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The high-molecular-mass kininogen deficient rat expresses all kininogen mRNA species, but does not export the high-molecular-mass kininogen synthesized

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The Katholiek substrain of Brown Norway (BN/Kat) rats exhibits a very low level of circulating high-molecular-mass (HMW) kininogen and a partial deficiency in plasma prekallikrein. Northern blot analysis of liver RNA revealed that HMW kininogen and prekallikrein mRNAs are present in these rats with a similar size and abundance compared to control Brown Norway (BN/Orl) rats. The low-molecular-mass kininogen mRNA, encoded by the same kininogen gene as HMW kininogen mRNA by alternative splicing, is detected in both strains by dideoxynucleotide limited primer extension analysis. Measurement of HMW kininogen by radioimmunoassay was performed in liver subcellular fractions. It reveals that, in contrast to its absence in the cytosolic fraction, HMW kininogen in deficients rats is slightly more abundant in the microsomal fraction, than in control rats. These observations exclude both an abnormality at the level of gene transcription and a major structural modification of the transcribed RNA and of the synthesized HMW kininogen. They favour the hypothesis of an abnormal intracellular transport of the HMW kininogen in deficient rats.

Northern blot; Subcellular fractionation; Primer extension; High-molecular-mass kininogen; RIA; Prekallikrein

1. INTRODUCTION

Kininogens are multifunctional multidomain proteins containing the sequence of bradykinin in their molecule. They are synthesized in the liver and circulate in plasma.

In most mammals, there are two different forms of kininogen: the high-molecular-mass (HMW) kininogen and the low-molecular-mass (LMW) kininogen, which differ in molecular size, sensitivity to kallikreins and physiological functions [1].

The HMW and LMW kininogens are single chain glycoproteins, consisting of three functional domains: an amino-terminal domain, called 'heavy chain', the bradykinin moiety released during proteolysis by kallikreins and a carboxyl-terminal domain, called 'light chain'. This last domain is of a reduced size in LMW kininogen, but in HMW kininogen it represents a large polypeptide chain responsible for the coagulation promoting activity of this molecule. The first and third domains are bound by a disulfide bridge.

HMW and LMW kininogen mRNAs in the rat are identical in nucleotide sequence up to the first 35 nucleotides residues of the light chains, after which their sequences differ completely. In fact, they are encoded from a single gene, the K kininogen gene bearing 11 exons. Part of the 10th and the 11th exons code for the specific sequences of HMW and LMW kininogen mRNAs, respectively [2]. Differential use of the two 3′-terminal exons is responsible for the generation of the two forms of kininogen mRNAs through alternative splicing of the gene transcript [12].

In the rat, in addition to HMW and LMW

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kininogens, there are two other low-molecular-mass kininogen species: T-kininogen I and II, which are not substrates for kallikreins. The T-I and T-II kininogen mRNAs are highly homologous to LMW kininogen (around 90%) [3]. They are generated from two other closely related but distinct genes (T-I and T-II kininogen genes).

Rare inherited deficiencies in HMW kininogen have been reported in man, associated with prolonged partial thromboplastic time and defective release in vitro of plasma kinins [4–6]. In the rat, a Brown Norway substrain (BN/Kat), bred in the Katholiek University of Leuven (Belgium), has been reported to be congenitally deficient in plasma HMW kininogen and unable to release kinins when treated with contact activating agents [7]. These animals have undetectable levels of immunoreactive HMW kininogen by crossed immunoelectrophoresis and their HMW kininogen concentration determined by a direct radioimmunoassay is less than 2% of the normal strain [8,18]. Like humans with HMW kininogen deficiency, these rats display coagulation abnormalities. They also exhibit reduced reactivity to inflammatory stimuli. Finally, they have been reported to also show a reduced plasma prekallikrein concentration [9].

In the present study, we analyzed transcription products of HMW kininogen, LMW kininogen and prekallikrein genes in BN/Kat compared with the control strain BN/Orl. Unexpectedly, HMW kininogen mRNA was detected at normal levels in the deficient rats. Immunoreactive HMW kininogen was studied in plasma and in liver subcellular fractions by direct radioimmunoassay (RIA).

2. MATERIALS AND METHODS

2.1. *Animals*

The two substrains of Brown Norway rats used were bred in our laboratory [8]: BN/Kat, deficient Brown Norway rats, from animals kindly supplied by Dr J. Damas, and originating from the inbred substrain of the Katholiek University of Leuven (Belgium) [7]; BN/Orl, normal Brown Norway rats, from a substrain originating in Orleans (France). Eight-week-old female rats were used.

2.2. *Northern blot*

2.2.1. RNA preparation

Livers were removed from anesthetized rats, immediately frozen in liquid nitrogen, and then stored at −70°C. Total RNA was extracted by the guanidine-cesium chloride method [10]. Total RNA concentration was determined by measurement of optical density at 260 nm.

2.2.2. Oligonucleotide and cDNA probes

Two different oligonucleotides were synthesized (fig.1): LK is a 38-mer complementary to nucleotides 1194–1231 common to the three low-molecular-mass kininogen mRNA species (K, T_1, T_2) [11]. HK is a 50-mer, corresponding to nucleotides 1848–1899 [12] of the HMW kininogen mRNA and is specific to this RNA species. The LK oligonucleotide was radioactively labelled at its 5′-ends with [γ-^{32}P]ATP using T_4 polynucleotide kinase. A DNA complementary to HK oligonucleotide was synthesized with [α-^{32}P]dCTP and the Klenow enzyme, using a 12-mer complementary to the 3′-end of HK as a primer. Rat liver prekallikrein cDNA (Seidah et al., in preparation) was labelled using the random primer DNA labelling reaction [13] and [α-^{32}P]dCTP.

2.2.3. Northern blot analysis

Northern blots were performed with glyoxalated RNA, according to the method of P. Thomas [14]. Hybridization of the blots with the different ^{32}P-labelled kininogen oligonucleotide and kallikrein cDNA probes was done at 42°C in 50% formamide for 24 h. The blots were then washed at 50°C in 0.1 × SSC, 0.1% $NaDodSO_4$ (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate, pH 7.0).

RNA transfer was checked by hybridization with a rat actin cDNA fragment.

2.3. *Dideoxynucleotide limited primer extension analysis*

In this procedure, in order to specifically distinguish between highly homologous LMW and T-kininogen mRNAs, we took advantage of base differences existing in the different transcripts for kininogens upstream from the sequence complementary to the LK oligonucleotide. Poly(A) mRNA (10 μg) was hybridized with 9×10^7 cpm of the labelled LK primer (specific activity: 4×10^7 cpm/μg). Primer extension was performed according to L.J. Field [15], using 29 units of AMV reverse transcriptase (Stehlin, Basel, Switzerland) and dideoxyATP. Samples were analyzed on 6% acrylamide/7 M urea sequencing gel.

2.4. *Western blot*

Plasma proteins were analyzed after reduction (5% 2-mercaptoethanol) and denaturation (3 min boiling) by electrophoresis in 8% polyacrylamide gel containing $NaDodSO_4$, according to the method of Laemmli [16]. Proteins were then transferred electrophoretically to nitrocellulose [17]. The blot was incubated for 12 h at 4°C with an anti-HMW kininogen antiserum [18] and then for 30 min at room temperature with a goat antirabbit immunoglobulin antibody conjugated to alkaline-phosphatase, and stained using the Protoblot system (Promega-Biotec, Madison, USA).

2.5. *Tissue fractionation*

Three BN/Orl and three BN/Kat rats were fasted for 12 h before the experiment. After collecting blood under ether anesthesia, livers were perfused with 80 ml of 0.9% NaCl, removed and immersed in a homogenization solution (20 mM Tris, pH 7.5/5 mM $MgCl_2$/0.25 M saccharose) containing a

protease inhibitor mixture (10 mg/ml phenylmethanesulfonyl fluoride, 1000 U/ml Trasylol and 10 mg/ml pepstatin A). Tissue fractionation studies were performed according to the procedure of De Duve [19]. Three fractions were separated: whole tissue homogenate, microsomal fraction and final supernatant (cytosoluble fraction). After Potter homogenization (2000 rpm), the homogenate was centrifuged at 13300 × *g* at 4°C for 15 min and the pellet discarded. After taking an aliquot (homogenate fraction), the supernatant was centrifuged again at 100000 × *g* for 1 h and collected (cytosoluble fraction). The pellet was then resuspended in the same buffer and submitted to an identical centrifugation. The final pellet (microsomal fraction) was resuspended in 20 mM Tris, pH 7.5/5 mM EDTA with the protease inhibitor mixture. After adding Chaps to 0.1% in order to solubilize proteins trapped in the membrane, all samples were stored at −80°C, until assayed. HMW kininogen was measured by RIA in all these fractions and in plasma. Protein content of all 3 fractions was quantified by the method of Lowry [20] using BSA as a standard.

2.6. *Radioimmunoassay*

The HMW kininogen was assayed by direct RIA, as previously described [18]. Antibodies raised against HMW kininogen did not display detectable levels of cross-reactivity with T kininogens in this RIA [18].

3. RESULTS AND DISCUSSION

The deficiency in HMW kininogen in BN/Kat is due to a genetic abnormality which may be located on either the regulatory or the structural part of the gene. Northern blot experiments of normal and deficient BN rats revealed the presence of HMW and low-molecular-mass kininogen mRNAs in the two strains of rats (fig.1). The HK synthetic probe, which corresponds to a sequence located inside the light chain specific to the HMW kininogen, hybridized with a single 2.2 kb RNA species, corresponding to that described by Kageyama et al. [21] for rat HMW kininogen. The HMW kininogen mRNA in both BN/Orl and BN/Kat has a similar size and is detected in similar amount. The LK probe, which has a sequence common to the three low-molecular-mass kininogen mRNA species but not present in HMW kininogen mRNA hybridized with a mRNA of lower size (1.6 kb) corresponding to the three low-molecular-mass kininogen mRNA species, T_1, T_2 and LMW. The band corresponding to these RNAs is equally present on Northern blot in the two BN strains.

The lack of regions showing sufficient base differences between the light chains of T kininogens and LMW kininogen gene transcripts precluded the preparation of gene specific hybridization probes, thus we used the dideoxynucleotide limited

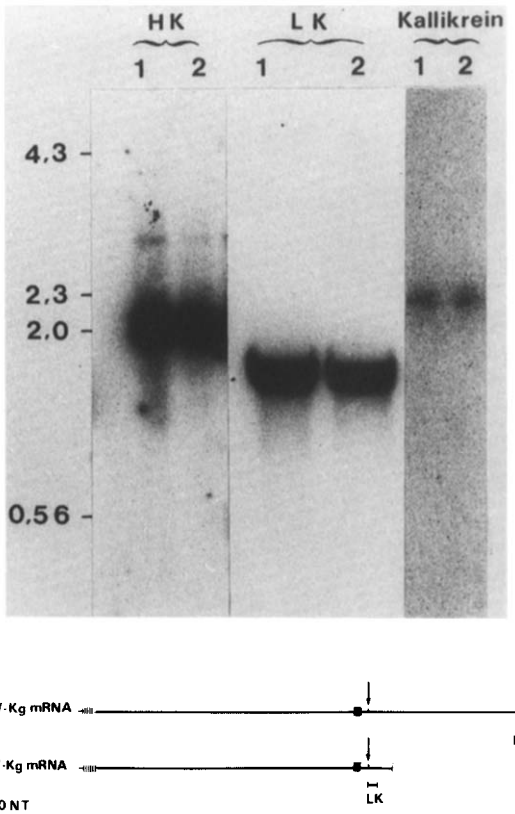

Fig.1. (Upper panel) Northern blot hybridization of 20 μg total liver RNA with HK, LK and prekallikrein cDNA probes. (Lower panel) Schematic localization of the oligonucleotide probes: HK probe (50 bases) and LK probe (38 bases) are indicated on the structure of HMW and LMW kininogen (Kg) mRNA, respectively. Hatched and solid boxes represent the signal peptide and the bradykinin sequence, respectively. The arrows indicate the beginning of the light chain of both kininogens.

primer extension analysis to investigate the specific presence of LMW kininogen mRNA. A base pair difference is located six bases upstream from the 3′-end of the LK primer on the mRNAs, with an adenine residue in the two T kininogen transcripts whereas there is a uracyl residue in the LMW kininogen transcript. The next uracyl residue is located 9 bases further upstream and is present in all three transcripts (see fig.2). The results of primer extension are shown in fig.2. A 44-base and a 53-base fragment were generated in the two strains showing that both the T kininogens and the LMW kininogen mRNAs were present in BN/Kat and BN/Orl rats.

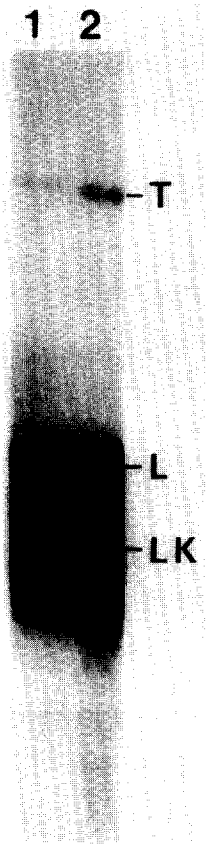

Fig.2. Dideoxynucleotide limited primer extension analysis of LMW kininogen transcripts of BN/Kat (1) and BN/Orl (2) rats. A sequence reaction was used as size marker. LK represents the non-extended 38 base oligomer used as primer, L band (44 bases) and T band (53 bases) correspond to fragments generated during primer extension by LMW and T kininogen mRNAs, respectively.

Damas previously reported that BN/Kat rats were also deficient in plasma prekallikrein [7] but later observed that their prekallikrein plasma levels were 25–30% of normal rats [9]. A deficiency in circulating prekallikrein could affect the stability of kininogen in plasma, as prekallikrein and HMW kininogen together form a circulating complex. We have investigated the presence of prekallikrein mRNA in the liver of the two BN strains by Northern hybridization using a rat prekallikrein cDNA. Fig.1 shows that a prekallikrein transcript is present in the BN/Kat and the BN/Orl strains, and has the same size and abundance in both strains.

Thus, in agreement with a recent study published by Fung et al. [22] at the time this work was being completed, transcription of HMW kininogen mRNA occurs in the BN deficient rats as well as in the non-deficient BN animals. This observation excludes the occurrence of an abnormality in the level of transcription as well as a major structural modification of the transcribed RNA. The LMW kininogen and liver kallikrein transcripts are also generated normally in both strains. These results prompted us to investigate further the presence and the structure of the HMW kininogen mRNA translation product.

The Western-blot technique was used to detect kininogen in plasma using a rat HMW kininogen antiserum. In BN/Orl plasma samples (fig.3), a major band, 120 kDa in size, was present, corresponding to the HMW kininogen. BN/Kat deficiency in plasma HMW kininogen was confirmed, although a very weak HMW kininogen band was detected.

HMW kininogen content of plasma and liver extracts was also measured by RIA (table 1). In contrast to the almost complete absence of immunoreactive HMW kininogen in plasma of BN/Kat, the HMW kininogen content in whole liver homogenate of BN/Kat was two-thirds that found in BN/Orl liver homogenate. We found that the RIA dilution curves of liver HMW kininogen was parallel to the standard curve obtained with pure Wistar rat HMW kininogen in both species (not shown). Therefore, the liver HMW kininogen appeared immunologically identical in BN/Kat rat to the HMW kininogen of other rats.

HMW kininogen content was also measured in microsomal and cytosolic fractions. In microsomes, HMW kininogen content was slightly higher in BN/Kat than in BN/Orl. However, HMW kininogen does not seem to accumulate in this compartment in either strain, since no enrichment is observed in comparison to the whole cellular homogenate. These observations, together with that of the presence of a lower amount of HMW kininogen in the cytosoluble fraction of BN/Kat rats in comparison to BN/Orl animals (table 1), is in favour of an abnormal intracellular traffic of the HMW kininogen in BN/Kat rats.

The presence of a very low amount of immunoreactive HMW kininogen in BN/Kat plasma, by RIA, confirmed by the result of Western blot,

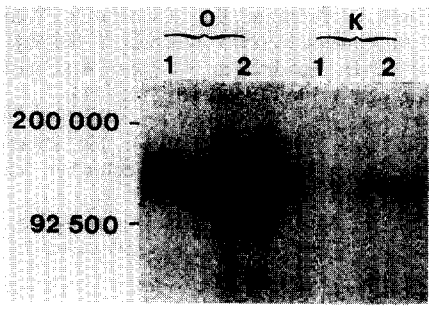

Fig.3. Western blot analysis of plasma HMW kininogen. A HMW kininogen antiserum was used for immunodetection. Two plasma concentrations of BN/Orl (O) and of BN/Kat (K) rats were applied on the gel (lane 1: 80 μg, lane 2: 400 μg protein).

strengthens the hypothesis that HMW kininogen deficiency is more likely due to an altered exportation than to a major structural modification of the protein.

Specific measurement of LMW kininogen in rat plasma is not possible due to the lack of specific antibodies to this kininogen species. A previous study reported that the BN/Kat rats were deficient in LMW kininogen [23]. However, results obtained in this laboratory indicate that the plasma of these animals is able to generate bradykinin upon treatment with rat urinary kallikrein and therefore contains a kallikrein sensitive kininogen. Moreover, the BN/Kat rats excrete normal levels of kinins in urine. These kinins most likely originating from a kallikrein sensitive kininogen detected in urine also in normal animals (Baussant, T. et al., in preparation). This kininogen is probably the LMW kininogen. These observations taken together suggest that the kininogen gene encoding for HMW and LMW kininogen could present a structural abnormality located after the region coding for bradykinin. The gene defect could be tentatively localized to the HMW kininogen specific exon 10. As this exon is also implicated in the formation of the complex with plasma prekallikrein, the observed partial deficiency in plasma prekallikrein could be due, at least in part, to the reduced stability in the circulation of the prekallikrein molecule not complexed to HMW kininogen.

Table 1

Liver tissue fractionation study: immunoreactive HMW-kininogen in subcellular fractions and plasma of normal (BN/Orl) and deficient (BN/Kat) Brown Norway rats

		HMW kininogen concentrations	
	Rat number	BN/Orl	BN/Kat
Homogenate	1	114.0	82.0
(ng/mg prot.)	2	136.9	82.9
	3	109.5	87.2
Microsomes	1	49.7	96.2
(ng/mg prot.)	2	96.3	100.0
	3	52.5	96.2
Cytosol	1	92.4	8.5
(ng/mg prot.)	2	111.8	7.7
	3	103.3	7.8
Plasma	1	51	1.8
(mg/ml)	2	46	1.5
	3	46	1.7

Values are expressed as ng/mg protein in each subfraction. Results obtained for 3 different animals in each substrain are given (rat number is indicated for each value)

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