily conserved. Active renin2 enzyme, a curious non-glycosylated renin derived from an evolutionarily recent gene duplication event unique to mice, as isolated from mouse submandibular gland, exhibits an N terminus homologous to human renal renin (15). In contrast, the sequence of active mouse renin1, as isolated from the As4.1 cell line (8), a putative model for mouse renal JG cells derived by transgene-targeted tumorigenesis, exhibits an N terminus that lies 7 amino acids downstream from the site reported for human kidney renin. Interestingly, this site is homologous to that observed for rat renal renin by Kim et al (11).

The primary amino acid sequences for rat, human and mouse renin 1 and 2 are shown in Fig 1 with the presumptive cleavage sites specified. It is interesting to note that the dibasic amino acid doublet corresponding to residues 65-66 of human preprorenin (42-43 of prorenin) is evolutionarily conserved although there is substitution of Lys-Lys in the case of the rat for the Lys-Arg found in mouse and human,
and there are potentially significant amino acid substitutions evident in the immediate vicinity of the
doublet. In particular mouse renin1 exhibits a proline for leucine substitution while the rat sequence bears
more similarity to the mouse renin2 sequence with a serine doublet in place of the leucine threonine of
human. As pointed out in the Discussion by Mecure et al, one explanation might be that the initial
cleavage in all 3 species is performed by Cathepsin B, but that the mouse and rat renins are subsequently
trimmed by other enzymes to generate the observed termini. Alternatively, different processing enzymes
might be involved in activating human renin versus rat and mouse renins. A lack of evolutionary
conservation for the processing of an enzyme which plays such a central role in physiology as renin is
unsettling, or at least intellectually unappealing, however, and the conservation of the dibasic doublet
despite the adjacent sequence variation could be taken to argue against this radical notion.

Consistent with the possibility that an initial cleavage at the dibasic site is followed by “nibbling” is a
report by Almeida et al who used an internally quenched fluorescent peptide assay to demonstrate that the
species specific Cathepsin B’s could appropriately cleave peptides mimicking the respective species
specific dibasic doublet processing sites for rat and human (1). At present it is unclear how the less
homologous substitution of proline in the case of mouse renin1 would effect cleavage at this site although
there are statements in previous papers (12) which suggest that three enzymes which have been found to
be capable of activating human renin, Cathepsin B, and the convertases PC1 and PC5, are incapable of
activating mouse renin1 and rat renin in the cell transfection assays employed. As a point of information
the convertases PC1 (2) and PC5 (14) have been shown to be capable of activating human renin and
mouse renin2, but it appears unclear that PC1 and PC5 exhibit appropriate cell specificity to fulfill the role
of the renal PPE in humans. They have been proposed to potentially perform this role at other sites, e.g.
adrenal, in humans. Again, however, there appears to be a disturbing lack of evolutionary conservation.

*In vivo* release of renin from juxtaglomerular cells is thought to be mediated via two pathways (20).
Processed active renin is secreted from dense modified lysosomal storage granules in response to physiological cues while the inactive zymogen is constitutively released via clear vesicle fusion with the plasma membrane. Support that the secretory granules are in fact secretory lysosomes has been provided by studying the influence on granule morphology by genetic mutations such as the Beige mutation which causes a lysosome secretory defect (7). In contrast, many of the cellular assays that have been described above have been performed in cultured cell lines which are characterized as having regulated secretory pathways characteristic of other tissue sites, e.g. rat pituitary GH4C1 cells (18) and mouse pituitary AtT20 cells (13). Renin release from juxtaglomerular cells is a highly regulated process (21). Renin release is stimulated by activation of the cAMP pathway and inhibited by elevation of intracellular Ca levels. It is unclear whether the cellular models which have been employed to study prorenin processing are genuinely representative of the processing/secretory pathway of JG cells. As pointed out by Jutras and Reudelhuber (9) in their cellular cotransfection studies of Cathepsin B and human prorenin in AtT20 cells, it appears that Cathepsin B is correctly sorted to lysosomes through modified mannose residues and does not appear to be co-secreted with renin from the regulated pathway. These observations raise the question of how Cathepsin B could be mediating the processing if the two proteins are not co-resident within the cells. It has been proposed that in the over-expression systems employed there may be sufficient activation of Cathepsin B in early stages of the secretory process, where the proteins are co-resident in the trans-Golgi and immature granules to achieve processing of prorenin before they segregate to their respective compartments.

In contrast there is a large body of evidence suggesting co-localization of renin and lysosomal enzymes in renal juxtaglomerular cells (24). Renin gene ablation studies in vivo have provided some interesting insights which suggest that the trafficking and maturation processes studied in the in vitro cell culture systems may not be mirroring the intact kidney. The Mullins laboratory differentially knocked out the Ren1d and Ren2d loci in a strain which harbors both loci. Ren1d gives rise to the glycosylated renin1
whereas Ren2d gives rise to the atypical non-glycosylated renin2. The Ren2 knockout resulted in no observable histomorphological alterations to juxtaglomerular cell morphology as monitored by electron microscopy (22), while ablating expression of Ren1d led to a change in immunostaining from a punctuate, abundant granular pattern to diffuse, weak cytoplasmic staining. Electron microscopy revealed a complete absence of dense granule formation in the Ren1d deficient mice (3). The results suggest that the signals required for sorting to the regulated pathway of juxtaglomerular cells in vivo are restricted to the renin1 protein, and further suggest that renin1 and renin2 are secreted by distinct pathways in vivo.

Parallel studies by Gomez’s laboratory in Ren1d-Ren2d mice (19) and Fukamizu’s laboratory in strains of mice harboring only the single Ren1c locus (26) exhibited a similar phenotype. In an elegant set of studies Mullins’s group went on to demonstrate that a BAC harboring wildtype Ren1d and Ren2d could completely rescue granulation and other aspects of the phenotype in otherwise renin deficient mice (16). On the contrary, a BAC harboring a Ren1d locus into which a Lac reporter cassette had been inserted, disrupting Ren transcription, was incapable of rescuing the granulation phenotype. Importantly, reporter expression clearly indicated expression in bonafide juxtaglomerular cells. These studies also demonstrated that overexpression of renin2 could not compensate for the loss of renin1 and indicate that an active Ren1 locus is essential and sufficient for normal morphology of the juxtaglomerular apparatus. They note that the salient difference between renin1 and renin2 lies in the three N linked glycosylation sites that characterize the renin1d and renin1c sequences in contrast to the renin2 sequence, and that human and rat renin also exhibit 2 of the 3 potential glycosylation sites. It is hypothesized that these may be the signal for trafficking and generation of dense granules, presumably via a modified lysosome pathway (5).

The present study by Mercure et al demonstrates by the most rigorous state-of-the-art means that Cathepsin B is not supported to be the relevant PPE in vivo for generation of renin in the mouse. In view of the disparities observed between human and mouse renin2 versus rat and mouse renin1 for in vitro processing by Cathepsin B it might be of interest to assess the effect of the Cathepsin B knockout on a
Ren1d knockout line where Ren2 is the only contributing locus, or a complete mouse Ren knockout
harboring a human Ren transgene (or humanized renin-angiotensin system). In the end, it's ironic that it
has taken so long to perform this critical test—in some respects it would appear to confirm that a negative
result needs to be performed in a sexier context in order to see the light of day in the literature. We can
thank the Reudlhuber lab for definitively reopening the search for the elusive PPE by reinforcing what is
known and not known and opening our thoughts to other explanations and possibilities.

REFERENCES

1. Almeida PC, Oliveira V, Chagas JR, Meldal M, Juliano MA, and Juliano L. Hydrolysis by

conversion is determined by a multiplicity of factors including convertase processing, substrate
specificity, and intracellular environment. Cell type-specific processing of human prorenin by the

3. Clark AF, Sharp MG, Morley SD, Fleming S, Peters J, and Mullins JJ. Renin-1 is essential for
normal renal juxtaglomerular cell granulation and macula densa morphology. *J Biol Chem* 272:

4. Do YS, Shinagawa T, Tam H, Inagami T, and Hsueh WA. Characterization of pure human renal

5. Faust PL, Chirgwin JM, and Kornfeld S. Renin, a secretory glycoprotein, acquires


7. Jensen BL, Rasch R, Nyengaard JR, and Skott O. Giant renin secretory granules in beige mouse


18. **Neves FA, Duncan KG, and Baxter JD.** Cathepsin B is a prorenin processing enzyme. *Hypertension* 27: 514-517, 1996.


Fig 1

Mouse (Ren-1) …TRLsAEWGVFTKRPSLTNLTPVVLTVLNYL…

Rat …TRISAEWGEFIIKSSFTNVTPVVLTVLNYL…

Human …ARLGPEWSQPMKRLTLGNTTSSVILTNYM…

Mouse (Ren-2) …TRLsAEWDVFTKRSSLTDISPVVLTVLNYL…

* Proposed N-linked glycosylation site
--- Dibasic doublet corresponding to residues 65-66 of human prepro renin
----- Adjacent sequence differences discussed in text