

# Prorenin is sorted into the regulated secretory pathway independent of its processing to renin in mouse pituitary AtT-20 cells

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A native human prorenin and a mutant of its processing site (Arg-43 to Gln) were expressed in mouse pituitary AtT-20 cells which process prorenin to renin and have both regulated and constitutive secretory pathways. The native prorenin was processed to renin and secreted in a regulated manner. Although the mutant precursor was not processed, it was also secreted in a regulated manner. These results suggest that prorenin is sorted into the regulated pathway, stored in secretory granules and released by stimulus whether it is processed to renin or not.

Renin; Prohormone processing; Gene expression; (AtT-20 cell)

## 1. INTRODUCTION

In neuronal and endocrine cells, many peptide hormones are synthesized from larger precursors, prohormones, through endoproteolytic cleavage at paired basic amino acids during intracellular transport [1,2]. These cells have two secretory pathways: a regulated pathway by which peptide hormones are selectively segregated into secretory granules and stored until their release is stimulated by secretagogues, and a constitutive pathway by which other proteins are secreted continuously without storage [3–5]. However, both the nature of the intracellular sorting signal of hormones and whether the hormones are sorted into the regulated pathway before or after processing are not yet well understood.

Renin is produced from a larger precursor, prorenin, in the juxtaglomerular cells of kidney [6]. Although this precursor is processed at paired basic amino acids in its N-terminal region and the resultant renin is stored in granules until its release is stimulated [7], the intracellular sorting signal of renin and where the precursor is processed are currently unknown. There are two possibilities: one is that prorenin is processed to renin and then sorted into the regulated pathway, while the

other is that prorenin is transported into secretory granules where it is processed to renin.

To solve this problem, a native human prorenin and a mutant of its processing site (Arg-43 to Gln) were expressed in AtT-20 cells which are known to process prorenin to renin and have both regulated and constitutive pathways [8,9].

## 2. MATERIALS AND METHODS

### 2.1. Plasmid construction and DNA transfection

Site-directed mutagenesis was performed as described by Morinaga et al. [10]. The oligonucleotide used to change the codon for Arg-43 is shown in fig.1a. An expression plasmid of native prorenin, pAGEHRn1, was constructed by subcloning of a cDNA fragment containing the entire coding region of human preprorenin [6] into the pAGE123 vector [11] (fig.1b). The pAGE123 vector was donated by T. Mizukami, Kyowa Hakko, Tokyo, Japan. A mutant plasmid, pAGFRQ43P, was constructed by exchanging the mutated fragment with the corresponding fragment of pAGEHRn1. AtT-20 cells (a generous gift from R.E. Mains and B.A. Eipper, Johns Hopkins University, Baltimore, MD) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C in 5% CO<sub>2</sub>. The expression plasmids were transfected into AtT-20 cells by calcium phosphate coprecipitation, and stable transfectants which were resistant to the neomycin analogue G418 were selected. Clonal cell lines were then screened for secretion of prorenin and renin into the culture media.

### 2.2. Renin assay

Culture media were assayed for active renin (direct assay) and total renin (active renin, and prorenin after activation with 100 µg/ml trypsin) content by an angiotensin I (AI) generating assay method as previously described [12]. Prorenin levels were derived by taking the difference between those two measurements.

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*Abbreviations:* DMEM, Dulbecco's modified Eagle's medium; 8-Br-cAMP, 8-bromo-cAMP; AI, angiotensin I

### 2.3. Radiolabeling and immunoprecipitation

Radiolabeling of renin/prorenin and their immunological identification were performed by the method of Fritz et al. [8]. Cells in 35 mm plates were incubated in 0.5 ml of methionine-free DMEM containing 0.4 mCi/ml of [<sup>35</sup>S]methionine (New England Nuclear, 660 Ci/mmol) and 10% dialyzed fetal calf serum. The culture media were collected after 8 h of incubation, and Nonidet P-40 and SDS were added to all of the collected samples to 1 and 0.1% by volume, respectively. The medium was then immunoprecipitated with anti-human renin or anti-human prorenin prosegment antiserum. The immune complexes were absorbed with protein-A Sepharose, spun in a microfuge through a cushion of 30% sucrose in 10 mM Tris, pH 7.5, containing 1% Nonidet P-40 and 0.1% SDS, washed once with 10 mM Tris, pH 7.5, and eluted from the resin by boiling in SDS gel sample buffer. Samples were electrophoresed on a 10% SDS-polyacrylamide gel and fluorographed.

### 3. RESULTS

Expression plasmids coding for human preprorenin and a mutant of its processing site were introduced into AtT-20 cells which process prorenin to renin and have both constitutive and regulated pathways [8,9]. Since the activity of renin promoter is tissue-specific, human preprorenin cDNA was placed under the Rous sarcoma virus enhancer/promotor which is active in AtT-20 cells [13] (fig.1b). Transfection with the native prorenin plasmid, pAGEHRn1, and with the mutant one,

pAGERQ43P, led to numerous G418-resistant colonies. Cell lines transfected with pAGEHRn1 secreted both prorenin and renin, but those transfected with pAGERQ43P secreted almost exclusively prorenin (table 1). The small amount of active renin in the media of the pAGERQ43P transfectants is probably due to the intrinsic activity of prorenin [9,14] and not due to specific intracellular processing since the labeling experiment did not reveal conversion of prorenin to renin (see below). Cell lines AtT-20/G4 and AtT-20/H4 which were transfectants of pAGEHRn1 and pAGERQ43P, respectively, were used for detailed studies.

Secretion of prorenin and renin from the transfectants was also investigated by radiolabeling with [<sup>35</sup>S]methionine followed by immunoprecipitation with either anti-renin or anti-prosegment antiserum. AtT-20/G4 cells secreted a major 48 kDa form of prorenin, which was immunoprecipitated with both anti-renin and anti-prosegment antisera, and a less prevalent 43 kDa form of renin, which was immunoprecipitated with only anti-renin antiserum (fig.2a, lanes 1 and 2). AtT-20/H4 cells secreted only prorenin (lanes 3 and 4).

A further investigation on the cellular pathway of prorenin and renin secretion was carried out by using 8-bromo-cAMP (8-Br-cAMP) to stimulate secretion via the regulated pathway [8,9,13]. After the labeling period, cells were chased for 3 h with unlabeled medium (first chase period) and, finally, replicated cultures were incubated for a further 3 h in unlabeled medium with or without 5 mM 8-Br-cAMP (second chase period). AtT-20/G4 cells secreted prorenin and renin for the first chase period (fig.2b, lane 2). However, for the second chase period, they secreted mainly renin whose secretion was greatly stimulated by 8-Br-cAMP (fig.2b, lanes 3 and 4). AtT-20/H4 cells secreted only prorenin whose secretion was stimulated by the secretagogue (fig.2b, lanes 6–8).

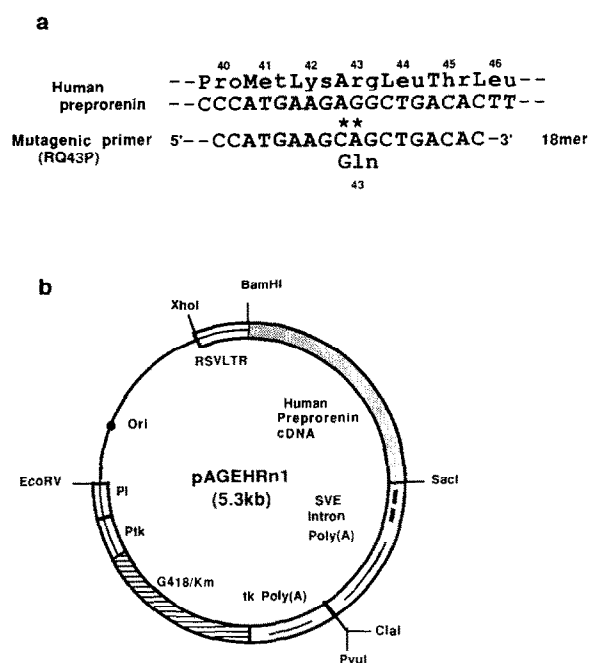


Fig.1. (a) Nucleotide sequence of the pertinent region of human preprorenin cDNA and the mutagenic primer. The oligonucleotide sequence of the primer is shown below the sequence of preprorenin cDNA. The nucleotides of the mutation site are indicated by asterisks. (b) Diagram of a preprorenin expression plasmid. RSVLTR, Rous sarcoma virus long terminal repeat; SVE Intron Poly(A), the intron and the polyadenylation signal from SV40 early gene; Ori, the replication origin of ColE1; PI, the pBR322 P1 promoter; Ptk, the thymidine kinase promoter from herpes simplex virus; G418/Km, Tn5 derived neomycin phosphotransferase gene; tk Poly(A), the polyadenylation signal from the thymidine kinase gene.

Table 1

Renin activity in the media of transfected AtT-20 cells

Clone designation	Plasmid	Active renin (ng AI/ml per h)	Prorenin (ng AI/ml per h)	Active renin (%)
AtT-20/G2	pAGEHRn1	33.4	111.3	23.1
AtT-20/G4	pAGEHRn1	53.4	84.3	38.8
AtT-20/G5	pAGEHRn1	5.7	10.4	35.3
AtT-20/G6	pAGEHRn1	1.8	4.9	27.3
AtT-20/H3	pAGERQ43P	1.5	17.0	8.1
AtT-20/H4	pAGERQ43P	4.6	> 150	< 3.1
AtT-20/H5	pAGERQ43P	11.3	> 150	< 7.5
AtT-20/H6	pAGERQ43P	5.2	143.6	3.5
AtT-20/F1	pAGE123	0	0	—

Cells were incubated in 24-well culture dishes containing 0.5 ml of medium. Culture media were harvested after 48 h, and renin activity was assayed

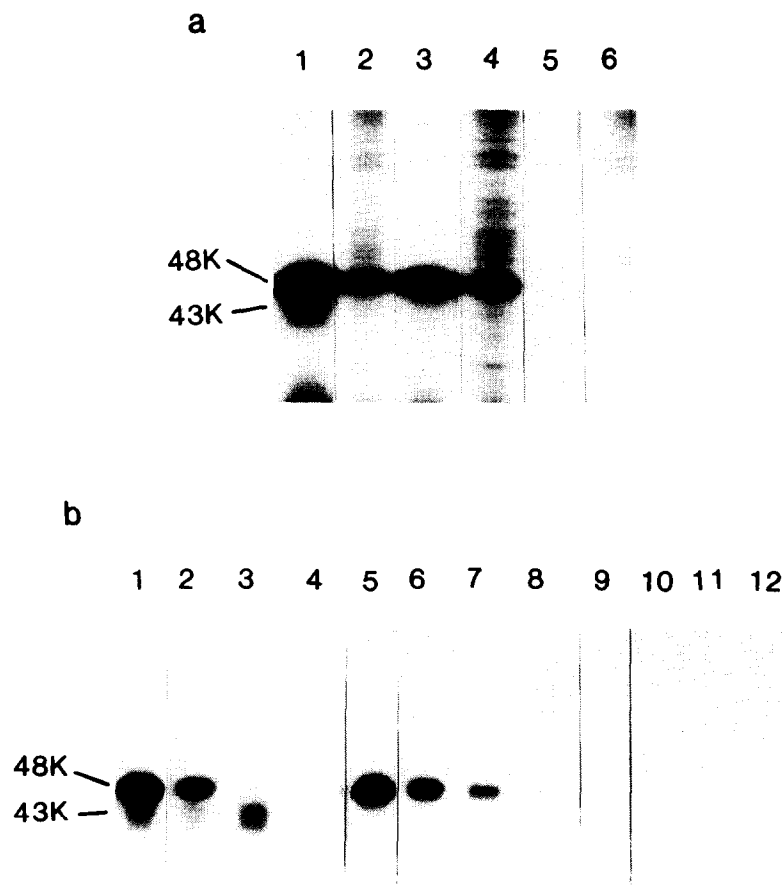


Fig.2. (a) Processing and secretion of prorenin and renin in AtT-20 cells. Lanes: AtT-20/G4 cells (1 and 2), AtT-20/H4 cells (3 and 4), and AtT-20/F1 control cells transfected with the pAGE123 vector (5 and 6) were labeled with [<sup>35</sup>S]methionine. The culture media were immunoprecipitated with anti-renin antiserum (1, 3 and 5) or with anti-prosegment antiserum (2, 4 and 6). (b) Stimulation of renin and prorenin secretion by a secretagogue. The culture media of AtT-20/G4 cells (1-4), AtT-20/H4 cells (5-8) and AtT-20/F1 cells (9-12) were immunoprecipitated with anti-renin antiserum. 1, 5 and 9, 8 h labeled media; 2, 6 and 10, first chase period; 3, 7 and 11, second chase period with 8-Br-cAMP; 4, 8 and 12, second chase period without 8-Br-cAMP. 1, 5 and 9 are derived from a shorter film exposure than the rest of the lanes.

#### 4. DISCUSSION

Many peptide hormones are produced from larger precursors which are processed at paired basic amino acids during transport. It is suggested that the precursors are processed after their transport into secretory granules since biologically inactive prosegment(s) are often colocalized there [2]. Alternatively, each cleaved product may possess a sorting signal and may be sorted individually after processing. In order to clarify this, the native prorenin and the mutant of its processing site were expressed in AtT-20 cells which process prorenin to renin and have both regulated and constitutive secretory pathways [8,9].

The cells which express the native prorenin secreted both prorenin and renin, while those expressing the mutant one secreted only prorenin. However, both renin from the former cells and prorenin from the latter ones were released in a regulated manner. These results suggest that prorenin is sorted into the regulated pathway,

stored in secretory granules and released by stimulus whether it is processed to renin or not.

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