Two rat homologues of human cystatin C

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Two immunochemically related forms of cystatin C-like inhibitors which differ in their $M_{r\,app}$ and isoelectric point have been found both in urine and seminal vesicles of rats. Amino-terminal sequences of these two cystatins are identical within the same fluid and exhibit a high degree of homology with that of human cystatin C. However, cystatins C purified from urine lack eight residues at their amino-terminal end when compared to those of seminal vesicles. The occurrence of two cystatin C-like components in rat fluids has been found to be due to the presence of a glycosylated form reported here as cystatin Cg which specifically binds concanavalin A and is susceptible to endo-β-N-acetylglucosaminidase treatment.

Cysteine proteinase; Glycosylation; Amino acid sequence; Cystatin C; Proteinase inhibitor; (Rat)

1. INTRODUCTION

Family 2 of the cystatin superfamily includes low-molecular-mass inhibitors of cysteine proteinases structurally related to chicken cystatin [1]. These molecules are characterized by the presence of two disulfide bonds and are mainly present in secretions or extracellular fluids such as egg white, seminal plasma, colostrum, saliva, cerebrospinal fluid and plasma [1,2]. In human, two related types of molecules belonging to this family, namely cystatin C and cystatins S, have been identified which differ in their immunochemical and physicochemical properties and their extracellular localization [1,3]. Homologues of human cystatin C have been found in other mammal species including monkey [4], mouse [5], dog [6], and beef [7]. Though being able to inhibit cysteine proteinases, little is known about the physiological role of these proteins. However, the presence of a peculiar tetrapeptide involved in the inhibition of phagocyte functions has been demonstrated in the amino-terminal part of human cystatin C [8,9]. Cystatin C is also implicated in a particular type of hereditary cerebrovascular amyloidosis characterized by deposits of an abnormal form of the protein [10].

We have previously reported on the purification of two immunologically related forms of cystatin belonging to family 2 in urine of nephropathic rats [11]. We provide evidence here that these molecules are structurally related to human cystatin C and that they differ essentially, if not exclusively, in the fact that one is glycosylated. The presence of a similar glycosylated cystatin C provisionally called cystatin Cg, in other tissues such as seminal vesicles lets one suppose that both forms of cystatin C occur physiologically. Analysis of the amino-terminal sequence of rat cystatins C also indicates that molecules purified from urine are partly degraded whereas those purified from seminal vesicles are most probably in a native form.

2. MATERIALS AND METHODS

Rat urinary cystatins first reported as inhibitors 17 kDa and 22 kDa were purified according to the procedure previously described [11]. Corresponding inhibitors in rat seminal vesicles have been purified using homogenates of seminal vesicle fluid

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Abbreviations: Endo F, endo-β-N-acetylglucosaminidase F; TFA, trifluoroacetic acid

as a starting material [11]. This homogenate was first submitted to fractionation by AcA 54 chromatography (2.6 × 40 cm column in 50 mM Tris-HCl, 150 mM NaCl = buffer A). Cystatin containing fractions were identified by their antigenic properties, pooled and dialysed against 50 mM potassium phosphate buffer, pH 6.0, then fractionated by FPLC on a Mono S column equilibrated in the same buffer and eluted with a linear NaCl (0–600 mM) gradient. Maximal elution of cystatin Cg and cystatin C was obtained at sodium chloride concentrations of 300 mM and 420 mM, respectively, and complete separation of the two forms was achieved by applying Mono S fractions onto a Superose 12 column equilibrated in buffer A. The final step of purification for each form of rat cystatin C was carried out using reverse-phase chromatography on an aquapore butyl BU-300 (Brownlee) cartridge eluted with a linear gradient from 0 to 60% acetonitrile in 0.07% TFA (flow rate 1 ml/min). After this last step, products issuing from either urine or seminal vesicles were used for sequence analysis.

Deglycosylation of rat cystatins was performed using endo-β-N-glucosaminidase F (EC 3.2.1.96) (Boehringer) [12] at concentrations ranging from 0.0025 to 5 U/ml. Fractions were incubated overnight in 20 mM potassium phosphate buffer, pH 6.5, 10 mM EDTA, 1% Triton X-100, 0.2% SDS in the presence of 1% 2-mercaptoethanol then analysed by SDS polyacrylamide gel (15%) electrophoresis [13].

Electroblotting on nitrocellulose sheets was performed as previously described [11] using either anti-rat urinary cystatin C raised in rabbits or peroxidase-linked concanavalin A (Sigma) at a final conjugate concentration of 0.08 mg/ml in 20 mM sodium phosphate buffer, pH 6.8, 150 mM NaCl, 1 mM $MnCl_2$, 1 mM $CaCl_2$.

N-terminal amino acid analyses were performed on an Applied Biosystems 477A protein sequencer and identification of PTH derivatives was made using an on-line associated model 120A analyser.

3. RESULTS AND DISCUSSION

We have previously demonstrated the presence of two cystatin C-like molecules in urine of rats treated with sodium chromate [11]. These inhibitors were purified, characterized by their $M_{r\ app}$, isoelectric point, and analysed for their cysteine proteinase inhibiting capacity. Since both inhibitors shared complete immunological identity towards anti-human cystatin C antibodies and antibodies raised in rabbits against each of them [11], the question was raised as to the structural relationship between these two molecules as well as to their biological occurrence.

Human and rat male sex glands are known to contain large amounts of various cystatin-like molecules [11,14]. Therefore rat seminal vesicle fluid was investigated for the presence of cystatins immunochemically related to those found in urine. Both inhibitors were found in homogenates of
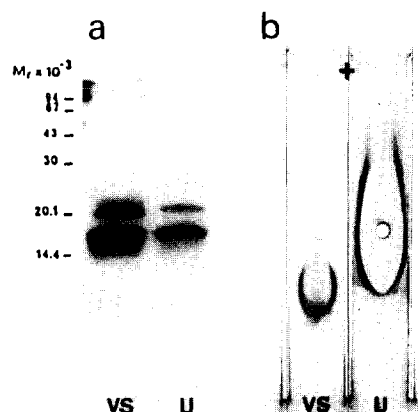

Fig.1. Electrophoretic pattern of rat cystatins C after SDS-PAGE (a) and immunoelectrophoresis (b). Samples were either homogenates of seminal vesicles (vs) or concentrated urine (u) after the first step of purification. Cystatins were revealed using anti-rat cystatin C (b) and after immunoblotting (a).

seminal vesicle fluid as shown in fig.1a and purified as described in section 2, thus suggesting that these two molecules occur naturally. However their electrophoretic mobility was significantly different from that of cystatins C present in urine as shown in fig.1b.

To explain this different electrophoretic behaviour, amino-terminal sequences of all cystatin varieties purified from either urine or seminal vesicles were determined. As shown in table 1 all sequences were identical with the noteworthy exception that cystatin C and cystatin Cg isolated from urine lack 8 residues at their N-terminal end when compared to their homologues in seminal vesicles. Taking into account the nature of the starting material and the method for sample collection it may be supposed that the presence of shorter molecules in urine is due to partial proteolysis of native cystatin C which is known to be highly susceptible to proteolysis by various exo- and endopeptidases in human [15,16]. Rat cystatin C also presents a high degree of homology with other cystatins of family 2 [4,7,17–19] as shown on table 2. Besides, the N-terminal sequence of rat cystatin isolated from seminal vesicles starts 10 amino acid residues upstream the highly conserved glycyl residue common to all members of the cystatin superfamily as does human cystatin C [20] suggesting this sequence most probably cor-

Table 1

Comparison of N-terminal amino acid sequence of rat cystatins C isolated from
seminal vesicles and from urine

Cystatin C (urine)	L L G A P Q E A D A S E E G V
Cystatin Cg (urine)	L L G A P Q E A D A S E E G V
Cystatin C (sem. ves.)	G T S R P P P R L L G A P Q E A D A S E E G V
Cystatin Cg (sem. ves.)	G T S R P P P R L L G A P Q E A D A S E E G V

responds to that of the native molecule. One can think therefore that such a N-terminal primary structure may be shared by other mammal cystatins C. This N-terminal part of cystatins susceptible to proteolysis could well be of major biological importance since it has recently been reported that in human it includes a biologically active tetrapeptide known as postin (KPPR) which may induce inhibition of the phagocyte function of monocytes and polymorphonuclear neutrophils [8,9]. The corresponding sequence in rat cystatin C differs from that in human only by one residue (PPPR) but it is not known as yet whether this peptide has similar biological effects.

Results from sequence analysis however, do not provide any explanation to the simultaneous presence of two molecular forms of rat cystatin C in urine as well as in seminal vesicles. Attempts have been made therefore, to demonstrate a possible glycosylation of the higher M_r component though cystatins from families 1 and 2 have been reported to be non-glycosylated [21]. Both inhibitors purified either from urine or seminal vesicles were incubated with endo F, which is specific for Asn linked glycans as described in section 2, then analysed by SDS-polyacrylamide gel electrophoresis. As shown on fig.2a the component of higher molecular mass (22 kDa) was completely transformed into a 17 kDa component indistinguishable from the low-molecular-mass
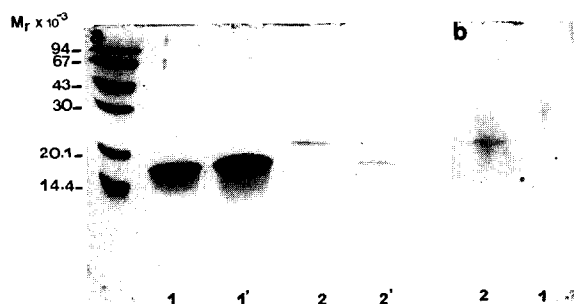

Fig.2. Rat cystatin C glycosylation. (a) SDS-PAGE of urinary cystatin C (about 2 μg) incubated without (1) and with (1') 0.0025 U of endo F and of urinary cystatin Cg (about 0.2 μg) without (2) and with (2') 0.0025 U of endo F. (b) Electroblot on nitrocellulose sheet of 2 μg urinary cystatin C (1) and 0.5 μg urinary cystatin Cg (2) after SDS-PAGE and incubation with peroxidase labeled concanavalin A. Enzyme activity was revealed using 4-chloro-1-naphthol as a substrate [11].

cystatin upon endo F treatment, whereas the low-molecular-mass cystatin was completely resistant to this treatment. One can suppose therefore that rat cystatin C occurs in part as a glycosylated form provisionally called cystatin Cg and this was confirmed after electroblotting of urine and seminal vesicle cystatins and revelation by peroxidase-linked concanavalin A. Results are shown in fig.2b which demonstrate that only cystatin Cg is able to bind the lectin. On the other hand, one can sup-

Table 2

N-terminal amino acid sequence of rat cystatin C aligned with those of other members of the cystatin family 2

Rat cystatin C	G T S R P P P R L L G A P Q E A D A S E E G V Q R A L D F A V S E Y N K G S N D A Y
Human cystatin C	S S P G K P P R L V G G P M D A S V E E E G V R R A L D F A V G E Y N K A S N D M Y
Beef colostrum cystatin	R L L G G L M E A D V N E E G V Q E A L S F A V S E F N K R S N D A Y
Chicken cystatin	S E D R S R L L G A P V P V D E N D E G L Q R A L Q F A M A E Y N R A S N D K Y
Human cystatin S	I I P G G I Y D A D L N D E W V Q R A L H F A I S E Y N K A T E D E Y

Residues identical to those in rat cystatin C are boxed

pose that glycosylation occurs at only one site of cystatin Cg since no stained band with intermediate mobility can be seen after SDS-PAGE following treatment by the smallest amount of endo F required to observe deglycosylation. No potential site of N-glycosylation is present in this part of the rat cystatin C sequence reported in this study, which covers about one third of the complete sequence. Neither is a potential site of N-glycosylation present in mammal cystatins, the complete sequence of which has been determined [1]. However occurrence for a glycosylated form of cystatin C in mammals agrees with the extracellular localization of these proteins as confirmed by the presence in human cystatin C of a hydrophobic leader sequence [22]. It is not known, however, whether the glycosylation of rat cystatin C induces any change of its biological functions. The cysteine proteinase inhibitory properties of rat cystatin C and of cystatin Cg were found to be similar using either papain or homologous cathepsin L as target proteinases, indicating that glycosylation does not significantly modify the inhibiting properties. Neither is the presence of the 8 amino-terminal residues essential for inhibition to occur. However the possible involvement of this N-terminal end in other biological functions such as that of precursor to biologically active peptides remains to be investigated further.

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