Post-translational processing of the inter- α -trypsin inhibitor in the human hepatoma HepG2 cell line

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In human hepatoma HepG2 cells, the serum inter- α -trypsin inhibitor (ITI)-like protein is synthesized from two protein precursors, the heavy chain (H) H2 and the light chain (L). Both of them carry sulphate groups involved in the chondroitin sulphate glycosaminoglycan (GAG) linkage, as demonstrated by [³⁵S]sulphate labelling, chondroitinase digestion and inhibition with β -D-xyloside, an artificial GAG acceptor. While inhibition of N-glycosylation prevented neither the maturation nor the secretion of the ITI-related entities, brefeldin A induced the accumulation of H and L precursors in the cells, therefore blocking subsequent association and maturation of the pre-

cursors before their secretion. The enzyme system involved in the ester linkage between H and L chains is localized in the trans-Golgi network since no ITI-like protein could be obtained in the presence of monensin; instead free heavy-chain protein forms and bikunin were secreted in culture supernatants. The ITI-like protein synthesized by HepG2 cells is therefore composed of two heavy chains HC2 linked to two bikunin chains by chondroitin sulphate bridges, although the GAG linkage between HC2 chains is presumably different. Further, a different maturation route leading to restricted heavy-chain forms, H_m and H_a, could be shown.

INTRODUCTION

Inter- α -trypsin inhibitor (ITI) is a plasma proteinase inhibitor that could act as an extracellular-matrix-stabilizing factor [1,2]. ITI is the major form of a family of proteins synthesized in the liver from four genes located on three different chromosomes [3]: three of them code for the heavy-chain polypeptides H1, H2 and H3 [3–6]; the last one codes for a hybrid polypeptide chain called L [7,8] which is cleaved into α 1-microglobulin and bikunin [9–11]. Mature ITI is composed of three chains HC1, HC2 and bikunin (maturation of H1, H2 and L precursors) cross-linked by chondroitin sulphate (CS) bridges [12,13]. Other plasma proteins related to ITI have also been described: the pre- α -trypsin inhibitor (P α I) [13], composed of HC3 and bikunin, and the HC2–bikunin complex [14–16]. In the latter, Enghild et al. [14,16] showed that a covalent glycosaminoglycan (GAG) bridge linked Ser¹⁰ of bikunin to the C-terminal Asp residue of HC3 or HC2. Indeed, the ITI molecule and ITI-related proteins are cleaved by chondroitinase into HC chains and bikunin [12–19].

The biosynthesis of GAG carbohydrate side chains involves an extensive series of post-translational modifications that occur during the intracellular transport of core proteins from the endoplasmic reticulum (ER) through the Golgi complex. For CS GAGs, the carbohydrate–protein linkage region is initiated by a xylosyltransferase and the sulphation involves 4- or 6-sulphotransferases. β -Xylosides are competitive inhibitors of reactions catalysing the addition of galactose to the xylosylated protein core [20]. The fungal metabolite, brefeldin A, inhibits the elongation and modification of the CS chains, whereas the initiation of the GAG linkage region is not affected [21]. Furthermore, enzymes normally resident in the cis-, medial- and

trans-Golgi are redistributed back to the ER, whereas components of the trans-Golgi network are not [22–24]. The Na⁺ ionophore monensin does not affect protein entry into the Golgi apparatus but inhibits exit from the Golgi complex into the trans-Golgi network [25].

Very little is known about the synthesis and mechanism of addition of the GAG chain to the heavy- and light-chain precursors of ITI. By combined treatments of HepG2 cells with β -D-xyloside, tunicamycin, brefeldin A or monensin, and digestions with chondroitinases, we here describe the post-translational processing of ITI H- and L-chain maturation in the ER, the Golgi complex and the trans-Golgi network, and propose an original molecular structure for the mature ITI-like protein synthesized by these cells.

MATERIALS AND METHODS

Materials

The human hepatoma HepG2 cell line (No. 85011430) was purchased from the European Collection of Animal Cell Cultures. Culture flasks (25 cm²), RPMI 1640 medium and fetal-calf serum, were from Costar and Gibco BRL. PBS, antibiotics and radiolabelled products were from BioMérieux, Boehringer and Amersham respectively. Tunicamycin, brefeldin A and chondroitinase ABC (EC 4.2.2.4) were from Boehringer. Monensin and *p*-nitrophenyl β -D-xylopyranoside were from Sigma, chondroitin AC lyase (EC 4.2.2.5) was from ICN.

Immunoprecipitations were performed using Protein-A–Sephareose from Pharmacia. All chemicals used were of analytical grade.

Abbreviations used: ITI, inter- α -trypsin inhibitor; P α I, pre- α -trypsin inhibitor; H, heavy chains; H1, H2, H3 and L, heavy- and light-chain precursors of ITI-related proteins; HC, mature heavy chains (HC1, HC2 and HC3); bik, bikunin; ER, endoplasmic reticulum; CS, chondroitin sulphate; GAG, glycosaminoglycan; PMSF, phenylmethanesulphonyl fluoride.

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Cell culture and preparation of homogenates

The HepG2 cells were propagated at 37 °C in air/CO₂ (19:1, v/v) in RPMI 1640 medium containing 2 mM glutamine, 1 mM sodium pyruvate, 500 µg/ml streptomycin, 100 units/ml penicillin, 10 % (v/v) fetal-calf serum, and passaged every 8–10 days. The proteins were labelled with [³⁵S]methionine and the homogenates were prepared according to [9]. Briefly, HepG2 cells were cultured until they reached 80–90 % confluence; the medium was then removed and the cells were washed twice in PBS before being placed in 2 ml of medium lacking fetal-calf serum and methionine/flask. After 1 h, 200 µCi of [³⁵S]methionine (1 mCi/mmol) were added to each flask and incorporation was allowed to proceed for 10 min (pulse). The labelling medium was removed and the cells were then washed in PBS and chased in a medium without fetal-calf serum supplemented by 3.35 mM unlabelled methionine. After various intervals, the culture medium containing the secreted labelled proteins was recovered. The cells were rinsed twice with ice-cold PBS, lysed by 2 ml of lysis buffer [100 mM Tris, pH 7.5, 1 % (v/v) Triton X-100, 0.5 % (w/v) sodium deoxycholate, 2 mM phenylmethanesulphonyl fluoride (PMSF) and 2 mM benzamidine] and centrifuged for 10 min at 12000 *g* at 4 °C. Cell lysates and culture media were used directly for immunoprecipitations. For labelling of CS GAG chains, the same protocol was used, except that [³⁵S]methionine was replaced by [³⁵S]sulphate (40 µCi/ml).

Enzymic digestions

For digestion with chondroitinases, culture supernatants obtained after 180 min of chase were used. A sample (1 ml) was incubated overnight at 4 °C in the presence of 50 µl of non-immune rabbit serum and 100 µl of Protein-A-Sepharose. The Protein-A-Sepharose was removed by pelleting and the supernatant was diluted 1:1 with 0.25 M Tris/HCl, 0.18 M sodium acetate, 0.25 M NaCl, 0.02 M EDTA, 0.05 % (v/v) BSA, pH 8.0, for chondroitinase ABC, or with 200 mM Tris/sodium acetate, pH 7.3, for chondroitin AC lyase. The mixtures were then incubated for 2 h at 37 °C with various quantities of enzyme, as described in the Figure legends.

Cultures in the presence of inhibitors

Stock solutions were made of β-D-xyloside (200 mM in dimethylsulphoxide), tunicamycin (0.5 mg/ml in 25 mM NaOH), brefeldin A (2 mg/ml in methanol) and monensin (0.35 mg/ml in water). Before labelling, the cells were preincubated for 1 h with β-D-xyloside 1 and 5 mM, tunicamycin 20 µg/ml (2.5 × 10⁻⁷ M) or monensin 0.35 µg/ml (5 × 10⁻⁷ M), whereas the preincubation with brefeldin A at a concentration of 1 µg/ml (3.5 × 10⁻⁶ M) was maintained for 4 h. The same concentrations were used throughout the labelling period.

Immunoprecipitations and SDS/PAGE analysis

Polyclonal anti-(H + L ITI) and anti-(L ITI) sera were prepared as described [26]. Specific anti-(H1, H2, or H3) sera were obtained by rabbit immunization with corresponding peptides produced in a bacterial expression system. Cell lysates and culture supernatants were immunoprecipitated by various antisera as in [27], then analysed by SDS/PAGE in discontinuous polyacrylamide gels (stacking gel 5 %, resolving gel 14 %) [28], followed by fluorography. The rainbow-coloured protein molecular-mass

markers from Amersham (myosin, 200 kDa; phosphorylase b, 92.5 kDa; BSA, 69 kDa; ovalbumin, 46 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 21.5 kDa; and lysozyme, 14.3 kDa) were routinely included in the run.

RESULTS

We have previously shown [9], through a time-course analysis, that the synthesis and maturation of ITI-related products in HepG2 cells followed two main steps: up to 30 min, precursors are mainly observed in cell lysates; then mature and assembled products are found in supernatants, with a maximum averaging 180 min. For the sake of clarity, although complete pulse-chase experiments were performed in the present study, only representative pictures will be given.

Culture in the presence of [³⁵S]sulphate

Contrary to what was seen with [³⁵S]methionine (Figure 1a, lane 1), only one band corresponding to the ITI-like protein (260 kDa) was obtained in the cell lysate (Figure 1b, lane 1, ITI) after pulse-labelling with [³⁵S]sulphate. With increasing chase times, this band strongly decreased while a very faint signal, corresponding to free light chain (L, 49.5 kDa), and a clear one, heavy chain (H, 100 kDa), became apparent (Figure 1b, lane 3). After 180 min of chase, cell supernatants labelled by [³⁵S]methionine showed the ITI-like protein and two heavy-chain protein forms (H_m, 120–125 kDa), (Figure 1a, lane 4), while only the ITI-like protein was detected by [³⁵S]sulphate label (Figure 1b, lane 4). A 220 kDa band (*) was observed in all supernatants, including after immunoprecipitation with non-immune rabbit serum (results not shown), thus constituting a permanent background noise. Both H- and L-chain precursors were therefore sulphated, as well as the mature ITI-like protein, although the mature heavy-chain forms (H_m) were not.

Treatment of the culture supernatants with chondroitinases

In order to study the chondroitin bridge in the ITI-like protein, HepG2 cell supernatants obtained after a 180-min-long chase were digested with increasing amounts of chondroitinase ABC (Figure 2a) or chondroitin AC lyase (Figure 2b) and immunoprecipitated with anti-(H + L ITI) serum or a mixture of anti-L

Figure 1 Sulphation of ITI-related proteins in HepG2 cell cultures

The cells were pulsed with [³⁵S]methionine (a) or [³⁵S]sulphate (b) for 10 min and chased for: 0 min (lanes 1), 10 min (lanes 2), 30 min (lanes 3) and 180 min (lanes 4). The cell lysates (lanes 1–3) or supernatant (lane 4) were immunoprecipitated by an anti-(H + L ITI) serum. Protein molecular-mass standards are indicated at left and right.

Figure 2 Action of chondroitinases on ITI-related proteins in HepG2 cell culture supernatants

The [35 S]methionine-labelled supernatants of HepG2 cell cultures obtained after 180 min of chase were digested by chondroitinases and immunoprecipitated by an anti-(H + L ITI) serum (a) or a mixture of anti-(L ITI) and anti-(H2 ITI) sera (b). The digestions were performed by addition to 200 μ l of culture medium of 0 munits/ml (control, lanes 1), 3 munits/ml (lanes 2), 30 munits/ml (lanes 3) and 150 munits/ml (lanes 4) of chondroitinase ABC (a) or chondroitin AC lyase (b).

Figure 3 Antigenic specificity of ITI-related proteins observed after chondroitinase digestion

The [35 S]methionine-labelled supernatants of HepG2 cell cultures obtained after 180 min of chase were digested by 30 munits/ml of chondroitinase and immunoprecipitated; the digestions were performed without enzyme (control, lanes 1), with chondroitinase ABC (lanes 2) or with chondroitin AC lyase (lanes 3) and revealed by anti-(H + L ITI) (a), anti-(L ITI) (b) and anti-(H2 ITI) (c) sera.

and anti-H2 sera. The control medium (Figures 2a and 2b, lanes 1) contained the mature ITI-like protein (ITI, 260 kDa), a heavy-chain protein form (H_d , 180 kDa) better seen with anti-H2 serum and two further heavy-protein forms (H_m , 120–125 kDa). The treatment with 3 munits/ml of chondroitinase ABC (Figure 2a, lane 2) or 30 munits/ml of chondroitin AC lyase (Figure 2b, lane 3) caused the disappearance of the ITI-like protein and the appearance of two new bands: a major one (HC/bik, 140 kDa) and a minor one of 190–200 kDa seen with chondroitin AC lyase. With higher enzyme concentrations, the latter species disappeared and two other protein groups became apparent: a

Figure 4 Effect of β -D-xyloside

The cells were pulsed with [35 S]methionine for 10 min in the presence of β -D-xyloside: 0 mM (control, lanes 1, 4 and 7), 1 mM (lanes 2, 5 and 8) and 5 mM (lanes 3, 6 and 9). The lysates, after 0 min of chase (lanes 1–3), and the supernatants, after 180 min of chase (lanes 4–9), were immunoprecipitated by anti-(H + L ITI) (lanes 1–6) and anti-(L ITI) (lanes 7–9) sera.

group of 86 kDa (Figure 2a, lanes 3 and 4; HC) resolved into two bands (80–85 kDa) with chondroitin AC lyase (Figure 2b, lanes 3 and 4) and another group which exhibited a double pattern at 27–28 kDa (bik). Furthermore, neither the 120–125 kDa (H_m) forms nor the minor 180 kDa form (H_d) were affected by chondroitinase digestion.

In order to determine the antigenic specificity of the new protein forms observed, supernatants obtained after a 30 munits/ml digestion were immunoprecipitated with specific antisera (Figure 3). Besides the well-known reactivity of the ITI-like protein (Figures 3a–3c, lanes 1; ITI), only the transient 140 kDa form (Figures 3a–3c, lanes 3; HC/bik) showed both ITI-H2 and -L antigenic reactivities. The new species observed displayed restricted antigenic specificities: the heavy-chain form (HC, 86 kDa) towards H2 (Figure 3c, lanes 2 and 3) and the two light-chain forms (bik, 27–28 kDa) towards L (Figure 3b, lane 2). The invariant forms (H_m , H_d) reacted only with anti-H2 serum.

The ITI-like protein synthesized by HepG2 cells was therefore cleaved by chondroitinases to give a half-molecule (HC/bik, 140 kDa), then its mature components HC (80–85 kDa) and bikunin (27–28 kDa).

Culture of HepG2 cells in the presence of β -D-xyloside

As a further way to study the CS linkage between ITI chains, β -D-xyloside, which is known as a competitive inhibitor of the reactions catalysing the addition of galactose to the xylosylated protein core, was used in HepG2 cell cultures labelled with [35 S]methionine (Figure 4). After a 10 min pulse, whatever the amount of the β -D-xyloside in the medium, cell lysates showed the same proteins, (Figure 4a, lanes 1–3): the heavy chain (H, 100 kDa) and the light chain (L, 49.5 kDa). After a 180-min-long chase, although no protein was observed in lysates (results not shown), the formation of the ITI-like protein (260 kDa) in supernatants was clearly inhibited by β -D-xyloside (Figure 4, lanes 5, 6, 8 and 9). Further, while 1 mM β -D-xyloside resulted in the appearance of a 140 kDa protein (HC–bik) with heavy- (Figure 4, lane 5) and light-chain (Figure 4, lane 8) antigenic properties, a 5 mM amount gave rise to a 25–28 kDa species displaying a light-chain immunoreactivity (Figure 4, lanes 6 and 9, bik). The level of the free heavy chains (H_m , 120–125 kDa) did not vary (Figure 4, lanes 4–6), although a possible increase was observed with 5 mM β -D-xyloside. Thus progressive inhibition of

Figure 5 Effects of tunicamycin

The cells were pulsed with [35 S]methionine for 10 min and chased for 0 min (lanes 1–4) or 180 min (lanes 5–10). Cell lysates (lanes 1–4) or culture supernatants (lanes 5–10) were produced in cultures in the absence (lanes 1, 3, 5, 7 and 9) or in the presence of tunicamycin (lanes 2, 4, 6, 8 and 10), and were immunoprecipitated by anti-(L ITI) (lanes 1, 2, 9 and 10) or anti-(H+L ITI) (lanes 3–8) sera. The culture supernatants (lanes 7–10) were digested by chondroitinase ABC.

the CS link in ITI led to the production of a half-molecule (HC–bik, 140 kDa) and then of non-assembled, free mature components (H_m and bikunin).

Study of the N-glycosylation of ITI products in HepG2 cells

In order to study the possible involvement of N-glycosylation of the precursors in the maturation processing of the ITI-like protein, pulse–chase experiments were performed with or without tunicamycin. Besides lowering of protein synthesis, tunicamycin caused a shift from 49.5 to 41 kDa for the light chain (Figure 5, lanes 1–4; L) and from 100 to 96 kDa for the heavy chain (Figure 5, lanes 3 and 4; H) in cell lysates. This was also observed in supernatants chased for 180 min, as the free heavy-chain forms (Figure 5, lanes 5 and 6; H_m) shifted from 120–125 to 115–120 kDa; the maturation of precursors into ITI-like protein was not affected by the lack of N-linked carbohydrate (Figure 5, lanes 5 and 6) although a possible shift of the observed proteins (~260 kDa) could not be detected in the SDS/PAGE system used. When the same products were, upon tunicamycin treatment, further submitted to chondroitinase ABC digestion, mature ITI components also showed an altered migration in the presence of tunicamycin: the HC chain shifted from 86 to 82 kDa (Figure 5, lanes 7 and 8) and the bikunin from 27–28 to 24–25 kDa (Figure 5, lanes 7–10). The aforementioned artefact protein (*) was also probably affected by tunicamycin treatment. All the ITI-related products therefore carried N-linked oligosaccharides, but such an addition was not required for their maturation and assembly into ITI-like protein, nor their secretion.

Figure 6 Effects of brefeldin A

The cells were pulsed with [35 S]methionine for 10 min and chased for 180 min in the presence of brefeldin A. The cell lysate was immunoprecipitated with anti-(H+L ITI) (lane 1), anti-(H1 ITI) (lane 2), anti-(H2 ITI) (lane 3) and anti-(H3 ITI) (lane 4) sera, and the supernatant (lane 5) with anti-(H+L ITI) serum.

Culture of HepG2 cells in the presence of brefeldin A

In the presence of brefeldin A, the only observable forms of ITI were its precursors: the 100 kDa heavy chain restricted to the H2 form (Figure 6, lanes 1–4; H) and the 49.5 kDa light chain (Figure 6, lane 1; L) which accumulated in the lysate where they could be observed up to 180 min. No protein antigenically related to ITI could be observed in supernatants (Figure 6, lane 5). Brefeldin A, which induces Golgi-specific changes and inhibits the GAG elongation, therefore locked ITI precursors in the ER.

Figure 7 Effects of monensin

The cells were pulsed with [35 S]methionine for 10 min and chased in the presence of monensin. The cell lysates (a) were obtained after a chase of 0 min and immunoprecipitated with anti-(H + L ITI) (lane 1), anti-(H1 ITI) (lane 2), anti-(H2 ITI) (lane 3) and anti-(H3 ITI) (lane 4) sera. The supernatant (b) obtained after 180 min of chase was immunoprecipitated with anti-(H + L ITI) (lane 1) and anti-(H2 ITI) (lane 2) sera.

Culture of HepG2 cells in the presence of monensin

HepG2 cell lysates obtained after treatment with monensin showed patterns identical with those obtained in normal conditions (Figure 1a, lanes 1–3) whatever the chase time: the light-chain (L, 49.5 kDa) and the heavy-chain form (H, 100 kDa) with restricted H2 specificity could be observed (Figure 7a, lanes 1–4). However, after 180 min of chase, unlike untreated cells (Figure 1a, lane 4), no ITI-like protein was observed in supernatants (Figure 7b, lanes 1 and 2) and a faint protein band (bik, 28 kDa) with L specificity was seen (Figure 7b, lane 1). The free heavy-chain forms H_m (120–125 kDa) and H_d (180 kDa), better evidenced with anti-H2 serum (Figure 7b, lane 2), corresponded to the free heavy-chain forms already seen in normal conditions (Figures 2b and 3c). Monensin, which inhibits the transport of proteins into the trans-Golgi network, therefore prevented the normal assembly of H and L precursors into ITI-like protein. The maturation of the heavy chain into H_m and H_d forms and that of the light chain into bikunin could, however, take place.

DISCUSSION

In order to obtain more details on the synthesis and maturation of the ITI which we previously studied in the human hepatoma HepG2 cell model [9], we have examined the sulphation and the subcellular localization of the maturation of the ITI heavy- and light-chain precursors before their assembly and secretion using several inhibitors and new antisera. As already described, ITI heavy- or light-chain precursors were first observed in lysates up to 30 min of chase (Figure 1a, lanes 1–3) then the ITI-like protein and two heavy-chain forms (H_m), resolved under optimal electrophoretic conditions, were secreted into the culture supernatants (Figure 1a, lane 4). However, as each heavy-chain-related molecule displayed an antigenic specificity restricted to H2 (Figures 6 and 7, lanes 2–4), HepG2 cells did not synthesize any H1- or H3-related forms. Furthermore, the faint 220 kDa band (*), previously called 'composite protein' [9] and observed in super-

natants regardless of the antiserum used, turned out to be an artefact related to non-specific binding to Protein-A-Sepharose [10].

As the GAG link between mature ITI components (HC and bikunin) involves CS [12,13], we used radiolabelling with [35 S]sulphate which clearly indicated that light- and heavy-chain precursors (H and L), as well as mature ITI, carried sulphated GAG chains (Figure 1b); this opposed a previous study [13,29] which found only sulphated light chain. When β -D-xyloside, a competitive acceptor of CS chain, was added to the medium, progressive inhibition of the ITI-like protein assembly was observed (Figure 4): at low competition, mature precursors were linked in a 140 kDa protein form with antigenic structure and size similar to the HC2-bikunin form reported by Enghild et al. [16]; at higher competition, ITI components were observed as free, non-assembled species (H_m and bikunin). However, while the light chain was observed in its mature form (bikunin), the heavy chain was not (no HC form). Furthermore, the H_m forms (120–125 kDa) lacked GAG chains as they were not labelled by [35 S]sulphate and they were secreted despite the presence of β -D-xyloside.

Endoglycosidases with various GAG specificities have been used to study ITI-related human serum proteins [12–19]. Digestion with ovine testicular hyaluronidase and chondroitinase ABC [12–17], or chondroitin AC lyase [18,19] showed that human ITI, P α I and HC2-bikunin released two heavy chains of 78 and 85 kDa and bikunin. ITI was therefore considered as composed of two heavy chains, HC1 and HC2, linked to a bikunin chain by CS bridges [13]. However, the present study demonstrates that HepG2 cells are able to synthesize a mature ITI-like protein, displaying electrophoretic characteristics identical with those of human serum although composed of L- and only H2-related species. Digestion of this ITI-like protein by chondroitinase ABC or chondroitin AC lyase at low concentration released a protein of 140 kDa with properties similar to those of the form obtained with 1 mM β -D-xyloside or those of the HC2-bikunin form reported by Enghild et al. [16]; in particular, no free HC2 form could be observed. With higher enzyme levels, two HC2 proteins (80 and 85 kDa) as well as two bikunin chains (27 and 28 kDa) were released. Furthermore, high levels of chondroitins A, B or C in no way modified the observed ITI-like protein structure (results not shown). Therefore, not only the heavy and bikunin chains are covalently linked by CS bridges but heavy chains are also linked by a CS GAG structure.

In the structural model proposed (Figure 8), ITI is composed of two identical heavy chains linked to two bikunins. A half-molecule is obtained by limited chondroitinase digestion or competition with low levels of β -D-xyloside. Free chains are observed under their mature forms (HC2 and bikunin) at complete digestion, while mature bikunin and heavy-chain form H_m are observed with 5 mM β -D-xyloside. The H–H and H–L GAG linkages are presumably different as the former is altered with low levels of xyloside and/or chondroitinase, although its exact nature and localization remain to be elucidated.

As depicted in Figure 9, the synthesis in HepG2 cells of ITI precursors and their assembly into ITI-like protein follow several steps which could be dissected out with inhibitors. Tunicamycin allowed us to show that ITI heavy- and light-chains were both N-glycosylated, as could be shown on precursor (H and L) as well as mature (H_m, HC and bikunin) components. As already proposed with respect to putative glycosylation sites, N-linked oligosaccharides are likely to be present in the HC part of the heavy chain [4], as well as in both components of the light chain (α 1-microglobulin and bikunin) [8]. However, HepG2 cells did

Figure 8 Structural model of the ITI-like protein and effects of digestion

ITI synthesized in the HepG2 cell line is composed of two identical heavy chains, HC2, and two bikunin chains. Low quantities of chondroitinase ABC or chondroitin AC lyase cleave this protein into two half-molecules composed of HC2–bikunin while higher amounts cleave the GAG bridge between HC2 and bikunin. The nature of the GAG bridge between HC2 chains is not known. (Y) N-linked oligosaccharides, (Z) glycosaminoglycan chains.

not exhibit a double pattern such as that observed for the light-chain precursor in the rat hepatocyte model and ascribed to variable rates of N-glycosylation [11,30]. Moreover, the absence of N-glycosylation did not prevent either the secretion or the maturation of the heavy chains and the ITI-like protein.

Brefeldin A totally blocked the maturation of ITI-related proteins in the cells: they were neither secreted nor degraded but accumulated in the ER as H and L precursors. Monensin was used at 5×10^{-7} M, a level known to have minimal effects on protein synthesis and not to inhibit the lysosomal degradation of proteins [31]. In such conditions, no ITI-like protein was secreted in the culture supernatants. The association of the heavy and bikunin chains probably requires specific enzyme activities present in the trans-Golgi network. However, the light-chain precursor matured in its usual way as it was secreted in the culture supernatant as a protein of 28 kDa, although the low level of this free bikunin form was probably related to a degradation process.

Overall, the synthesis and assembly of ITI precursors followed two main paths. The first one, leading to the ITI-like protein, involved the addition of GAG chains, which is considered to be a Golgi complex-related event [32–34]. The very low labelling

observed on the light-chain precursor with [35 S]sulphate may correspond to the fact that the GAG linkage on the light chain is a limiting factor, the heavy chain being afterwards quickly assembled into the ITI-like protein. This GAG-chain route is inhibited by β -D-xyloside and monensin. Conversely, the 120–125 kDa H_m forms are likely to represent another process of maturation restricted to heavy-chain precursors: they are normally secreted in supernatants after maturation in the ER/proximal part of the Golgi compartment where they could also be observed (Figure 1a, lane 3). Furthermore, these species did not carry sulphate groups, were not modified either by β -D-xyloside or by chondroitinases, or any of the inhibitors studied. How H_m forms may be associated in the 180 kDa H_d species has to be determined, as the latter is the only possible linked form observed in the presence of monensin.

In the human hepatoma HepG2 cells, the ITI-like protein is composed of two mature HC2 and two bikunin chains the structure and maturation of which are respectively schematized in Figures 8 and 9. No P α I or HC2–bikunin were evident in HepG2 cells, as opposed to rat hepatocytes where P α I was observed in the cell lysate and culture supernatant [11]. The

Figure 9 Model of maturation of ITI protein chains in HepG2 cells

Tunicamycin blocks the N-glycosylation of heavy- and light-chain precursors leading to the secretion of normally assembled but unglycosylated forms of ITI-like protein and H_m heavy chains. The redistribution of ER, cis-, medial-, and trans-Golgi induced by brefeldin A locks the precursor chains in the cells. Monensin prevents the linkage between heavy and light chains which undergo normal maturation in the ER/proximal part of the Golgi complex leading to the secretion of heavy-chain forms (H_m and H_d) and bikunin. In normal conditions, the heavy- and light-chain precursors are N-glycosylated in the ER and transported to the cis-Golgi apparatus where GAG and sulphate are added; the precursors are then cleaved into HC2 and bikunin and the mature proteins are assembled into ITI in the trans-Golgi network and secreted; heavy chains are also secreted as free (H_m) or associated (H_d) forms. (Y) N-linked oligosaccharides, (Z) glycosaminoglycan chains.

existence of HC1 and HC2 in serum ITI, as reported by Enghild et al. [13] and Malki et al. [19], could correspond to a mixing of two ITI isotypic protein forms composed of two identical heavy chains, HC1 or HC2, linked to two bikunin chains. The P α I and HC2-bikunin derivatives found in normal human serum may be related to a degradation of the labile CS bridges herein described between the heavy chains.

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