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Identification of a Protein Increasing in Serum of Nagase Analbuminemic Rats Bearing Intestinal Tumors as an Isotype of T-Kininogen

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Increase in an unidentified protein was observed in serum of Nagase analbuminemic rats (NAR) bearing intestinal tumors induced by azoxymethane. This protein seemed to be a polymer of a protein of 73 kDa as estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and so was tentatively named 73K-protein. The serum concentration of 73K-protein in NAR bearing intestinal tumors was 11.9±2.2 mg/ml (mean±SD, n=5), whereas that in control NAR was 2.0±0.2 mg/ml. Increase of the serum 73K-protein level was also observed in Sprague-Dawley rats bearing intestinal tumors, skin tumors, subcutaneous sarcomas, or mammary tumors and in ACI rats bearing urinary bladder tumors. On double immunodiffusion analysis, the 73K-protein was not detected in mouse, guinea pig, pig, horse, or human serum. A cDNA clone bearing the sequence encoding 73K-protein was isolated from a cDNA library constructed from rat liver mRNA. The nucleotide sequence of the 73K-protein showed 98.8% and 96.9% homologies with the sequences of the 3′-proximal domains of the cDNAs for TI- and TII-kininogen, respectively. Therefore, the 73K-protein was concluded to be an isotype of T-kininogen.

Key words: Analbuminemic rats — Intestinal tumor — Serum protein — T-kininogen — Nucleotide sequence analysis

Nagase analbuminemic rats (NAR)[5] are a mutant strain established in our laboratory from a stock of Sprague-Dawley rats (SDR) and characterized by the absence of serum albumin and hyperlipidemia.[1] The absence of serum albumin in NAR is compensated for by an increase in the concentration of globulins,[2] and thus NAR is suitable for studies on serum globulins, whose concentrations are very low in normal rats, as well as for studies on the biological functions of albumin.

Recently, carcinogenicity experiments have been performed in this mutant.[3–10] In a series of studies, we observed increase of an unidentified protein in the serum of NAR bearing intestinal tumors induced by azoxymethane. Increase of this protein was observed not only in NAR but also in other strains of rats bearing various tumors. In this work, we studied the properties of this protein. From nucleotide sequence analysis we concluded that it is an isotype of the T-kininogen reported by Furuto-Kato *et al.*[11]

T-Kininogen is a novel kininogen liberating T-kinin (Ile-Ser-bradykinin) and differing from high- or low-molecular-weight kininogens.[11–13] It is present only in rats[11,14] and is identical or similar to the α_1-major acute phase protein (MAP).[11,15–17] However, its biological functions are still unknown. The present study suggests the participation of T-kininogens in tumor biology.

MATERIALS AND METHODS

Azoxymethane and 3-methylcholanthrene were purchased from Sigma Chemical Co., St. Louis, MO. 7,12-Dimethylbenz[*a*]anthracene was from Wako Junyaku Co., Tokyo. N-Butyl-N-(4-hydroxybutyl)nitrosamine was from Izumi Chemicals Co., Yokohama. Polyacrylamide gradient gel (PAA 4/30), cyanogen bromide-activated Sepharose 4B, oligo-(dT) cellulose, and a cDNA synthesis kit were from Pharmacia Fine Chemicals, Uppsala, Sweden. A DNA *in vitro* packaging kit (Gigapack, Plus & Gold) was from Stratagene, San Diego, CA. A DNA sequencing kit (7-DEAZA sequencing kit) was from Takara Shuzo Co., Kyoto.

Animals NAR and rats of an analbuminemic congenic strain with the ACI genetic background (ACI-alb)[18] were bred in our laboratory. SDR and ACI were purchased from Clea Japan, Inc., Tokyo. They were kept 5 to a cage (265×425×200 mm) with wood shavings and placed on an Iso-rack (Sanki Scientific Co., Tokyo) under conventional conditions in an animal room with air-conditioning (23±2°C and 55±5% relative humid-

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[5] Abbreviations used: NAR, Nagase analbuminemic rats; SDR, Sprague-Dawley rats; MAP, α_1-major acute phase protein; ACI-alb, analbuminemic strain of rats with ACI genetic background; SDS, sodium dodecyl sulfate.

ity) and artificial lighting from 7:00 a.m. to 7:00 p.m. Throughout the experiments, they were provided with a standard diet, CE-2 (Clea Japan, Inc.) and tap water *ad libitum*.

Sample collection Intestinal tumors, skin tumors, subcutaneous tumors, urinary bladder tumors, and mammary tumors were induced with azoxymethane, 3-methylcholanthrene, 3-methylcholanthrene, N-butyl-N-(4-hydroxybutyl)nitrosamine, and 7,12-dimethylbenz[*a*]anthracene, respectively, as described previously.[3-9] Blood was drawn from a tail vein periodically throughout the experiment and from the abdominal aorta at the end of the experiment. The samples were allowed to clot at room temperature and then serum was obtained by centrifugation at 3,000*g* for 15 min.

Electrophoresis Gradient gel electrophoresis was carried out in a GE 2/4 vertical electrophoresis system (Pharmacia Fine Chemicals) with PAA 4/30 gradient gel covering the acrylamide range of 4–30%T. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was carried out by the method of Laemmli[19] in 7.5% gel. Proteins were stained with Coomassie Brilliant Blue R-250.

Preparation of antiserum After polyacrylamide gradient gel electrophoresis, the protein was recovered from the gel electrophoretically by the method of Posner[20] in a Maxyield GP apparatus (Type AE-3590, Atto Corporation, Tokyo). Samples of 0.5 and 0.2 mg of the extracted protein were injected sc into rabbits with Freund's complete adjuvant at intervals of 2 weeks. One month after the first injection, blood was withdrawn from the rabbits and the resulting antiserum was used for assay of the serum concentration of protein by the single radial immunodiffusion method.[21] The IgG fraction of antiserum was obtained by fractionation with 33% saturated ammonium sulfate and CM-cellulose column chromatography, and was subjected to immunoaffinity purification by the method of van Eijk and Noort.[22] The coupling gel used was cyanogen bromide-activated Sepharose 4B and the column size was 25×200 mm.

Nucleotide sequence determination Total cellular RNA was extracted from about 1 g of NAR liver (1 week old) by the guanidinium thiocyanate/cesium chloride procedure of Chirgwin *et al.*[23] Poly(A)$^+$RNA was selected by oligo(dT)-cellulose chromatography. cDNA was synthesized from 3 μg of poly(A)$^+$RNA as described by Gubler and Hoffman[24] with a cDNA synthesis kit and introduced into λgt11 as described by Young and Davis[25] with Giga pack, Plus & Gold. The recombinant phages producing strong signals were isolated from the library by the immunological screening method of Young and Davis.[25,26] The cDNA insert of the phage DNA was subcloned into pUC18 plasmid DNA[27] and the nucleotide sequence was determined by the dideoxy chain termination method[28] as modified by Mizusawa *et al.*[29] with a DNA sequencing kit.

RESULTS

Properties of the unidentified protein in serum of NAR bearing intestinal tumors Fig. 1 shows the electrophoretic pattern of serum protein of NAR bearing intestinal tumors separated by polyacrylamide gradient gel electrophoresis. The serum of tumor-bearing NAR contained an unidentified protein which was scarcely detected in serum of control NAR. This protein was extracted from the gel electrophoretically (Fig. 1, lane 5) and administered to rabbits to raise antibody. The antiserum obtained reacted specifically with the unidentified protein. Therefore, we purified the protein from pooled serum of tumor-bearing NAR by immunoaffinity column chromatography using this antiserum. The purified protein had higher mobility than that of the unidentified protein on polyacrylamide gradient gel electrophoresis (Fig. 1, lane 6). This faster-moving protein was also observed in the serum of control NAR and increased

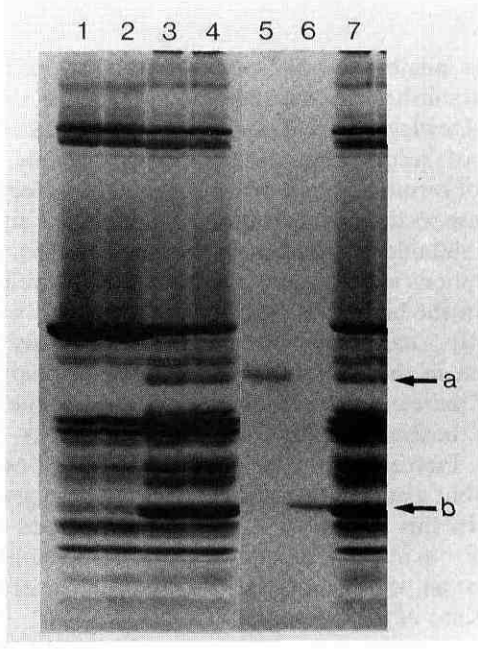

Fig. 1. Electrophoretic analysis of serum proteins of NAR bearing intestinal tumors induced by azoxymethane on a polyacrylamide gradient gel. Arrows: a, slow-moving protein; b, fast-moving protein. Lanes 1 and 2, sera from control NAR; lanes 3, 4, and 7, sera from tumor-bearing NAR; lane 5, slow-moving protein extracted from the gel; lane 6, protein purified by immunoaffinity chromatography with antiserum against the unidentified protein extracted from the gradient gel.

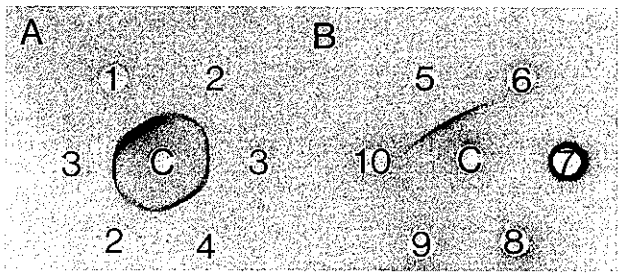

Fig. 2. Double immunodiffusion analysis of the unidentified protein (A) and sera of other animals (B). C, anti-unidentified protein antiserum; 1, serum of tumor-bearing NAR; 2, unidentified protein extracted from polyacrylamide gradient gel; 3, protein purified by immunoaffinity column chromatography; 4, serum of control NAR; 5, serum of control SDR; 6, mouse serum; 7, pig serum; 8, guinea pig serum; 9, horse serum; 10, human serum.

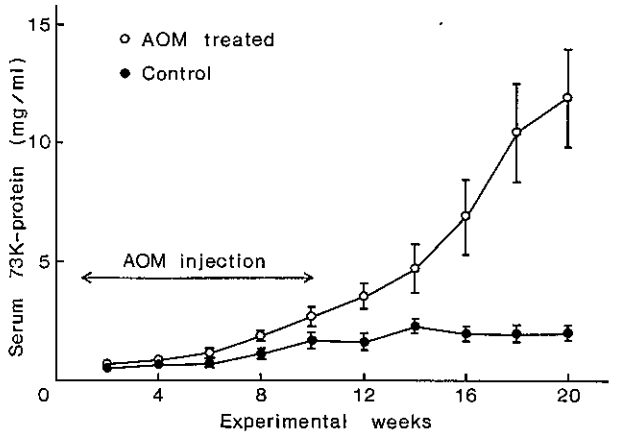

Fig. 3. Change of 73K-protein in the serum of NAR associated with induction of intestinal tumors by azoxymethane (AOM). Points and bars are means and standard deviations for values in five animals.

markedly in the serum of tumor-bearing NAR. The unidentified protein extracted from the gradient gel and the faster-moving protein purified by immunoaffinity chromatography were identical immunogenically as shown by double immunodiffusion analysis (Fig. 2, A) and had the same molecular weight of 73 kDa on SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol. Furthermore, the unidentified protein extracted from the gel was gradually converted to the faster-moving protein by repeated freeze-thawing. These results suggest that this protein may be a polymer of the faster-moving protein and may appear with increase in the serum concentration of the faster-moving protein. Therefore, we tentatively termed both these proteins 73K-protein. On double immunodiffusion analysis, this 73K-protein was not detected in mouse, guinea pig, pig, horse, or human serum (Fig. 2, B).

73K-Protein in the serum of tumor-bearing rats The concentration of 73K-protein in the serum of tumor bearers and control rats was assayed by the single radial immunodiffusion method. Fig. 3 shows the changes in the serum concentration of 73K-protein in NAR during the induction of intestinal tumors by azoxymethane. The concentration of this protein increased gradually from soon after the end of azoxymethane injection to 11.9± 2.2 mg/ml (mean±SD) in week 20, which was 6 times that in controls (2.0±0.2 mg/ml). A slight increase of serum 73K-protein with age was observed in control NAR, but this increase was considerably smaller than that in tumor bearers. Fig. 4 shows a comparison of the serum concentrations of 73K-protein in NAR, SDR, ACI-alb, and ACI with and without tumors. All tumor bearers had higher serum concentrations of 73K-protein than non-tumor bearers. The highest ratio of the serum concentration of 73K-protein in tumor bearers to that in

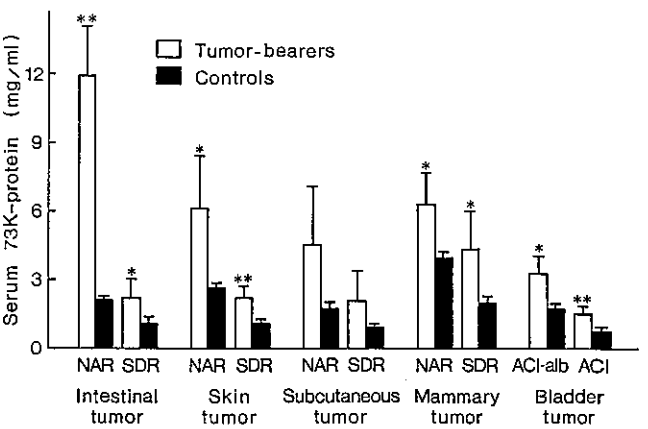

Fig. 4. Comparison of serum levels of 73K-protein in tumor-bearing and non-tumor-bearing rats. Sera were obtained at 21, 46, 17, 20, and 23 weeks after the beginning of administrations of carcinogens for induction of intestinal tumors, skin tumors, subcutaneous tumors, bladder tumors, and mammary tumors, respectively. Columns and bars are means and standard deviations for values in five animals. No symbol, $P<0.1$; *, $P<0.05$; **, $P<0.01$ (Student's t test).

non-tumor bearers was observed in NAR bearing intestinal tumors. The serum concentrations of 73K-protein in rats with other tumors were about double those in non-tumor bearers.

Nucleotide sequence of a 73K-protein cDNA clone Three recombinant clones bearing the sequence for 73K-protein were isolated from an NAR liver cDNA library (approximately 9,000 PFU) by immunological screening with a polyclonal antibody against 73K-protein. A

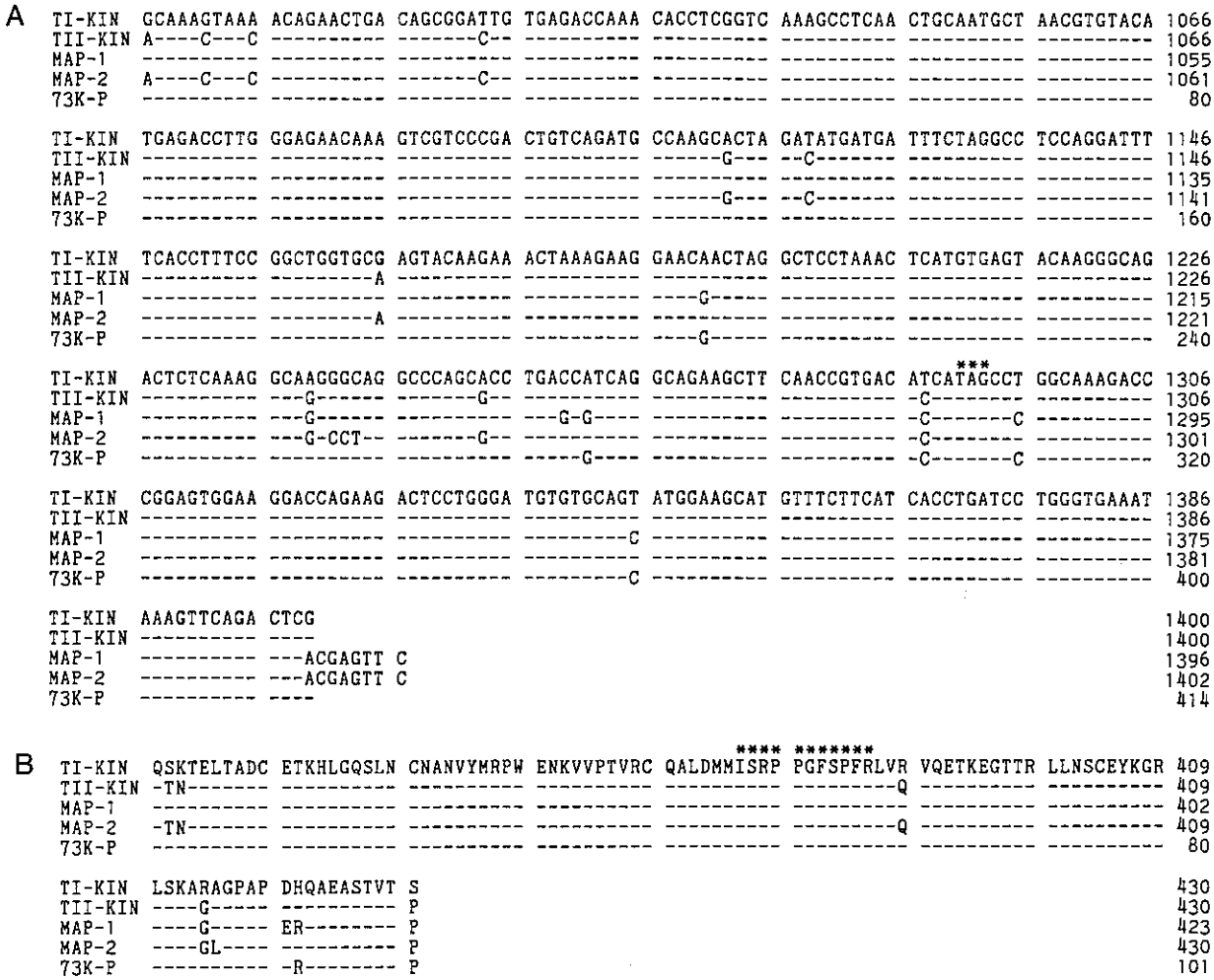

Fig. 5. Comparison of nucleotide (A) and amino acid (B) sequences of T-kininogens, MAPs and 73K-protein. The sequences of TI- and TII-kininogen (TI- and TII-KIN) are from Furuto-Kato *et al.*[11] and those of MAP-1 and -2 from Cole *et al.*[15] and Anderson and Heath,[16] respectively. 73K-P means 73K-protein. Only different residues from those in TI-kininogen are shown: identical residues are indicated by hyphens. Asterisks in A and B indicate the terminator codon and the amino acid sequence for T-kinin, respectively.

cDNA insert of 414 base pairs was isolated from one of the recombinant phages, and subcloned into the pUC18 vector. Fig. 5 shows the nucleotide sequence of this recombinant plasmid and the deduced amino acid sequence in comparison with those of rat TI- and TII-kininogens reported by Furuto-Kato *et al.*[11] and MAPs reported by Cole *et al.* (MAP-1)[15] and Anderson and Heath (MAP-2).[16] The cloned cDNA for 73K-protein showed 98.8% homology with the nucleotide sequence of the carboxyl proximal domain of rat TI-kininogen. The only mismatches between 73K-protein and TI-kininogen are single base-pair changes at five locations. Most of these mismatches are T to C (3 points) or A to G (2 points) substitutions. The amino acid coding region of the cDNA was 304 base pairs in length. The amino acid sequence of the 73K-protein deduced from the nucleotide sequence of the cDNA was almost identical to that of TI-kininogen, with differences in only two of 101 amino acids, as shown in Fig. 5, B. Exactly the same differences between TI-kininogen and 73K-protein in both nucleotide and amino acid sequences were observed between TI-kininogen and MAP-1, which is considered to be an isotype of TI-kininogen.[13] The homology of the nucleotide sequences of TII-kininogen and 73K-protein was 96.9% and that of the sequences of MAP-2 and 73K-protein was 95.9%.

DISCUSSION

Enjyoji *et al.*[13] reported two types of T-kininogens, TI- and TII-kininogen, and at least two variants of TI-kininogen, TIα- and TIβ-kininogen. Their TIα- and TIβ-kininogen corresponded to TI-kininogen reported by Furuto-Kato *et al.*[11] and MAP (MAP-1) reported by Cole *et al.*,[16] respectively. The cDNA for 73K-protein cloned here showed 98.8% homology of base sequence with TI-kininogen reported by Furuto-Kato *et al.*[11] Of five different base pairs, three were present in the amino acid coding region and resulted in substitutions of 2 of 101 amino acids. All these differences were also observed between TI-kininogen and MAP-1. These results indicate that 73K-protein is intermediate between TI-kininogen and MAP-1, and is an isotype of TI-kininogen, like MAP-1. However, we used polyclonal antiserum for screening the cDNA clone for 73K-protein, and the TI- and TII-kininogens were indistinguishable immunogenically.[13] Therefore, 73K-protein may be an isotype of T-kininogen rather than of TI-kininogen.

The molecular weights of T-kininogens and the low-molecular-weight kininogen purified from rat plasma by Enjyoji *et al.* were 68 and 73 kDa, respectively.[12] On the other hand, the molecular weight of the 73K-protein, which is considered to be an isotype of T-kininogen, is 73 kDa. The reason for this difference in the molecular weights of 73K-protein and T-kininogen is unknown. The antiserum used here gave a single precipitin line against the serum of tumor-bearing NAR and normal SDR, as shown in Fig. 2, A and on immunoelectrophoresis (data not shown). No precipitin line was observed against serum of other animals which contained low-molecular-weight kininogens, as shown in Fig. 2, B. Therefore, the antiserum was considered to be specific for T-kininogens.

In the present study, 73K-protein was found to consist of two forms with different mobilities on polyacrylamide gradient gel electrophoresis, and the form with lower mobility seemed to be a polymer of the other. Enjyoji *et al.*[13] reported a slower moving form with a faster moving (true) T-kininogen on polyacrylamide gel electrophoresis, which they supposed to be a polymer of the latter. The aggregation of a single oligometric low-molecular-weight kininogen of rats has also been reported.[30]

The serum concentration of 73K-protein was highest in NAR bearing intestinal tumors, while the serum concentration of 73K-protein in SDR bearing intestinal tumors was almost the same as those of rats with other tumors. This difference seemed to be caused by a difference in susceptibilities to induction of intestinal tumors. The intestinal tumors induced by azoxymethane were more numerous and larger in NAR than in SDR.[5] Moreover, the concentration of 73K-protein in the serum clearly increased with tumor growth, as shown in Fig. 3. On the other hand, the control levels of serum 73K-protein were higher in NAR than in SDR. This difference was also observed between ACI-alb and ACI, and thus is considered to be a genetic difference.

Matsumura *et al.*[31] reported that a kinin-generating cascade, especially bradykinin cleaved off from high-molecular-weight kininogen, seemed to play a significant role in enhanced permeability and retention in tumor tissue, resulting in more effective accumulation of plasma proteins and macromolecular drugs than in normal tissue. On the other hand, Itoh *et al.*[32] reported that the low-molecular-weight kininogen, which releases bradykinin, increased in the plasma and ascites of tumor-bearing mice and may regulate tumor growth by inhibiting tumor-related proteolytic activity. These reports suggest that kininogens may be important in tumor development. However, there seems to be no report concerning the relation of T-kininogens to tumors.

T-Kininogens are known to be thiol proteinase inhibitors and to increase under conditions of inflammation.[13, 33, 34] However, the natural enzyme(s) that acts on T-kininogens is unknown. Moreover, T-kininogen seems to be present only in rats.[11, 14] We could not detect 73K-protein in mouse, guinea pig, pig, horse, or human serum. Therefore, the biological functions of T-kininogens and T-kinins in tumor biology are unknown and require study.

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