Human inter- α -trypsin inhibitor

Synthesis and maturation in hepatoma HepG2 cells

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In hepatoma HepG2 cells, human inter- α -trypsin inhibitor (ITI) was synthesized as three heavy chains, H-1 (100 kDa), H-2 (110 kDa) and H-3 (113 kDa), and light hybrid chain (49.5 kDa) composed of α_1 -microglobulin and HI-30 (ITI derivative, human inhibitor of 30 kDa). The association of at least two heavy chains, H-1 and H-3, with the HI-30 part of the light chain gave rise to a molecule similar to serum ITI. A composite protein ($\sim$ 250 kDa) including heavy and light chains was also secreted, while α_1 -microglobulin and ITI H-2 protein were released as separate entities. Light chain synthesis could be the limiting factor for ITI maturation.

INTRODUCTION

Human inter- α -trypsin inhibitor (ITI) is one of the plasma protease inhibitors. Since the apparent molecular mass of this glycoprotein is 180 kDa on SDS/polyacrylamide-gel electrophoresis (PAGE), even with strong reducing/dissociating agents, it has long been thought to be a single polypeptide chain with 8.5% carbohydrate [1,2]. Smaller molecules with inhibitory activity are antigenically related to ITI; these so-called ITI derivatives are found in normal plasma, urine and bronchial mucus [3]. We previously reported that ITI is synthesized by separate mRNAs that code for heavy (H) and light (L) chains [4]. The L chain cDNA was isolated and its deduced amino acid sequence corresponded to two proteins: α_1 -microglobulin and the ITI derivative HI-30 (human inhibitor of 30 kDa) [5,6]. Recently, three separate cDNA clone families corresponding to ITI H chains have been isolated and sequenced; they displayed highly similar sequences [7–9] and coded for three H polypeptides named H₁ (92 kDa), H₂ (98 kDa) and H₃ (107 kDa) [9]. The H₃ and H₁ genes are located on chromosome 3p211–212 and the H₂ gene on chromosome 10p15 [9]. Although the number and assembly of H and L chains in the mature ITI molecule remain unclear, it has recently been demonstrated that H and L chains are likely to be cross-linked by a covalent chondroitin sulphate bridge [10].

In the present work, we study the different proteins that cross-react immunochemically with ITI in cultures of human hepatoma HepG2 cells. As intracellular ITI-related proteins are different from those excreted, a maturation process is very likely to take place before ITI secretion.

MATERIALS AND METHODS

Cell cultures and preparation of homogenates

The synthesis of ITI was analysed in human hepatoma

HepG2 cell cultures. These cells were maintained in RPMI 1640 medium (Gibco) containing 10% heat inactivated fetal calf serum plus antibiotics. They were passaged every 8–10 days. Cells were plated in 25 cm² culture flasks until they reached 80–100% confluency [(4–5) $\times$ 10⁶ cells per flask]. The growth medium was then removed and replaced with 2 ml of methionine-deficient medium. After 1 h, 200 μ Ci of [³⁵S]L-methionine (Amersham; 1 mCi/mmol) were added to each flask and incorporation was allowed to proceed for 10 min (pulse). The cells were then washed and chased for various times with 3.35 mM unlabelled methionine in normal medium. After collection of the supernatant medium, the cultures were washed and lysed in 2 ml of 10 mM-Na₂EDTA, pH 7.2, containing 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM-phenylmethanesulphonyl fluoride and 0.15 M-NaCl, and centrifuged for 10 min at 12000 g.

Immunoprecipitations

Rabbit anti-[(H + L) ITI], anti-(H ITI) and anti-(L ITI) antisera were prepared as described in a previous report [11]; rabbit anti- α_1 -microglobulin antiserum was obtained from Accurate Chemical and Scientific Corporation. Lysates and supernatants were immunoprecipitated by various antisera as in [12]. Briefly, 25 μ l of non-immune rabbit serum and excess (100 μ l) Protein A-Sepharose (Pharmacia) were added to 500 μ l of lysate or supernatant and the mixture was incubated overnight at 4 °C. After collection of the liquid phase by centrifugation, 5 μ l of antiserum and new Protein A-Sepharose (30 μ l) were added. After overnight incubation at 4 °C, the Protein A-bound immune complexes were washed ten times in 10 mM-Na/K phosphate buffer, pH 7.0/1 M-NaCl/2% Triton X-100/1% sodium deoxycholate/4 mM-EDTA/2% bovine serum albumin, and then twice in 10 mM-Na/K phosphate buffer, pH 7.0/150 mM-NaCl. Protein desorption and final analysis were performed as in [12].

Abbreviations used: ITI, human inter- α -trypsin inhibitor; PAGE, polyacrylamide-gel electrophoresis; HI-30, human inhibitor of 30 kDa (ITI derivative); H, heavy; L, light.

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SDS/PAGE and Western blot

After immunoprecipitation, translation products, cell lysates and supernatants were analysed in SDS/PAGE (15% gels) followed by autoradiography [13]. Densitometric analysis of the lanes was performed on a Gelman DCD 16 densitometer. Samples of fresh human serum were electrophoresed in the same SDS/PAGE, then transferred to nitrocellulose filters (BA 85, Schleicher and Schüll) according to [14]. Proteins were detected with the unlabelled antibody enzyme method [15].

RESULTS

ITI mRNA translation products

As already described [9], immunoprecipitation of the translation products *in vitro* of human liver poly-(A)⁺ RNAs by anti-[(H + L) ITI] antiserum revealed four different polypeptide chains (Fig. 1a). Three of these corresponded to ITI H chains: H₁ (92 kDa), H₂ (98 kDa) and H₃ (107 kDa). The remaining one, ITI L chain (45 kDa) [4], was composed of two tandemly synthesized proteins: α_1 -microglobulin and HI-30 [6].

ITI-related products observed in hepatoma HepG2 cells

Hepatoma HepG2 cells metabolically labelled with [³⁵S]methionine during a 10 min pulse exhibited multiple forms of ITI-related immunoprecipitable protein. Their complete characterization was hindered by: (i) their variation according to chase times, (ii) their differences in antigenic reactivity, and (iii) the high background level occasionally observed in immunoprecipitates.

Up to seven bands could be immunoprecipitated by anti-[(H + L) ITI] antiserum (Fig. 1b, lane L3). An L protein (49.5 kDa) was observed very early in lysates (Fig. 1b, lanes L1–L3), but not in supernatants (Fig. 1b,

Fig. 1. Analysis of ITI-related proteins by autoradiography of SDS/PAGE

(a) [³⁵S]Methionine-labelled translation products from total human liver mRNAs [4] were immunoprecipitated by anti-[(H + L) ITI] antiserum. (b) Pulse-chase analysis of HepG2 cell culture products was performed on lysates (lanes L) and supernatants (lanes S) after immunoprecipitation by anti-[(H + L) ITI] antiserum. After a 10 min pulse, the chase was conducted during: 0 min (lanes 1), 10 min (lanes 2), 30 min (lanes 3), 1 h (lanes 4), 2 h (lanes 5), 5 h (lanes 6) or 8 h (lanes 7). (c) Immunoblot of total serum proteins after SDS/PAGE was probed by anti-[(H + L) ITI] antiserum. Abbreviations: ITI-like; ITI-like protein; CP; composite protein; 180: 180 kDa protein; H-3, -2, -1; heavy chains; L; light hybrid chain (α_1 -microglobulin/HI-30).

lanes S1–S7). In the group of three proteins in the 100–110 kDa region, the H-1 (100 kDa) band was detected only in lysates (Fig. 1b, lanes L1–L6), while the H-2 (110 kDa) and H-3 (113 kDa) bands were seen in lysates (Fig. 1b, lanes L2–L4) and in supernatants (Fig. 1b, lanes S3–S7). Three heavier components were observed with similar patterns: (i) a protein (180 kDa) restricted to lysates (Fig. 1b, lanes L2–L4); (ii) a protein (~250 kDa) named composite protein (Fig. 1b, lanes L1–L3 and S3–S7) and (iii) a very heavy protein (>300 kDa) (Fig. 1b, lanes L3 and S3–S7) with a picture similar to that of the major band detected after immunoblotting of human serum electrophoresed in the same conditions (Fig. 1c); the latter was therefore called ITI-like protein.

Antigenic properties of hepatoma HepG2 cell products

The bands observed with anti-[(H + L) ITI] antiserum displayed different antigenic reactivities: the L protein was immunoprecipitated by anti-(L ITI) antiserum (Fig. 2, lane L1) while the H-1, H-2 and H-3 proteins were detected with anti-(H ITI) antiserum (Fig. 2, lanes L2 and S2). Conversely, the heaviest bands (composite protein and ITI-like) were immunoprecipitated by anti-(L ITI) as well as anti-(H ITI) antisera. Several other bands could occasionally be observed but were probably artefacts since they were also detected with non-immune rabbit serum (Fig. 2, lanes L0 and S0).

When anti- α_1 -microglobulin antiserum was substituted for anti-[(H + L) ITI] antiserum, two bands only displayed the same pattern: the L protein (Fig. 3, lanes L1–L3) and the composite protein (Fig. 3, lanes S3–S7). A further broad band (α_1 -microglobulin, 32 kDa) was observed after a chase of 30 min (Fig. 3, lanes L3 and S3–S7).

Time-course analysis

The data obtained from the pulse-chase experiment depicted in Figs. 1–3 are summarized in Fig. 4 with respect to time and cellular location of components. The L component (49.5 kDa) was synthesized first, with a maximum at the beginning of the chase (Fig. 4a); it was not detectable after 1 h and could not be observed in

Fig. 2. Immunochemical reactivity of HepG2 cell culture products in SDS/PAGE

Lysates (lanes L) and supernatants (lanes S) of HepG2 cell cultures were immunoprecipitated by non-immune rabbit serum (lanes 0), anti-(L ITI) antiserum (lanes 1) and anti-(H ITI) antiserum (lanes 2). Pulse time was 10 min and chase times were 30 min (lanes L) and 1 h (lanes S). Abbreviations as in the legend to Fig. 1.

Fig. 3. Analysis of α_1 -microglobulin-related proteins by autoradiography of SDS/PAGE

Pulse-chase analysis of HepG2 cell culture products was performed on lysates (lanes L) and supernatants (lanes S) after immunoprecipitation by anti- α_1 -microglobulin antiserum. After a 10 min pulse, the chase was conducted during: 0 min (lanes 1), 10 min (lanes 2), 30 min (lanes 3), 1 h (lanes 4), 2 h (lanes 5), 5 h (lanes 6) or 8 h (lanes 7). Abbreviations: α_1 M, α_1 -microglobulin; otherwise as in the legend to Fig. 1.

Fig. 4. Results of densitometric scanning after pulse-chase analysis of HepG2 cell culture products

After autoradiography of SDS/PAGE, bands were quantified by densitometry and their respective intensity was plotted (ordinate, arbitrary units) according to chase times (abscissa). Bars correspond to lysates and lines to supernatants. (a) Light proteins immunoprecipitated by anti- α_1 -microglobulin or anti-(L ITI) antiserum, (b) heavy proteins immunoprecipitated by anti-(H ITI) antiserum, (c) very heavy and composite proteins immunoprecipitated by anti-(H + L), anti-H or anti-L ITI antiserum, or by all antisera. Abbreviations as in the legends to Figs. 1 and 3.

supernatants. The synthesis of α_1 -microglobulin was slightly delayed, but it was quickly observed primarily in supernatants.

Among the H proteins (Fig. 4b), the H-1 component (100 kDa) was the most prominent; high amounts were already present at the beginning of the chase and it was still detectable after 2 h. All three H proteins reached a maximum at 30 min of chase, but H-2 (110 kDa) and H-3 (113 kDa) could not be observed after 2 h. In supernatants, very low levels of H-1 could be detected, whereas H-2 and H-3 reached a plateau.

The largest molecules (> 300 kDa, ~ 250 kDa) were mainly obtained during the first 60 min of chase, but at levels lower than the H components (Fig. 4c). Their excretion levels reached a maximum between 2 and 5 h, and then decreased slightly.

DISCUSSION

Clear-cut immunochemical and molecular mass differences were observed between the ITI-related proteins synthesized inside the cells and those secreted by cultures of hepatoma HepG2 cells. However, their characterization was hindered, mostly because of non-specific background observed in immunoprecipitates (Fig. 2, lanes L0–S0), despite multiple and stringent washings, as detailed in the Materials and methods section. Several minor bands could easily be considered non-specific because: (i) they were observed with all antisera as well as with non-immune rabbit serum and (ii) their intensity was very low and constant among different lanes. The specificity of our ITI antisera could be questioned but, since their original description [11], they have allowed us to: (i) describe two distinct mRNA families coding for ITI [4], (ii) select and characterize cDNA clones coding for ITI H and L chains [8], and finally (iii) describe one L and three H ITI chains encoded by four genes on separate chromosomes [9]. Furthermore, these results have been confirmed by other groups [6,7] and in particular, the cDNA-deduced amino-acid sequences have proved to match perfectly with serum ITI-derived peptides [7]. Moreover, it must be emphasized that one should be very careful in interpreting the present data since (i) the antigenic reactivity of the proteins under study may differ, depending upon the antiserum used and their associations within one product, and (ii) the radioactive signal obtained is also highly dependent upon the methionine content of the proteins and the sharpness of the bands observed.

Translation products of human liver mRNAs *in vitro* have been characterized as H₁ (92 kDa), H₂ (98 kDa), H₃ (107 kDa) and L (45 kDa) ITI polypeptide chains [9]. The large immunological similarity of these products with HepG2 cell lysate H-1 (100 kDa), H-2 (110 kDa), H-3 (113 kDa) and L (49.5 kDa) proteins suggests a clear relationship between them. In fact, taking an average 10% glycosylation rate from translation products to cell lysate proteins would explain the observed differences in molecular mass.

The L (49.5 kDa) cellular lysate polypeptide chain reacted with anti-(L ITI) as well as with anti- α_1 -microglobulin antisera, which is in complete agreement with the fact that a hybrid protein (α_1 -microglobulin/HI-30) was encoded by the cDNA reported by Kaumeyer *et al.* [6]. As opposed to free HI-30 protein which could be observed neither in lysates nor in supernatants, free

α_1 -microglobulin protein was found in the culture lysate as a broad band; in fact, up to three distinct forms, with molecular masses of 30, 32 and 36 kDa, could be observed on more concentrated gels (results not shown). The present values were slightly higher than those determined in another study [16], but the observed heterogeneity could be attributed to different glycosylation stages, only the 36 kDa protein being present in the culture supernatants.

An intermediate product, the composite protein (~ 250 kDa), was observed at early stages in lysates, just before the appearance of the serum ITI-like molecule. Both proteins were then quickly secreted in the cell medium. However, the composite protein displayed a peculiar antigenic reactivity, being recognized by all antisera used, which led to the conclusion that it was composed of ITI heavy chain(s) and complete light chains, i.e. α_1 -microglobulin HI-30 hybrid molecule(s). Another intermediate product, with ITI-restricted antigenicity, may exist (180 kDa); however, given the faintness of the corresponding band and the existence of similar products with non-immune rabbit serum (Fig. 2, lane L0), it was considered to be an artefact.

The very low level of heavy chain H-1, and the complete absence of light chain and free HI-30 in supernatants, argue for their involvement in the constitution of the heavy (> 300 kDa) ITI-like molecule also observed in serum, whereas free, glycosylated α_1 -microglobulin was released. The H-3 component was also very likely to be involved in the ITI-like protein (> 300 kDa), since it was obtained in constant amount in supernatants and synthesized at a higher level in lysates (Fig. 4b). Conversely, the amounts of H-2 in supernatants gradually increased in a manner roughly parallel to that of the ITI-like protein (> 300 kDa). The time for ITI protein maturation in hepatoma HepG2 cell cultures was about 40 min. At the beginning of the chase (30 min), the L protein (49.5 kDa) decreased considerably in the lysate and did not appear in the supernatant, whereas H-1, H-2 and H-3 increased and the amount of ITI-like protein (> 300 kDa) remained low.

In a detailed study devoted to the characterization of α_1 -microglobulin [16], it was demonstrated that results obtained on hepatoma cell lines were identical to those given by normal liver, thus justifying the use of such cultures. In the present work, the ITI protein could only be obtained within hepatoma cells by association of glycosylated chains, as demonstrated by its absence among products obtained by cell-free translation of normal human liver mRNAs. This suggests that the covalent linkage between ITI H and L chains involving a chondroitin sulphate bridge [10] requires a hepatic factor, or glycosylated chains, or both.

Recently, a 12-amino-acid residue sequence of the ITI N-terminal H chain has been reported, unambiguously identifying two possible residues in positions 9–11 [10]. Such data argue for the presence of two heavy chains with highly similar sequences within the mature ITI molecule. Serum ITI could therefore contain two complete or partial H chains, namely H-3 and H-1, plus one or two light (HI-30) chains covalently linked. A possible scheme for the synthesis and assembly of human ITI would therefore be the following: H-1 and L (α_1 -microglobulin/HI-30 hybrid) proteins are first synthesized and give rise to the composite protein (~ 250 kDa); after the appearance of H-2 and H-3 components

(10 min of chase), the serum ITI-like molecule can be observed as well as free α_1 -microglobulin, possibly released from the α_1 -microglobulin/HI-30 hybrid and/or composite protein (~ 250 kDa). After 30 min of chase, free H-2 and α_1 -microglobulin molecules are secreted, as well as composite and serum-like proteins. A possible serum equivalent to the heavy chain H-2 (110 kDa) and the composite protein (~ 250 kDa) excreted in culture supernatants remains to be identified. Furthermore, we reported that the H₃ and H₁ chains were encoded by genes located on the same chromosome whereas the H₂ chain was encoded by a gene located on a different one [9]. This is in good agreement with common control of the expression of H₃ and H₁ genes, whereas the regulation of the H₂ gene expression would be separate. The present study also argues for L chain synthesis being the limiting factor for maturation of the ITI-like protein (> 300 kDa).

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