Involvement of the three inter-α-trypsin inhibitor (ITI) heavy chains in each member of the serum ITI family

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Abstract Partial cDNAs coding for each of the three human inter-α-trypsin inhibitor (ITI) heavy chains were expressed in a bacterial plasmid system and rabbits were immunised with the fusion peptides obtained. Despite the strong sequence homology of these chains, the antisera turned out to be highly specific in the analysis of corresponding mRNA translation products or partially digested serum ITI. Besides classical serum ITI members, their use in Western blotting made it possible to evidence an H3-related ITI form and a low-amount H1-related HC/bikunin component. The relative levels of ITI family members was further studied in baboon and foetal calf sera.

Key words: Inter-α-trypsin inhibitor; Pre-α-trypsin inhibitor; Recombinant DNA; Hybrid protein; Polyclonal antiserum; Western blotting

1. Introduction

Inter-α-trypsin inhibitor (ITI) is a human plasma proteinase inhibitor with an apparent molecular mass of 220 kDa. Besides its proteinase inhibitory function, little is presently known concerning its actual physiological role, even though it has been recently reported that ITI-related proteins may act as extracellular matrix stabilizing factors [1–3]. Its multichain structure was demonstrated through the identification of two different mRNA populations, one of them coding for heavy chains (H) and the other for a light chain (L) [4]. The peptide sequence of the light chain, deduced from that of the cDNA [5,6], corresponds to the precursor of two tandemly arranged proteins, α1-microglobulin and bikunin (responsible for the protease inhibitory activity), which separate at the time of ITI maturation [7,8]. Three mRNAs coding for distinct heavy-chain peptides, called H1, H2 and H3, were then described [9–11]; they showed highly similar amino acid sequences. Further, the ITI-related proteins are synthesized in the liver by four genes located on three different chromosomes [12].

The purification and N-terminal micro-sequencing of serum ITI-related proteins led to the description of two components [13]: (1) ITI (225 kDa) composed of HC1, HC2 (maturation of H1 and H2 precursors) and bikunin; (2) pre-α-trypsin inhibitor (PαI, 125 kDa) composed of HC3 (maturation of H3) and bikunin. A further one, described as being composed of HC2 + bikunin [13] with a mass close to that of PαI, was recently called inter-α-like inhibitor (IαLI) [14]. As for ITI, these proteins cannot be dissociated into their constituent chains under reducing conditions. The assembly between heavy and light chains has been shown to be mediated through chondroitin sulphate linkage in the ITI molecule [15], an unusual structure in plasma proteins. Indeed, a glycosaminoglycan (GAG) chain carried by bikunin covalently cross-links the two chains in PαI and HC2/bikunin [16,17] and the three chains in ITI [18].

In the present study, we used cDNA fragments corresponding to part of ITI H1, H2 and H3 chains in order to produce immunogenic peptides in a bacterial plasmid expression system. The highly specific polyclonal antibodies obtained allowed a specific analysis of ITI-family members in serum and led to the postulate that each heavy chain contributes to the ITI and HC/bikunin composition.

2. Materials and methods

2.1. Chemicals and materials

The pMALc vector, *E. coli* TB1 strain, amylose resin and anti-MBP antiserum were obtained with the 'Protein fusion and purification system' kit purchased from New England Biolabs. The cDNA inserts were prepared using the Geneclean II kit from BIO 101; restriction enzymes and ligase from GIBCO BRL were used according to the manufacturer's instructions. Isopropyl β-D-thiogalactoside (IPTG), Freund's complete and incomplete adjuvants were from Boehringer and Difco Laboratories. For the antiserum specificity characterization, radiolabeled translation products of human liver mRNAs were obtained using the Translation kit and [^{35}S]methionine from GIBCO BRL and Amersham, respectively. Protein-A Sepharose from Pharmacia was used for immunoprecipitations. The rainbow-coloured protein high-molecular weight markers (14.3–200 kDa) from Amersham were routinely included in the run. For the ITI-related serum protein analysis, chondroitin AC lyase from *Arthrobacter aurescens* (EC 4.2.2.5) was from ICN Biomedicals. Western blot was achieved using nitrocellulose sheets from Shleicher and Schüll with a 0.45-μm pore size. Rabbit anti-[(H + L) ITI], anti-L ITI antisera were prepared as previously described [19]. The goat antirabbit IgG antiserum was from Dako, the horseradish peroxidase antiperoxidase (PAP) complex prepared in rabbit was from ICN and 3,3′-diaminobenzidine tetrahydrochloride (DAB) was purchased from Sigma. The molecular mass standards used (30–212 kDa) were from Pharmacia. All chemicals used were of analytical grade.

2.2. Production of immunogenic ITI-H peptides

The isolation of cDNA fragments was performed from the λgt11 clones named λHuHITI-44 (analogous to λHuHITI-19), λHuHITI-9, λHuHITI-13, originally immunoscreened for the expression of either

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Abbreviations: ITI, inter-α-trypsin inhibitor; PαI, pre-α-trypsin inhibitor; IαLI, inter-α-like inhibitor; H1, H2, H3 and L, heavy- and light-chain precursors of ITI-related proteins; HC1, HC2 and HC3, mature heavy chains of ITI-related proteins; GAG, glycosaminoglycan; MBP, maltose-binding protein; IPTG, isopropyl β-D-thiogalactoside; PAP, peroxidase antiperoxidase; DAB, diaminobenzidine; EGTA, ethyleneglycoltetraacetic acid; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

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ITI H chain [12,20], H1, H2 and H3, respectively. Each fragment was subcloned into the *Eco*RI site of the pMALc expression vector polylinker, in fusion with the *malE* gene-coding for the maltose-binding protein (MBP). The recombinant vectors were used to transfect the TB1 strain of *E. coli* [21]. The transfected cells were grown up to 4×10^8 cells/ml. The expression of the fusion peptides was induced with IPTG, which was added to the culture to a final concentration of 0.3 mM. Cell lysates were prepared in lysis buffer (10 mM di-sodium hydrogenophosphate, 30 mM NaCl, 0.25% (v/v) Tween-20, 10 mM β-mercaptoethanol, 10 mM ethylenediamine-tetraacetic acid, 10 mM ethyleneglycoltetraacetic acid (EGTA), 100 mM phenylmethyl sulphonyl fluoride, pH 7.0) containing 1 mg/ml lysozyme. After one freeze-thaw cycle, NaCl was added to a final concentration of 500 mM and the cellular debris were pelleted by centrifugation at 20,000 rpm for 30 min at 4°C (SW 27 Beckman rotor). The supernatant was diluted 1/5 with column buffer (10 mM sodium phosphate, 30 mM NaCl, 1 mM sodium azide, 10 mM β-mercaptoethanol, 1 mM EGTA, 0.25% (v/v) Tween-20, pH 7.0) and was applied to an amylose resin column. The hybrid proteins linked to the resin were eluted with 10 mM maltose in column buffer. The proteins were concentrated by ultrafiltration on PM 30 membranes (Amicon) and their level was estimated on microtiter plates by the Bradford method [22].

Anti-hybrid protein antibodies were raised in rabbits. Primary immunizations were carried out by intramuscular injections of 1 mg of hybrid protein in Freund's complete adjuvant. Booster injections consisting of 1 mg of hybrid protein in Freund's incomplete adjuvant were given by the same route after 1 month and then every 2 weeks. Blood was taken 1 week after each injection and antisera were stored at −20°C.

2.3. Characterization of anti-MBP-H antisera

The specificity of the anti-MBP-H antisera was investigated by: (1) immunoprecipitation of the translation products of human liver mRNAs obtained in mRNA-dependent rabbit reticulocyte lysates in the presence of [^{35}S]methionine as previously described [4]; (2) analysis by Western blotting of serum proteins submitted to chondroitin AC lyase digestion according to [23], except that 4 mU of enzyme was used.

2.4. SDS-PAGE and Western blotting

Immunoprecipitated products were analysed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) in discontinuous polyacrylamide gels (stacking gel 5%, separating gel 14%) [24]. After electrophoresis and fixing, the gel was soaked in 20% (w/v) 2,5-diphenyloxazole scintillation grade in dimethyl sulphoxide, dried and fluorographed with Kodak XAR films at room temperature for 1–5 days [25].

The ITI-related serum proteins and purified ITI [26] were analysed by SDS-PAGE in ExcelGels (Pharmacia), except that home-made 5.5–15% gradient gels were used, or in discontinuous polyacrylamide gels (stacking gel 5%, separating gel 7.5%). After electrophoretic transfer of polyacrylamide gels to nitrocellulose [27], proteins were revealed by the unlabeled antibody enzyme method [28]: each step was performed for 30 min at room temperature in PBSTM (8 mM Na_2HPO_4, 2 mM KH_2PO_4, 500 mM NaCl, 0.1 g/l merthiolate, 0.05% (v/v) Tween-20, pH 7.0); nitrocellulose sheets were successively incubated in specific antiserum diluted 300-fold (anti-[(H + L) ITI], anti-L ITI) or 100-fold (anti-MBP-H), goat antirabbit IgG antiserum diluted 300-fold and PAP complex diluted 200-fold. Each step was followed by three 10-min washes. Membranes were finally stained using 0.6 g/l DAB and 0.01% (v/v) hydrogen peroxide in PBSTM.

3. Results

3.1. Obtaining of H-specific antisera from bacterial expression of ITI-H cDNAs

The cDNA fragments excised from λgt11 clones (*Eco*RI-*Sma*I for H1, *Eco*RI-*Eco*RI for H2 and *Eco*RI-*Acc*I for H3) corresponded to the central and/or the 3′ end of ITI heavy-chain mRNAs. Their respective sizes were 1163, 1190 and 1634 bp and they coded for three peptides with a theoretical mass of 42,146, 43,731 and 61,136 Da. With respect to the mature heavy chains (HC), they contained the 138, 320 and 304 C-terminal amino acids, respectively. At their 5′ end, all these inserts included an *Eco*RI restriction site present either in the cDNA (H1) or in the form of an artificial cloning site (H2 and H3). Such a site being in the same reading frame in pMALc and λgt11 vectors, proteins fused with the *malE* gene product (MBP) could be directly obtained. These fusion proteins could be further purified as a result of the affinity of the carrier protein (MBP) for an amylose resin. When studied in SDS-PAGE, they turned out to be heterogeneous with masses ranging from 42 kDa (MBP alone) to 105 kDa (MBP-H3). Western blotting and probing with an anti-[(H + L) ITI] antiserum only evidenced the heaviest fusion proteins, 84 kDa for MBP-H1, 85 kDa for MBP-H2 and 105 kDa for MBP-H3 (results not shown).

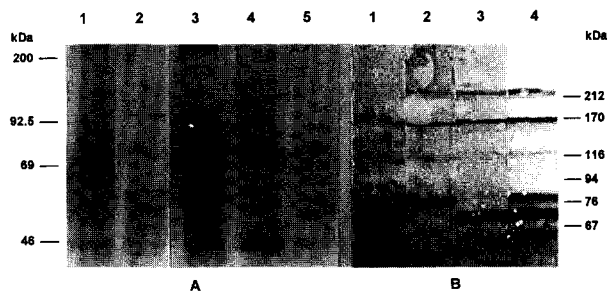

Fig. 1. Analysis of anti-MBP-H antisera. (A) Immunoprecipitations of translation products from human liver mRNAs characterized by SDS-PAGE (14% acrylamide) and autoradiography. Immunoprecipitation was performed with anti-MBP-H1 (lane 1), anti-MBP-H2 (lane 2), anti-MBP-H3 (lane 3), anti-[(H + L) ITI] antiserum (lane 4) and non-immune rabbit serum (lane 5). (B) Human serum submitted to limited digestion with chondroitin AC lyase was analysed by SDS-PAGE (ExcelGel 5.5–15% gradient), electrotransferred to nitrocellulose and probed with anti-MBP-H3 (lane 1), anti-MBP-H2 (lane 2), anti-MBP-H1 (lane 3) and anti-[H + L] ITI (lane 4) antisera. The scales correspond to molecular weight standards. Cathode at the top.

After immunization of rabbits with the concentrated fusion protein preparations (1–2 mg/ml), the polyclonal antisera obtained were analysed by immunoprecipitation of the translation products from human liver mRNAs (Fig. 1A). As previously described [12], the anti-[(H + L) ITI] antiserum revealed three heavy chains H1 (92 kDa), H2 (98 kDa), H3 (107 kDa) and one light chain (45 kDa) (Fig. 1A, lane 4). Immunoprecipitation of the same products with anti-MBP-H1, anti-MBP-H2 and anti-MBP-H3 specifically detected H1, H2 and H3, respectively. Similar results were obtained with translation products from baboon liver mRNAs. No reaction was observed with a non-immunised rabbit serum (Fig. 1A, lane 5) or an anti-MBP antiserum (result not shown).

Serum submitted to limited digestion by chondroitin AC lyase, a procedure known to give rise to free ITI heavy chains [23], was analysed by SDS-PAGE and Western blotting with anti-ITI antisera. As shown in Fig. 1B, anti-[(H + L) ITI] antiserum detected four bands corresponding to native undigested ITI (220 kDa), an intermediary product (170 kDa) and two bands between 70 and 85 kDa. Each anti-MBP-H antiserum evidenced a particular free H form with respective masses of 72 kDa (anti-MBP-H1), 84 kDa (anti-MBP-H2) and 75 kDa (anti-MBP-H3).

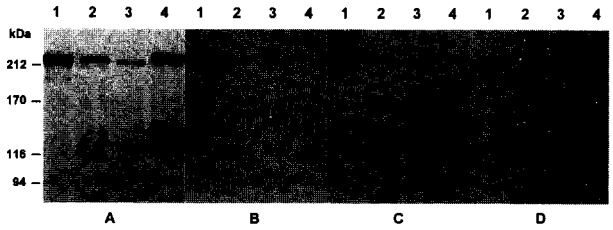

Fig. 2. Western blot analysis of serum ITI-related proteins. Purified human ITI (lane 1), human (lane 2), baboon (lane 3) or foetal calf serum (lane 4) were analysed by SDS-PAGE (7.5% acrylamide), electrotransferred to nitrocellulose and probed with anti-L ITI (A), anti-MBP-H1 (B), anti-MBP-H2 (C) and anti-MBP-H3 (D) antisera. The scale corresponds to molecular weight standards. Cathode at the top.

3.2. *Immunochemical analysis of serum ITI-related proteins*

Human, baboon and foetal calf serum as well as a purified human ITI fraction were analysed by SDS-PAGE followed by electrotransfer on nitrocellulose and immunochemical detection with anti-L ITI and anti-MBP-H antisera (Fig. 2). In human serum, three components (115, 125 and 220 kDa) were revealed with anti-L ITI (Fig. 2A, lane 2). The 115–125-kDa proteins reacted with anti-MBP-H2 and anti-MBP-H3 (Fig. 2C,D, lane 2) and occasionally as a faint signal with anti-MBP-H1. The 220-kDa protein gave similar signals with anti-MBP-H1 (Fig. 2B, lane 2) and anti-MBP-H2 (Fig. 2C, lane 2); the reaction with anti-MBP-H3 was weak though present in a concentrated fraction (Fig. 2D, lane 1). Several individual human serum samples were tested and gave identical results (data not shown).

In baboon serum, the results obtained with the same antisera were similar: among the two major forms (120 and 200 kDa) evidenced with anti-L ITI (Fig. 2A, lane 3), the former reacted with anti-MBP-H2 (Fig. 2C, lane 3), and particularly with anti-MBP-H3 (Fig. 2D, lane 3), while the latter was marked by anti-MBP-H1 and anti-MBP-H2 antisera (Fig. 2B,C, lane 3). In foetal calf serum, two major forms (130 and 240 kDa) were evidenced with anti-L ITI (Fig. 2A, lane 4). The 130-kDa form only reacted with anti-MBP-H2 (Fig. 2C, lane 4). The 240-kDa protein was clearly marked by anti-MBP-H2 (Fig. 2C, lane 4) and more weakly by anti-MBP-H3 (Fig. 2D, lane 4) but failed to react with anti-MBP-H1. Elsewhere, an anti-MBP antiserum did not give any immunochemical reaction with human, baboon and foetal calf sera or the ITI preparation (results not shown).

4. Discussion

The proteins of the ITI family are basically composed of four polypeptide chains, H1, H2, H3 and L. After maturation processing, these components are thought to be assembled through carbohydrate cross-links to yield different covalent structures among which only three, ITI (HC1/HC2/bikunin), PαI (HC3/bikunin) and HC2/bikunin, have been characterized [13,16,17]. Since the distribution of heavy chains within the ITI family is not rigorously defined, we chose to produce immunogenic peptides from partial cDNAs of H1, H2 and H3 with a bacterial expression system in order to obtain antisera specifically directed against these chains. The observed masses of the hybrid proteins MBP-H obtained and their reactivity with an anti-[(H + L) ITI] antiserum showed that the reading frame of the inserts was preserved in the recombinant plasmids and led to the synthesis in *E. coli* of an ITI-related peptide domain. The immunization of rabbits with the hybrid proteins MBP-H1, MBP-H2 and MBP-H3 resulted in highly specific H chain polyclonal antisera. As MBP is a strictly bacterial protein, the anti-MBP activity of these new antisera was unlikely to restrict in any way their use for the analysis of human products and this turned out to be true. Despite the high sequence similarity of ITI heavy chains [11], the anti-MBP-H antisera did not show any cross-reaction between them (Fig. 1). Within the framework of the present study, they may, therefore, be considered as anti-H1-, anti-H2- and anti-H3-specific antisera.

The study of ITI-related human serum proteins showed two main groups displaying an immunochemical reactivity with both anti-L (bikunin) and anti-H (heavy chain) antisera. The first one (220 kDa), corresponding to ITI, showed expected reactivities towards the anti-L, H1 and H2 antisera; a further H3 reactivity could be clearly demonstrated in the purified ITI preparation and was occasionally observed in serum as a very weak signal. The second group (115–125 kDa) corresponded to two-chain structures: (1) the PαI (125 kDa), composed of HC3 and bikunin [16]; (2) the HC2/bikunin complex (130 kDa) [17], also called IαLI [14]; and (3) a 115-kDa protein occasionally seen with both anti-L and anti-H1 antisera, therefore, corresponding to an HC1/bikunin form, whose existence has been previously postulated [17].

Baboon serum gave results virtually identical with those of human serum, except that the PαI/IαLI forms (120 kDa) were primarily marked by the anti-H3 antiserum and the observed size of baboon ITI was smaller, in keeping with earlier findings [5,12]. Contrasting results were obtained in foetal calf serum: besides a slightly higher molecular mass, ITI only displayed L, H2 and H3 reactivities and the 120-kDa protein group was restricted to HC2/bikunin. Despite the low reactivity of our antisera towards bovine proteins, these results are in total agreement with data obtained by protein-sequencing [29] where ITI (236 kDa) was composed of H2 and H3 heavy chains linked to bikunin and a 126-kDa form involved one H2 heavy chain + bikunin; the latter complex would correspond to ESF, the extracellular matrix stabilizing factor previously reported [1].

In the originally described composition of ITI [13] as well as in a further study [23], chemical treatment and/or enzymatic digestion of ITI were used before the identification of its components by N-terminal-sequencing; the number and nature of chains involved in the native molecule could, therefore, not be accurately determined. Indeed, several elements challenge the commonly accepted composition of ITI, viz. only two heavy chains (HC1 and HC2) + bikunin: (1) in foetal calf serum, ITI is devoid of the HC1 chain and composed of HC2, HC3 and bikunin chains as herein described in agreement with the protein-sequencing results [29]; (2) the H3 chain is found in human serum ITI, although in very low amounts, thus explaining why it had not been previously detected by protein-sequencing; (3) acute-phase mediators induce variations in the transcription rates of ITI genes, including opposite effects on ITI H1 and H2 genes [30]; (4) Wisniewski et al. [31] recently purified a serum protein with high affinity for TSG-6, a member of the hyaladherin family; this protein was shown to be an ITI molecule which only displayed H2 and bikunin chains by protein-se-

quencing; furthermore, the same component was found in supernatants of HepG2 cells, which in fact synthesise an ITI form only composed of HC2 and bikunin chains [32]. We, therefore, propose a multichain structure for serum ITI involving two identical or two different heavy chains, with a major involvement of H1 and H2. Given the molecular mass of ITI compared with that of its components (heavy chains, bikunin and the GAG link) and the nature of the ITI-like protein produced by HepG2 cells, native ITI is likely to be composed of two heavy chains linked to two bikunin chains.

Each heavy chain may also associate to bikunin to give two-chain structures corresponding to HC1/bikunin, HC2/bikunin (IαLI) and HC3/bikunin (PαI) complexes, respectively. Since the major form (PαI) corresponds to the H3 chain, as opposed to its low representation among ITI molecules, the ITI-H3 molecule could be a form of low stability giving rise to high levels of PαI. Indeed, the observed differences in the respective serum concentrations of ITI family members may result from a balance between ITI synthesis and metabolic pathways with a low stability of particular components, as demonstrated, for instance, with human leukocyte elastase [33]. In this respect, it is worth noting that HepG2 cells do not synthesise two-chain structures which may only be found after partial digestion of the ITI molecule with chondroitinase [32] although it has been proposed that interleukin 6 induces in HepG2 cells the synthesis of a PαI form whose composition has not been ascertained [34].

The present ITI heavy-chain antisera were shown to be unexpectedly highly specific as further confirmed by the results obtained on foetal calf sera (ITI, HC2/bikunin). They allowed a better understanding of ITI synthesis and maturation in cellular models [32,35] and moreover permitted a very simple analysis of all ITI family members in complex mixtures, thereby suggesting alternatives to their structuring. However, the mechanism responsible for the coexistence of ITI and HC/bikunin forms in serum, i.e. direct synthesis or transition/degradation, has yet to be elucidated.

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