Inter-alpha-trypsin-inhibitor (ITI): two distinct mRNAs in baboon liver argue for a discrete synthesis of ITI and ITI derivatives

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Human serum inter-alpha-trypsin-inhibitor (ITI) has so far been assumed to be comprised of a single polypeptide chain which can undergo fragmentation, whereby inhibitory ITI derivatives are released into the blood stream. In contrast, the analysis of the baboon liver mRNA translation products showed that ITI is made up of heavy and light chain(s). The latter may be excreted independently and very likely corresponds to the so-called ITI derivatives.

Inter-alpha-trypsin-inhibitor *Serum protease inhibitor* *mRNA translation* *Protein structure* *Baboon*

1. INTRODUCTION

Inter-alpha-trypsin-inhibitor (ITI) is a serine protease inhibitor present in human serum as a 180-kDa glycoprotein which consists of a single polypeptide chain and 8.5% carbohydrates [1,2]. A partial (14-kDa) amino acid sequence of ITI, termed HI-14, has been published; it corresponds to the trypsin inhibitory active part of the molecule which displays two tandem Kunitz-type domains of similar sequence (3). The primary sequence of ITI outside HI-14 is not known. Physiological ITI-related molecules of smaller M_r (ITI derivatives) have been found in body fluids such as serum, bronchial mucus and urine [4–7]; they display immunological cross-reactivity with 180-kDa ITI. In particular, a 30-kDa molecule has been purified from human serum and urine; it is referred to as HI-30 and is basically composed of HI-14 and carbohydrates [8]. An outstanding feature of ITI is its possible ability to act as pro-inhibitor: as yet, 180-kDa ITI has been commonly considered a carrier which releases ITI derivatives into the blood stream [6].

We here report the characterization of the translation products from two distinct mRNAs coding for ITI which both originate from baboon liver. These results provide new clues on the primary structure of ITI and argue against the 180-kDa ITI being a pro-inhibitor; rather, the so-called ITI derivatives may be excreted independently after synthesis by a single mRNA population.

Abbreviations: ITI, inter-alpha-trypsin inhibitor; HI-14/HI-30, human ITI C-terminal (14/30 kDa) inhibitory active parts; UID, urinary ITI derivative; NHS, normal human serum; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CIE, crossed immunoelectrophoresis

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2. MATERIALS AND METHODS

2.1. *Proteins and antisera*

Highly purified human 180-kDa ITI devoid of derivatives and ITI-free human serum were prepared as in [9]. A urinary ITI derivative (UID) of M_r 43 000 has been purified from normal human

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urine and previously characterized; it displays considerable immunological cross-reactivity with purified HI-30 [10]. Anti-total ITI antiserum was made available [11]; it reacted with 180-kDa ITI in serum and ITT derivatives in serum and urine. Anti-180-kDa ITI and anti-UID antisera were prepared and characterized as in [10]; the former reacted with the 180-kDa ITI exclusively, whereas the latter reacted strondly with UID and was also able to bind 180-kDa ITI at least by virtue of its HI-14 domain.

2.2. *Isolation of mRNAs*

Liquid nitrogen-stored liver tissue was first pulverized in nitrogen and then homogenized in a tissue grinder (Duall Kontes) with Tris buffer (200 mM Tris-HCl, pH 8.5, 250 mM sucrose, 25 mM $MgCl_2$, 50 mM KCl, 5 mM beta-mercaptoethanol, 0.5 mg/ml heparin, 4 mg/ml yeast tRNAs) at a ratio of 3 ml buffer/g tissue and then adjusted to 1% Na deoxycholate and 1% Triton X-100. The homogenate was centrifuged (5000 rev-/min, 15 min, 4°C, rotor SW27 Beckman); the post mitochondrial supernatant was diluted (1:1) with 0.1 M Na acetate buffer (pH 5.0), 1% SDS, and RNAs were obtained by phenol-chloroform extraction. Finally, poly(A)-RNAs were purified by 2 successive oligo(dT)cellulose (Collaborative research) chromatographies [12] of ethanol-concentrated RNAs. Fractionation of poly(A)-RNAs by sucrose gradient centrifugation was accomplished as in [13].

2.3. *Cell-free translation system*

mRNA-Dependent rabbit reticulocyte lysates were prepared as in [14]. Translation of baboon mRNAs in the presence of [^{35}S]methionine (Amersham) and radioactivity counting of the trichloroacetic acid-precipitable translation products have been detailed in [15].

2.4. *Immunoprecipitation on translation products and inhibition*

This was performed as in [15]. Briefly, 15–20 μl lysate were added with 5 μl antiserum supplemented or not with 10 μl inhibitor (nomal human serum (NHS), or purified proteins, see section 3); excess (20 μl) protein A–Sepharose (Pharmacia) was added and the mixture was incubated overnight at 4°C. The protein A-bound immune complexes were finaly eluted and further analyzed [15].

2.5. *Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)*

Total translation and immunoprecipitated products were analyzed by SDS-PAGE [16] in discontinuous polyacrylamide gel slabs (stacking gel 5%, 2 cm long; cathodic separating gel 5%, 10 cm long; anodic separating gel 15%, 20 cm long). [^{14}C]M_r markers were obtained from Amersham. Gel slabs were treated for fluorography [17]. Autoradiography was made on Fuji X-ray films for 3–10 days at −80°C.

3. RESULTS

3.1. *Immunochemical comparison of baboon and human ITIs*

When analyzed by crossed immunoelectrophoresis with the 3 anti-ITI antisera mentioned in section 2, baboon and human sera displayed a major

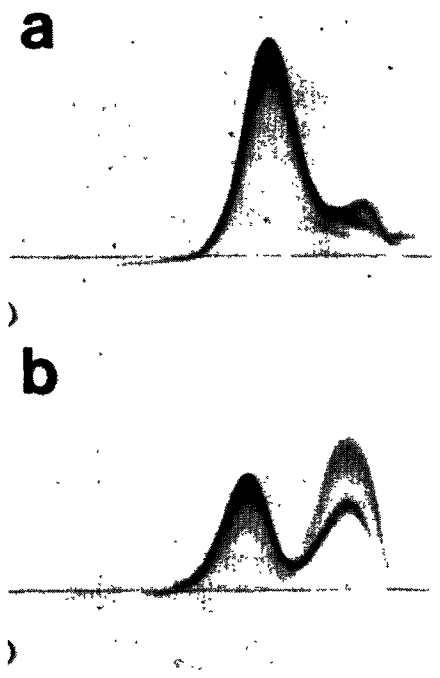

Fig.1. Crossed immuno-electrophoresis (CIE) of human (plate a) and baboon (plate b) sera. CIE was performed at pH 8.6 as in [10] with an anti-total ITI antiserum. 1st dimension: 7 μl serum, anode at right; 2nd dimension: 2 $\mu l \cdot cm^{-2}$ antiserum, anode at the top.

cathodic 180-kDa ITI peak, as in [9]; furthermore, two anodic ITI derivatives in partial immunological identity with the 180-kDa ITI could be seen with anti-total ITI (fig.1) or anti-UID. Baboon and human serum ITIs differed by slower migration of the 180-kDa ITI and higher amounts in ITI derivatives in the former. A similar analysis of urine (not shown) indicated that baboon and human UIDs behaved identically with respect to immunoreactivity and fast electrophoretic migration. Finally, baboon serum ITI and derivatives, purified as in [11], displayed M_r-values (175000 for ITI) which were quite similar to those found for the human counterparts in SDS–PAGE (not shown).

3.2. *Characterization of ITI mRNA translation products*

Poly(A)-RNAs from baboon liver and purified rabbit globin mRNAs stimulated a 15- and 30-fold incorporation of [^{35}S]methionine into trichloroacetic acid precipitable translation products, respectively, as compared with control translation without any exogenous mRNAs.

Immunoprecipitation of the translation products from baboon liver mRNAs were analyzed with 3 distinct anti-ITI antisera (fig.2); in all 3 instances, inhibition of immunoprecipitation was performed with the same panel of human reagents (NHS; ITI-free serum; UID; 180-kDa ITI). Three polypeptide chains of 95, 90 and 40 kDa, respec-

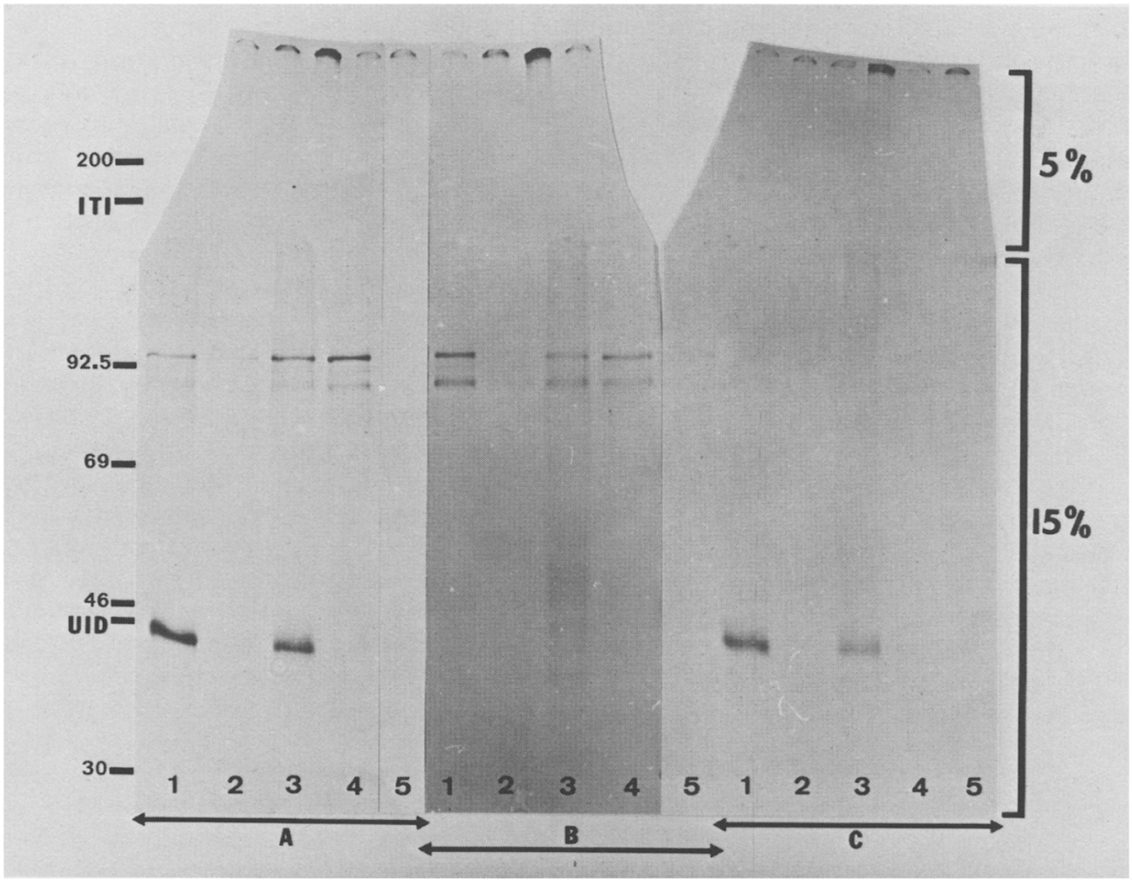

Fig.2. SDS–PAGE autoradiography of immunoprecipitated translation products from baboon liver mRNAs. Antisera: A, anti-total ITI antiserum; B, anti-180-kDa ITI antiserum; C, anti-UID antiserum. Inhibitors: 1, none; 2, NHS; 3, ITI-free serum; 4, human UID; 5, human 180-kDa ITI. Left scale, M_r markers (kDa); right scale, acrylamide %. Anode at the bottom.

tively, were detected with an anti-total ITI antiserum. The two heaviest chains were bound by the anti-180-kDa ITI antiserum and not the anti-UID antiserum; this reaction could only be inhibited by the 180-kDa ITI (or NHS). In contrast, the 40-kDa polypeptide chains was bound by anti-UID antiserum, this reaction being inhibited by UID and 180-kDA ITI, whereas anti-180 kDa ITI antiserum did not react with this polypeptide chain. With all 3 anti-ITI antisera, NHS abolished the immune precipitation whereas ITI-free serum did not; this argues that all immune precipitations are strictly ITI-specific.

The 95-, 90- and 40-kDa polypeptide chains corresponded to 11, 1 and 88% of the radioactivity immunoprecipitated by anti-total ITI antiserum, respectively as measured by densitometry (not shown). When separating baboon liver mRNAs by centrifugation on a 5–25% sucrose gradient and immunoprecipitating the translation products from each fraction with an anti-total ITI antiserum, two populations of mRNAs emerged (fig.3); mRNAs coding for the 40-kDa polypeptide sedimented more slowly than 18 S rRNAs, whereas mRNAs coding for 95- and 90-kDa polypeptides were located near the bottom of the gradient.

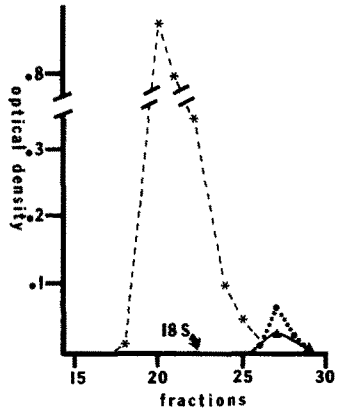

Fig.3. Densitometric analysis of autoradiographies after SDS-PAGE of the translation products from two ITI encoding mRNAs separated by centrifugation on a 5–25% sucrose gradient (bottom at right). (··●··) 95-kDa polypeptide band. (—▲—) 90-kDa polypeptide band. (– –*– –) 40-kDa polypeptide band.

4. DISCUSSION

On biochemical and immunochemical grounds, the strong similarity observed between human and baboon ITI and ITI derivatives prompted us to analyze the mRNA-dependent translation products from baboon liver to seek further insights on human ITI biochemistry and physiology.

ITI is described as a single polypeptide chain with 8.5% carbohydrates [1,2]. As mRNA translation yields unglycosylated proteins, we expected ITI to be translated as a single chain of about 160 kDa. This was clearly not the case, as 3 chains of 95, 90 and 40 kDa were obtained instead. That the mRNA purification and translation procedures were not responsible for an artefactual lack of heavier translation products was indicated by the presence of several heavy polypeptides (up to 150 kDa) in the 5% gel in SDS-PAGE when analyzing the total translation products (not shown). The 90-kDa polypeptide represented only 1% of the ITI-related translation products. This minor component may originate from cleaved and/or poorly translated mRNA, or represent a final product from the 95-kDa protein considered a precursor; it will not be discussed further.

The light and major heavy ITI polypeptide chains are clearly distinct: they are coded for by two different mRNA populations and do not cross-react as demonstrated by their unique reactivity with anti-UID and anit-180-kDa ITI antisera, respectively. We can infer that the heavy chain is present in 180-kDa ITI and not in UID, whereas the light chain is present in both UID and 180-kDa ITI, since the latter inhibited the binding of anti-UID antiserum to this light chain. On the basis of the large similarity between light chain and UID, the latter containing HI-14 [10], we can infer further that the light chain contains HI-14 sequence; i.e., the inhibitory active part of 180-kDa ITI [3].

The presence of two polypeptide chains with differing immunochemical properties encoded by two discrete mRNAs strongly suggests that ITI is not a single polypeptide chain. We conclude now that the 180-kDa ITI contains both heavy and light chains. Given the 8.5% carbohydrate content and a final M_r of 175000, the ITI molecule is assumed to be tentatively comprised of one heavy (90 kDa) and two light (40 kDa) chains, which is also consis-

tent with the higher synthesis level for the light chain as compared to the heavy one. As yet, the assumption of a single polypeptide chain in the 180-kDa ITI relied upon the behaviour or ITI in dissociating/reducing media, as neither SDS nor beta-mercaptoethanol disrupts the molecule into smaller proteins [1,9]. A urea-SDS-beta-mercaptoethanol mixture is likewise without effect (not detailed). This suggests that very strong inter-chain linkages are involved in the model proposed here; their nature remains to be elucidated. The present model of the 180-kDa ITI contains one or more light chains; i.e., UID-like protein sequences. As UID displays 4 trypsin inhibitory sites [10], this raises the question as to whether several inhibitory sites are present in the 180-kDa ITI molecule; the 1:1 stoichiometry published for 180-kDa ITI and trypsin [18] leads us to consider that some sites would then remain inactive because of steric hindrance or oxidation of amino acids.

UID and serum ITI derivatives do not react with anti-180-kDa ITI anti-serum which binds the 95-kDa polypeptide. Therefore, the latter is found exclusively as a component of the 180-kDA ITI and all ITI derivatives originate from the 40-kDa chain-encoding mRNA population. Derivative heterogeneity in serum may result from polymerization and/or enzyme complexation. This picture argues against the 180-kDa ITI being a pro-inhibitor, unless it is assumed that heavy-chain clearance occurs at a very high rate immediately after ITI fragmentation, which seems unlikely. The observation of an increased level of serum ITI derivatives in inflammation has been attributed by others to accelerated fragmentation of the 180-kDa ITI [3]. In contrast, we recently confirmed the increase in ITI derivative during inflammation but observed no concomitant decrease in the 180-kDa ITI; we then hypothesized a discrete synthesis of the 180-kDa ITI and its derivatives [10]. These results are consistent with such a process as two different mRNAs would enable the cell to synthesize heavy and light chains simultaneously, part of the latter being excreted as such. In this respect, the unbalanced light and heavy chain syntheses observed from in vitro mRNA translation could correspond to the very high levels of ITI-derivatives consistenly found in baboon sera. Finally this study demonstrates that heptocytes synthesize ITI. Analysis of mRNAs obtained from non-hepatic tissues should clarify the origin of pulmonary and urinary ITI-related inhibitors which may be synthesized in loco.

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