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Role of *N*-linked oligosaccharides attached to human renin expressed in COS cells

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One or both of two putative *N*-glycosylation sites (at asparagine-5 and -75) of human renin was eliminated by amino acid replacement of the asparagine residue with an alanine residue using site-directed mutagenesis. The three glycosylation-deficient renins (Asn-5, Asn-75, Asn-5 and -75 mutants) were expressed in COS cells and secreted into the conditioned media. The secreted amounts of the three mutants were different from one another, although the mutant and wild-type renins had practically the same specific activity. An Asn-5 and -75 mutant which did not contain any glycosylation sites was unstable in the medium, suggesting that the *N*-linked oligosaccharides play an important role in stabilization of human renin.

Renin; *N*-linked oligosaccharide; Site-directed mutagenesis; Gene expression; (COS cell)

1. INTRODUCTION

Renin (EC 3.4.23.15), an aspartyl proteinase, is a key enzyme of the renin-angiotensin-aldosterone system in that it catalyzes the first and rate-limiting step in the generation of angiotensin II, which is a major pressor substance and stimulates aldosterone secretion [1]. Human [2], hog [3], rat [4], and bovine [5] renal renins are all glycoproteins, although two of the known extrarenal renins, from mouse submaxillary gland [6] and bovine pituitary [5], are free of carbohydrate moieties.

Carbohydrate moieties of glycoproteins have been implicated in many functions, such as signalling for protein transport, maintenance of correct polypeptide conformation, and protection from proteolytic degradation (review [7]). However, little is as yet known of the properties and function of carbohydrate moieties of renal renins. To examine the role of oligosaccharides attached to human renin, we generated glycosylation-deficient mutants of the renin in which one or both of two putative *N*-glycosylation sites was eliminated by amino acid substitution using site-directed mutagenesis. This approach has permitted us to assess the contribution of each oligosaccharide chain to the function of human renin.

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Abbreviations: SDS-PAGE, SDS-polyacrylamide gel electrophoresis; DMEM, Dulbecco's modified Eagle medium; AI, angiotensin I

2. MATERIALS AND METHODS

Site-directed mutagenesis was carried out according to the method of Morinaga et al. [8]. The oligonucleotides used to change the codon for Asn at the glycosylation site are shown in fig.1. The cDNA containing both mutations was constructed by the fragment recombination between the single mutant cDNAs. The nucleotide sequence in the coding region of each mutant renin cDNA was determined by the methods of Hattori and Sakaki [9] and Maxam and Gilbert [10] to verify that only the desired mutation had occurred. A 0.9-kb *AvaI*/*Eco*RV fragment was cut out of each mutant cDNA and exchanged with the corresponding fragment of pSVDPRnPA33 [11], an expression plasmid of wild-type human renin, to yield the expression plasmid of each mutant renin. The expression plasmids were transfected into parallel cultures of COS-7 cells by DEAE-dextran method with chloroquine treatment [12]. The transfected cells were cultured in serum-free DMEM.

After trypsin treatment of conditioned medium, renin activi-

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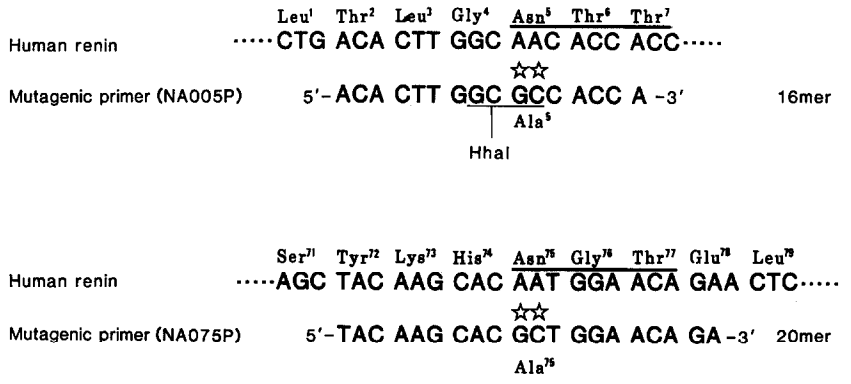

Fig.1. Nucleotide sequence of the pertinent regions of human renin cDNA and synthetic oligonucleotide sequences used for mutagenesis. The synthetic 16-mer oligonucleotide (NA005P) was used to change the codon for Asn-5 (AAC) to the codon for Ala (GGC) at the first glycosylation site. The 20-mer oligonucleotide (NA075P) was used to change the codon for Asn-75 (ATT) to the codon for Ala (GCT) in the second site. These oligonucleotide sequences are shown below the sequence of human renin cDNA. The nucleotides of the mutation site are indicated by a star (*). The location of new restriction endonuclease (*Hha*I) recognition site is indicated by the underlined nucleotides.

ty was determined by radioimmunoassay of angiotensin I (AI) generated. Renin content in the medium was determined by using a Renin RIA Pasteur kit (Diagnostics Pasteur, France). Specific activity was evaluated from the value of renin activity and renin content in the conditioned medium. SDS-PAGE was performed essentially as described [13] and Western blot analysis was carried out as described [14].

3. RESULTS AND DISCUSSION

Human renal renin is a glycoprotein [2] which has two putative *N*-glycosylation sites in its amino acid sequence [15], i.e. Asn-5 and Asn-75 (fig.1). To examine the role of *N*-linked oligosaccharides attached to human renin, we eliminated one or both of the putative *N*-glycosylation sites in human renin cDNA by replacing the codon for Asn with that for Ala using oligonucleotide-directed site-specific mutagenesis. Using these mutated cDNAs we constructed three mutant renin expression plasmids, i.e. pNA005SV (Asn-5 to Ala), pNA075SV (Asn-75 to Ala), and pNA575SV (Asn-5 and -75 to Ala). These expression plasmids as well as pSVDPRnPA33 [11], containing wild-type human renin cDNA, were transfected separately into parallel cultures of COS-7 cells. The transfected cells were incubated with serum-free DMEM for 72 h, and then the conditioned medium was harvested.

Western blot analysis showed that each conditioned medium contained a single protein reactive with anti-human renin antibodies and the molecular masses estimated for these proteins were different from one another (fig.2 and table 1). The molecular mass estimated for the double mutant NA575 (42 kDa) was in good agreement with that expected from nonglycosylated human prorenin [15,16], indicating that in this mutant the two *N*-glycosylation sites had been completely eliminated. In addition, the double mutant, NA575, had no affinity for either concanavalin A or wheat germ lectin (not shown). This provided another line of evidence that no carbohydrate moiety had attached to the mutated glycosylation sites. The average M_r values of the oligosaccharide chain attached at Asn-5 and Asn-75 were estimated to be approximately 3000 and 1000, respectively (table 1), suggesting that position-specific glycosylation had taken place in human renin expressed in COS cells.

By determining the activity and content of renin in each conditioned medium, we calculated the specific activity of each mutant renin (table 1). The specific activities of all the mutant renins were practically identical to that of the wild-type enzyme, and the value (around 15 mg AI/mg per h) was similar to that of purified human renin from kidney [17]. It is thus evident that the oligosaccharides are not required for the enzymatic activity of human renin.

The mutant renin activities in the conditioned media varied remarkably depending on the position of eliminated glycosylation sites (table 1). The

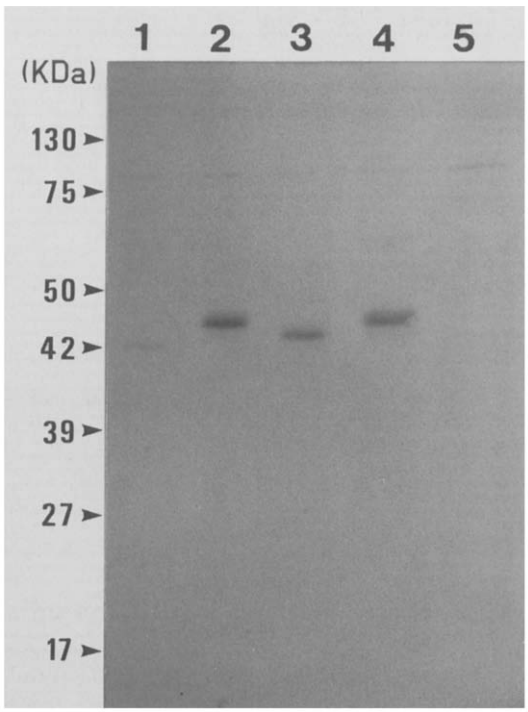

Fig.2. Western blot analysis of wild-type and mutant renins. The serum-free conditioned medium, which was incubated for 72 h, was used for the sample. The media conditioned by COS cells transfected with pNA075SV and with pNA575SV were concentrated 5-fold on an Amicon Centricon 10 concentrator. Lanes: 1, NA575 (Asn-5 and -75 to Ala); 2, NA075 (Asn-75 to Ala); 3, NA005 (Asn-5 to Ala); 4, wild-type; 5, the conditioned medium of untransfected COS cells.

mutant renin, NA005, which contained one *N*-linked oligosaccharide chain at Asn-75 was secreted into the conditioned medium nearly as efficiently as the wild-type enzyme which contained both oligosaccharide chains.

In contrast, NA075 which contained one oligosaccharide chain at Asn-5 and double mutant, NA575 which had no *N*-linked oligosaccharide, were secreted at a much lower level than the wild-type. In particular, the renin activity of NA575 in 72 h-incubated medium was only 4% of that of the wild-type (table 1).

It is noteworthy that a smaller oligosaccharide chain at Asn-75 (M_r about 1000) seems to be much more important to accumulate renin in the medium than a larger oligosaccharide chain at Asn-5 (M_r about 3000). The lower renin activity of mutant renins in the medium was assumed to be attributed to their lower concentration since their specific activity was practically same. Thus, effect of the mutation of renin cDNA on the gene expression level was examined.

Northern blot analysis showed that all mutant renin cDNAs were transcribed at similar levels to the wild-type (data not shown). This suggested that the translational level of mutant renins was also similar to that of the wild-type, indicating a possibility that a low concentration of renin in the conditioned medium of NA075 or especially of NA575 was attributed to a low rate of secretion of the mutant renins from the COS cells. But this was not the case because more than 90% of all mutant renins produced in COS cells were secreted into the medium.

To understand the reason for the decline in renin activity of NA075 and NA575, we estimated the renin activity of all mutants in the media at intervals (12–72 h). The renin activity of NA075 and especially of NA575 was very low at 12–72 h incubation compared with that of NA005 and the wild-type (fig.3). In particular, NA575 was unstable in the conditioned medium (fig.3, right-

Table 1

Properties of wild-type and mutant renins expressed in COS cells

Renin	Molecular Mass (kDa)	Size of attached oligosaccharide (kDa)	Renin activity in conditioned medium (ng AI/ml per h)	Specific activity (mg AI/mg per h)
Wild-type	46	4	111.9 ± 4.8	14.5 ± 4.76
NA005	43	1	105.6 ± 2.6	15.0 ± 2.62
NA075	45	3	49.2 ± 3.7	15.3 ± 3.74
NA575	42	–	4.5 ± 0.5	12.7 ± 0.45

After a 72 h incubation, the serum-free conditioned medium was harvested, and renin activity and renin content were assayed. Values are the means ± SD for three experiments

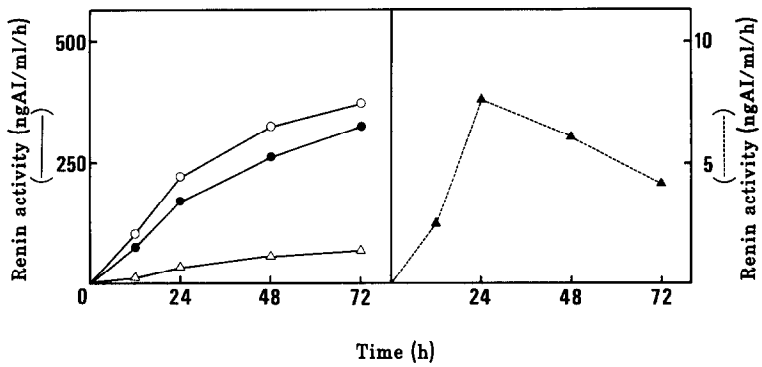

Fig.3. Time course of renin activity in the conditioned medium. COS cells were transfected with each expression plasmid, incubated for 24 h with DMEM containing 10% fetal calf serum, and then the medium was changed to the serum-free DMEM. Samples were taken at 12, 24, 48, and 72 h, and renin activity was assayed. (○—○), wild type; (●—●)NA005 (Asn-5 to Ala); (△—△), NA075 (Asn-75 to Ala); (▲—▲) (right-hand panel), NA575 (Asn-5 and -75 to Ala).

hand panel). This instability of NA575 is one of the reasons for its low activity in the medium and probably in the cells. All the results in the present investigation indicate that the carbohydrate moiety is important for the stabilization of the renin molecule.

However, the decrease in renin activity of NA575 and NA075 in the conditioned media may not be rationalized only in terms of the loss of their stability. Further studies are needed to understand how the oligosaccharides contribute to intracellular transportation of human renin.

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