Effects of propeptide deletion on human renin secretion from mouse pituitary AtT-20 cells

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To study the role of the N-terminal propeptide in the secretory process of renin, mouse pituitary AtT-20 cells were transfected with expression plasmids of human preprorenin and a mutant deleted of its propeptide. The transfectant of the native construct secreted inactive prorenin and active renin, and renin secretion was stimulated by a secretagogue, 8-Br-cAMP. On the contrary, the transfectant of the deleted construct secreted only active renin, whose release was also stimulated by the secretagogue. The amount of renin molecule secreted from the latter transfectant was lower than that from the former one, although a significant amount of fully active renin could be produced. These results suggest that the propeptide plays an important role in the secretory process of renin, probably folding and/or stabilizing the renin molecule, but it does not contain the signal for intracellular sorting to target renin to secretory granules.

Renin; Propeptide; Oligonucleotide-directed mutagenesis; Protein folding; AtT-20 cell

1. INTRODUCTION

Many proteins are produced from larger precursors by removal of the 'propeptides', whose structures and locations in the precursors are different from one protein to another [1–4]. There are several possible functions of propeptide, such as providing a source of zymogen for local activation [4], acting as a linker between the signal peptide and the mature protein to ensure cleavage of the signal peptide [5,6], guiding correct folding of the protein [7,8], and acting as a sorting signal through the secretory pathways [9,10].

In neuroendocrine cells, many peptide hormones are also produced from larger, inactive precursors, prohormones, by removal of propeptides by endoproteolysis usually at paired basic amino acids during intracellular transport [1–3]. These cells have two secretory pathways [11–13]: one is the regulated pathway by which mature hormones are produced by processing of prohormones and targeted to secretory granules where they are stored until the release is stimulated; the other is the constitutive pathway by which other proteins are secreted continuously without storage. The nature of the sorting signal of hormones to be targeted to secretory granules is now debatable. There are some reports that the mature hormone sequence has the sorting information [14,15], while there are also some reports implicating the propeptide in the sorting [9,10].

Renin, an aspartyl protease, is synthesized mainly in the juxtaglomerular cells of kidney and plays a pivotal role in the regulation of blood pressure and sodium balance [16]. It is produced from an inactive precursor, prorenin, by endoproteolytic cleavage of the N-terminal propeptide at paired basic amino acids during intracellular transport, stored in secretory granules and released by stimulus [17–20]. We have recently shown that prorenin is sorted into the granules independent of its processing in mouse pituitary AtT-20 cells [21] and that deletion of the propeptide remarkably reduces the amount of renin molecule secreted from Chinese hamster ovary (CHO) cells [22].

In this study, to further examine the role of the propeptide in the secretory process of renin, we have transfected AtT-20 cells, which can process prorenin to renin and have both regulated and constitutive pathways [23–26], with expression plasmids of human preprorenin and of a mutant deleted in its propeptide.

2. MATERIALS AND METHODS

2.1. *Plasmid construction and transfection*

Oligonucleotide-directed mutagenesis to delete the nucleotide sequence encoding the 43 amino acid N-terminal propeptide of human preprorenin was described previously [22]. A mutant plasmid, pAGEΔPRn, was constructed by exchanging the corresponding fragment of pAGEHRn1 [21], an expression plasmid of native human preprorenin, with the mutated one. AtT-20 cells grown in Dulbecco's

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Abbreviations: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; AngI, angiotensin I

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modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) at 37°C in 15% CO_2 were transfected with the expression plasmids by calcium phosphate coprecipitation, and then stable transfectants resistant to a neomycin analog G418 were selected. When indicated, the highest renin-secreting clonal cell lines were used for experiments.

2.2. *Assays*

Culture media were assayed for active renin and total renin (active renin, and prorenin after activation with 0.1 mg/ml trypsin) activities by an angiotensin I (AngI) generating assay method as described previously [27]. Prorenin levels were deduced by taking the difference between these two measurements. Total renin concentration was determined with the Total Renin IRMA Kit (Daiichi Radioisotope). Specific activity was evaluated from the values of total renin activity and total renin content in the medium.

2.3. *Radiolabeling and immunoprecipitation*

Radiolabeling and immunological identification of renin molecules were performed as described previously [21]. Briefly, cells at about 70% confluency in a 35-mm plate were incubated with 0.5 ml of methionine-free DMEM containing 0.4 mCi/ml of [^{35}S]methionine and 10% dialyzed FCS. The media were collected after 10 h and then subjected to immunoprecipitation with an appropriate antiserum and protein A-Sepharose. The precipitated proteins were analyzed by SDS-PAGE followed by fluorography.

2.4. *Northern blot analysis*

mRNA levels of renin and β-actin of transfected cells were compared by Northern blot analysis as described previously [22].

3. RESULTS

AtT-20 cells, which can process prorenin and other prohormones and have both regulated and constitutive pathways [23–26], were transfected with the expression plasmids of human preprorenin and a mutant deleted of its propeptide. As shown in Table I, the transfectant of the native construct, pAGEHRn1, secreted inactive prorenin and active renin as previously described [21]. On the contrary, the transfectant of the deleted construct, pAGEΔPRn, secreted only active renin, whose activity was about 10-fold lower than the total renin activity from the pAGEHRn1 transfectant. The total renin content in the medium of the pAGEΔPRn transfectant was also about 10-fold lower than that of the pAGEHRn1 transfectant. Thus, the renin molecules secreted from these two transfectants had no difference in the specific activity of total renin.

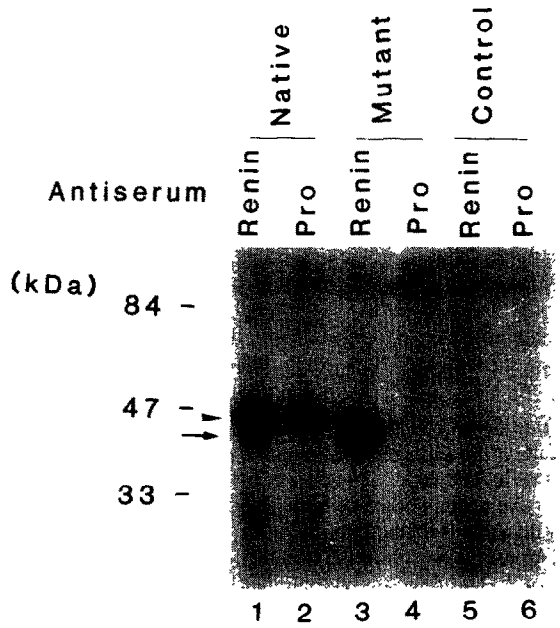

Fig. 1. Secretion of prorenin and renin from the transfected AtT-20 cells. AtT-20 cells transfected with pAGEHRn1 (lanes 1 and 2), pAGEΔPRn (lanes 3 and 4), and pAGE123 (lanes 5 and 6) were labeled with [^{35}S]methionine for 10 h. The culture media were immunoprecipitated with anti-renin serum (lanes 1, 3 and 5) or anti-propeptide serum (lanes 2, 4 and 6). 0.3 and 0.1 ml of media of the pAGEΔPRn transfectant and other transfectants, respectively, were used for immunoprecipitation. The arrowhead and arrow indicate the position of prorenin and renin, respectively.

Secretion of renin molecules from these transfectants was also examined by radiolabeling with [^{35}S]methionine followed by immunoprecipitation with anti-human renin or anti-human prorenin propeptide serum (Fig. 1). The pAGEHRn1 transfectant secreted a

Table I

Renin activities and renin contents in the media of the transfected AtT-20 cells

Introduced plasmid	Activity (ng AngI/ml per h)			Total renin concentration (ng/ml)	Specific activity[a] (ng AngI/ng per h)
	Active renin	Prorenin	Total renin		
pAGEHRn1	125.2 ± 10.2	274.8 ± 27.1	400.0 ± 27.1	10.6 ± 1.3	38.5 ± 5.6
pAGEΔPRn	37.5 ± 2.2	0	37.5 ± 2.2	1.01 ± 0.06	37.3 ± 3.7
pAGE123	0	0	0	0	–

[a] Specific activity of total renin

Cells were incubated with 1 ml of medium in a 35-mm plate for 48 h. The culture medium was then removed and renin activity and renin content were determined. Values are the means ± SD of three experiments. pAGE123 is the expression vector which does not contain preprorenin cDNA

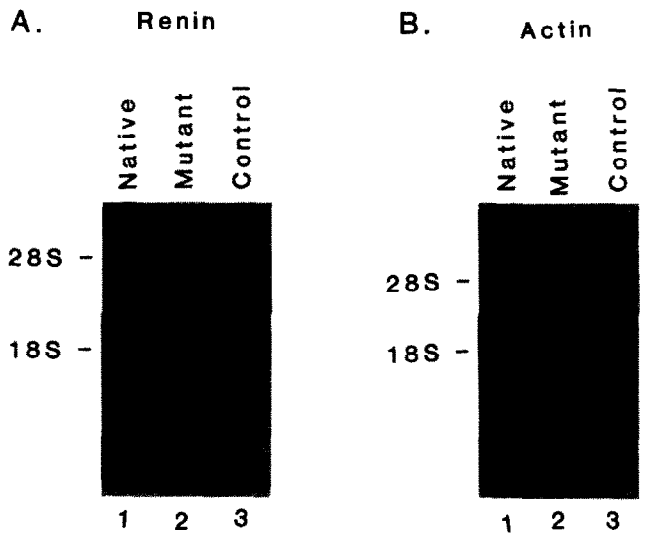

Fig. 2. Northern blot analysis. Total RNA (10 μg) was isolated from the AtT-20 cells transfected with pAGEHRn1 (lane 1), pAGEΔPRn (lane 2) and pAGE123 (lane 3), and analyzed by Northern blotting using [α-^{32}P]dCTP-labeled human preprorenin cDNA (A) or mouse β-actin cDNA (B) as a probe. In (A) doublets of the hybridizing RNA in lanes 1 and 2 are probably derived from the heterogeneity of polyadenylation of mRNAs.

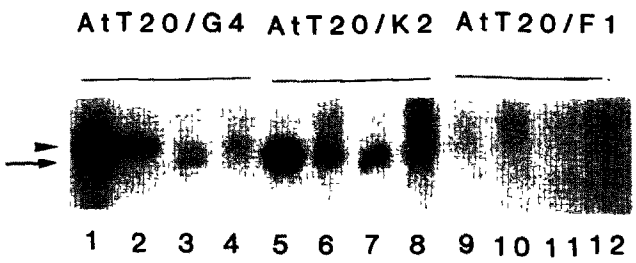

Fig. 3. Stimulation of renin secretion by a secretagogue. The culture media of AtT-20/G4 cells (lanes 1–4), AtT-20/K2 cells (lanes 5–8), and AtT-20/F1 cells which are cloned cells transfected with pAGE123 (lanes 9–12) were immunoprecipitated with anti-renin serum. Lanes 1, 5 and 9, 10 h-labeled media; lanes 2, 6 and 10, first chase period; lanes 3, 7 and 11, second chase period with 8-Br-cAMP; lanes 4, 8 and 12, second chase period without 8-Br-cAMP. The arrowhead and arrow indicate the positions of prorenin and renin, respectively.

major 46 kDa protein which reacted with both anti-renin and anti-propeptide sera, and a less prevalent 42 kDa protein which reacted only with anti-renin serum (lanes 1 and 2) as previously described [21]. On the contrary, the pAGEΔPRn transfectant secreted only a 42 kDa protein which reacted only with anti-renin serum (lanes 3 and 4). These results indicate that the transfectant of the native construct secretes prorenin and renin and that of the deleted construct secretes only renin. Densitometric scanning revealed that the secreted amount of renin molecule from the pAGEΔPRn transfectant was about 8-fold lower than that from the pAGEHRn1 transfectant.

To assume that the difference in renin secretion observed between these two transfectants was not due to the difference in transfection efficiency nor in transcription efficiency of the introduced renin cDNA, mRNA levels in the cells were compared by Northern blot analysis (Fig. 2). The mRNA levels of both renin and β-actin as control were similar between these two transfectants by densitometric scanning. These results indicate that the difference in renin secretion between these two transfectants was derived from the difference(s) in the process after translation of renin mRNA.

An investigation of the intracellular pathway of prorenin and renin secretion in AtT-20 cells was performed by using 8-Br-cAMP to stimulate secretion via the regulated pathway [23–26]. To facilitate analyses, the highest renin-secreting clonal cell lines, AtT-20/G4 and AtT-20/K2 which are transfectants of pAGEHRn1 and pAGEΔPRn, respectively, were used for the following experiments. After the labeling period, cells were chased for 2 h with unlabeled medium (first chase period) and, finally, replicated cultures were incubated for a further 2 h with unlabeled medium in the presence or absence of 5 mM 8-Br-cAMP (second chase period). As shown in Fig. 3, AtT-20/G4 cells secreted prorenin and renin until the first chase period (lanes 1 and 2), however, for the second chase period, they secreted mainly renin whose secretion was stimulated by 8-Br-cAMP (lanes 3 and 4). AtT-20/K2 cells secreted only renin whose secretion was also stimulated by the secretagogue (lanes 5–8). These results suggest that renin is sorted into the regulated secretory pathway in spite of the absence of the propeptide.

4. DISCUSSION

The propeptides of many pro-proteins have been suggested to have several functions. To know the role of the N-terminal propeptide of prorenin in secretion, we have transfected AtT-20 cells with the expression plasmids of human preprorenin and of a mutant deleted in its propeptide. The deletion results in a great reduction in the amount of renin secreted from the cells. One possible function of the propeptide in renin secretion is that it could act as a linker between the signal peptide and mature renin to ensure cleavage of the signal peptide. It has been reported that the N-terminal propeptides of proparathyroid hormone [5] and proapolipoprotein AII [6] facilitate efficient signal peptide function. In the case of renin, we will use a cell-free translation and translocation system to solve this problem, because it is difficult to solve this problem by using an intact cell system.

There is also a potential role for the propeptide in folding and/or stabilization of renin molecule. It has been shown that the propeptides of prosubtilisin [7] and pro-α-lytic protease [8], both of which are bacterial enzymes, are essential for guiding active conformation

of the enzymes. In the case of renin, we have recently shown that some amino acid substitutions in the propeptide reduce the amount of prorenin secreted from monkey kidney COS cells probably due to the inefficiency of its folding [28]. In the present and previous [22] studies using AtT-20 and CHO cells, respectively, we have shown that deletion of the propeptide greatly reduces the secreted amount of renin from these cells, even if a significant amount of the fully active enzyme can be produced. These results suggest that the propeptide could be important, although not necessary, for correct folding and/or stabilization of the renin molecule.

Another possible function of the propeptide is to act as an intracellular sorting signal to target renin to secretory granules. There are some reports about the role of the propeptides of other pro-proteins in the sorting. The propeptide of prosomatostatin has been shown to direct its targeting to the granules [9,10]. On the contrary, the C-peptide of proinsulin [15] and the propeptide of proapolipoprotein AI [29] have been reported to not contain the sorting information. In this study, we have demonstrated that deletion of the propeptide does not affect the intracellular sorting of the renin molecule. We could speculate that the mature renin portion itself contains the sorting signal. Further mutagenesis of the mature renin portion could be helpful to identify the amino acids which contribute to the sorting signal.

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