Secretion of human intestinal angiotensin-converting enzyme and its association with the differentiation state of intestinal cells

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Human angiotensin I-converting enzyme (ACE) exists in intestinal epithelial cells as a membrane-bound (ACEm) and secretory glycoprotein (ACEsec). The electrophoretic mobilities of ACEsec and ACEm on SDS/polyacrylamide gels are similar; the N-deglycosylated ACEsec and ACEm, in contrast, display slight differences in their apparent molecular masses, indicating that the carbohydrate contents of ACEsec and ACEm are different. Moreover, ACEsec is solely N-glycosylated whereas ACEm is N- and O-glycosylated, assessed by lectin binding studies. Spontaneous release of ACEsec is achieved by incubation of brush border membranes at 37 °C. Aprotinin, leupeptin and EDTA partly inhibit the generation of ACEsec, strongly suggesting that ACEsec is generated from ACEm by proteolytic

cleavage. The expression levels of ACEsec in the intestine may be associated with the differentiation state of mucosal cells. Thus ACEsec is more abundant than ACEm in immature non-epithelial crypt cells of patients with coeliac disease. Well-differentiated epithelial cells, by contrast, contain predominantly ACEm. The variations in the proportions of cleaved ACEsec in differentiated and non-differentiated cells may be due to varying levels of the cleaving protease. Alternatively, because epithelial cell differentiation is accompanied by alterations in the levels of oligosaccharyltransferases, the results suggest a critical role for the glycosylation pattern of ACEm in its susceptibility to the putative cleaving protease.

INTRODUCTION

The epithelial cells of the small intestine are implicated in a variety of physiological functions that are essential and indispensable for the normal growth and development of mammals. One of the main events constantly taking place at the luminal surface of the intestinal cells is the terminal digestion of micro-molecular nutrients. This process is accomplished by a fabric of molecules, digestive enzymes, which are found in the brush border or microvillar membrane of the intestinal epithelial cells (reviewed in [1,2]). These enzymes are glycoproteins that, in most cases, are found associated with the membrane via trans-membrane domains. In two cases, secreted forms of membrane-bound enzymes have also been identified: angiotensin I-converting enzyme (ACE) (kininase II, EC 3.4.15.1) [3–9] and *p*-aminobenzoic acid-peptide hydrolase [10]. The mechanisms that lead to the generation of the secreted forms of *p*-aminobenzoic acid-peptide hydrolase and ACE in the small intestine have not yet been elucidated. Nevertheless a proteolytic processing of membrane-bound ACE (ACEm) to hydrophilic secreted ACE (ACEsec) taking place at the cell surface has been proposed [6]. A proteolytic processing of ACEm to ACEsec has been shown to be associated with a detectable shift (almost 5 kDa) in the apparent molecular mass of the secreted species [6], as a consequence of the elimination of the membrane-anchoring domain and cytoplasmic tail. Additionally, recent results with testicular ACE have also proposed a proteolytic mechanism, and sequence data of the C-terminus of the cleaved enzyme supported this idea [11].

Intestinal ACEm [8], as other ACE molecules [12–14], is associated with the membrane via a membrane-anchoring domain. Surprisingly, the size of intestinal ACEm is found to be electrophoretically indistinguishable from that of ACEsec [9]. This suggested that either (1) proteolytic cleavage of ACEm to

ACEsec is not a likely mechanism, otherwise a marked shift of at least 5 kDa should be discerned, or (2) ACEsec and ACEm differ in their glycosylation patterns and could be therefore distinguished electrophoretically.

In this paper I investigated the secretion of ACE and provide evidence that ACEsec is most probably generated by post-translational proteolytic cleavage from ACEm and that this process is strongly dependent on the differentiation state of intestinal cells.

EXPERIMENTAL

Intestinal tissue and antibodies

Human small-intestinal biopsy specimens (approx. 5–10 mg wet weight) were obtained from patients biopsied for diagnostic purposes. Examination of the tissue by light microscopy revealed normal intestinal morphology with villi structures. Further, the enzymic activities of standard brush border glycoproteins were within the normal range (sucrase 28–80 units/g; lactase 16–49 units/g, determined by the method of Asp et al. [15]). Another source for the organ culture studies was biopsy specimens from patients with acute coeliac disease (CD). When examined by light microscopy the specimens revealed features typical of CD characterized by a totally flattened surface with clear crypt structures and no villi [16]. The activities of sucrase and lactase were below the detection limit.

Monoclonal antibodies against ACE, sucrase–isomaltase (SI), dipeptidyl peptidase IV (DPP-IV) and aminopeptidase N (ApN) were gifts from Dr. Hans-Peter Hauri (Biocenter, Basel) and Dr. Erwin E. Sterchi (University of Bern, Switzerland) [17] and used in the form of ascites prepared from hybridoma-bearing pristane-primed Balb/c mice. For immunoprecipitation experiments,

1–2 μ l of each antibody was used in a single immunoprecipitation. Here the antibody was mixed with 40 μ l of a suspension of Protein A–Sepharose (Pharmacia) [50 % (w/v) in PBS] and 1 ml of 0.1 M phosphate buffer, pH 8.0, for 1 h at 4 °C. The beads (denoted hereafter as immunobeads) were washed with phosphate buffer and further used for immunoisolation of the corresponding antigen.

Biosynthetic labelling of biopsy specimens

The biopsy samples were biosynthetically labelled essentially as described by Naim et al. [18]. Briefly, each biopsy specimen was placed on stainless-steel grids in organ culture dishes (Falcon) [19] and labelled with 1 ml of an incubation medium containing methionine-free RPMI 1640, streptomycin (100 μ g/ml), penicillin (100 i.u./ml), 10 % dialysed fetal calf serum (FCS) (all from Gibco) and 150 μ Ci of [35 S]methionine (ICN). Labelling was performed continuously for 6 h. The coeliac biopsy specimen was labelled for 2 h and chased for 20 h with unlabelled methionine. The cell viability after 20 h of chase was assessed by the analysis of the subunit structure and biosynthesis of control glycoproteins, such as ApN (see Figure 4) or DPPIV (results not shown). The results demonstrated that these proteins were processed normally and were not degraded, indicating that the tissue was viable. After labelling, the incubation media were collected and protease inhibitors (1 mM PMSF, 1 μ g of pepstatin, 5 μ g of leupeptin, 17.4 μ g of benzamidine, 5 μ g of antipain and 1 μ g of aprotinin) (all from Boehringer-Mannheim) were immediately added. The media were then centrifuged at 100 000 g for 30 min at 4 °C and 800 μ l (instead of 1 ml) of the supernatants were recovered to avoid contamination with possibly existing membrane pellets. The biopsy specimens were cooled to 4 °C, washed three times in cold PBS and homogenized with a Teflon–glass homogenizer in 1 ml of 25 mM Tris/HCl (pH 8.1)/50 mM NaCl, and the mixture of protease inhibitors mentioned above (buffer A). The homogenates were either further processed directly for immunoprecipitation or kept frozen at –20 °C until used. In some experiments labellings were performed in the presence of leupeptin (5 μ g) and aprotinin (1 μ g) and 5 mM EDTA. The tissue integrity after labelling in the presence of EDTA was assessed by light microscopy and was found to be similar to the control biopsy sample.

Incubation of 35 S-labelled brush border membranes

Brush border membranes were purified from biopsy specimens that were labelled with [35 S]methionine for 8 h. The membranes were homogenized with 1 ml of 25 mM Tris/HCl, 50 mM NaCl, pH 8.1. The membranes were then incubated for 1 h at 37 °C and subsequently centrifuged at 100 000 g for 1 h at 4 °C. The pellets and supernatants were further processed for immunoprecipitation with anti-ACE and anti-SI antibodies.

Lectin binding studies

Lectin affinity studies of purified ACE molecules was slightly modified from the procedure previously described for SI [20] and lactase–phlorizin hydrolase [21]. Briefly, the immunoprecipitated proteins were eluted from Protein A Sepharose beads in 50 μ l of 0.1 M Tris/HCl, pH 7.5, containing 1 % (w/v) SDS at 37 °C for 1 h and precipitated with three volumes of ice-cold acetone at –20 °C. The protein pellet was recovered by centrifugation, dried, dissolved in 500 μ l of PBS containing 1 % (w/v) Triton X-100 (TX-100) and finally subjected to lectin–Sepharose beads. TX-100 was omitted when secretory ACE

(ACEsec) was analysed. Usually 200 μ l of the lectin–Sepharose beads were used, which were pre-equilibrated before use with 10 volumes of PBS and 0.5 % TX-100 (or without TX-100 in the case of ACEsec). The protein solution was mixed with lectin–Sepharose beads for 4 h at 4 °C with continuous shaking. The lectin–Sepharose beads carrying the bound protein were washed 10 times with 1 ml of PBS/0.5 % TX-100. The bound material was finally eluted with the specific sugar in 500 μ l of PBS/0.5 % TX-100. The following lectins were used: *Helix pomatia* lectin, wheat germ agglutinin and *Lens culinaris* lectin (all from Pharmacia). The following sugars were used to elute specifically the bound glycoproteins: 10 mM *N*-acetyl-D-galactosamine (Sigma) (for *Helix pomatia* lectin), 100 mM *N*-acetyl-D-glucosamine (Sigma) (for wheat germ agglutinin) and 100 mM α -methylmannoside (Sigma) (for *Lens culinaris* lectin). The eluates, as well as the material not bound by the beads, were precipitated with ice-cold acetone and the protein pellets dissolved in electrophoresis sample buffer. When *Helix pomatia* lectin–Sepharose was used, pretreatment of the immunoprecipitated protein with neuraminidase was performed as described elsewhere [21]. Elution and processing of the beads were then as described above.

Other procedures

Subcellular fractionation of biosynthetically labelled biopsy specimens into brush border membranes and intracellular membranes was by the method of Naim et al. [20]. Immunoprecipitations were performed essentially by the method of Naim et al. [18]. Digestion of 35 S-labelled immunoprecipitates with peptide:*N*-glycopeptidase F (PN Gase F) or endoglycosidase F/glycopeptidase F (New England Bio Labs) was in accordance with the manufacturer's instructions. SDS/PAGE was performed in 6 % (w/v) slab gels by the method of Laemmli [22] and fluorography as described previously [18].

RESULTS

Spontaneous release of a secretory form of ACE from brush border membrane ACE

The existence of hydrophilic forms of ACE in intestinal cells [9] raises questions related to the molecular mechanisms involved in the generation of these forms. For example, is hydrophilic ACE derived from membrane-bound ACE by post-translational proteolytic cleavage of the membrane anchor by endopeptidases, as in the pig kidney [6] or lung [23,24]?

To examine this possibility, brush border membrane fractions from biosynthetically labelled biopsy samples (35 S-labelled brush border membranes) were incubated in homogenization buffer for 1 h at 37 °C and centrifuged. The pellet and the supernatant were subjected to immunoprecipitation with anti-ACE antibodies. Figure 1 shows that ACE immunoprecipitated from the membranes contained only the 184 kDa brush border membrane form of ACE (ACE_m) (lane 1) [8,9]. The supernatant contained an ACE species of an apparent molecular mass indistinguishable from ACE_m (Figure 1, lane 2). The intensity of this band comprised almost 25 % of total ACE initially found in the brush border membranes (the sum of ACE found in the pellet and the supernatant). Longer incubations at 37 °C did not lead to substantial changes in the intensity of this polypeptide (results not shown). Analysis of integral proteins of the brush border membrane demonstrated that the observed release at 37 °C is restricted to ACE. In fact, SI was found exclusively in the membrane pellet (Figure 1, lane 3) and not in the incubation buffer (Figure 1, lane 4); similarly lactase–phlorizin hydrolase,

Figure 1 Release of ACE from brush border membranes

Biopsy samples were biosynthetically labelled for 6 h with [35 S]methionine, homogenized and brush border membranes were prepared. The membranes were homogenized, incubated for 1 h at 37 °C and centrifuged at high speed. The supernatant (lanes 2 and 4) and detergent extracts of the pellet (lanes 1 and 3) were processed for immunoprecipitation with anti-ACE (lanes 1 and 2) and anti-SI (lanes 3 and 4) antibodies. The immunoprecipitates were analysed by SDS/PAGE on 5% gels. Shown is a fluorogram of the gel. Abbreviations used: Lys, lysates; Sup, supernatant.

Figure 2 Effect of protease inhibitors on the secretion of ACE

Biopsy samples were biosynthetically labelled for 6 h with [35 S]methionine in the absence (lanes 1 and 2) or presence of EDTA (lanes 3 and 4) or leupeptin and aprotinin (lanes 5 and 6). Detergent extracts of the biopsy specimens (lanes 1, 3 and 5) or culture media (lanes 2, 4 and 6) were immunoprecipitated with anti-ACE (upper panel) or anti-DPPIV (lower panel) antibodies. The immunoprecipitates were analysed by SDS/PAGE on 5% (upper panel) or 6% (lower panel) slab gels. Shown are fluorograms of the gels. Abbreviation used: apr/leu, aprotinin/leupeptin.

another integral brush border protein, was recovered only in the pellet (results not shown).

A secretase activity has been demonstrated to be responsible for the generation of secreted ACE in the kidney [25]. This activity could be inhibited by EDTA, but not by inhibitors of serine proteases, thiol proteases or aspartate proteases. To

determine whether the proteolytic cleavage of ACEm in the intestine has characteristics comparable with that in the kidney, biopsy samples were labelled biosynthetically in the presence of a mixture of serine protease inhibitors or EDTA.

Figure 2 depicts the electrophoretic analysis of one representative experiment. The serine protease inhibitors aprotinin and leupeptin had some effect on the secretion of ACEsec, because less ACEsec was found in the culture medium (Figure 2, upper panel, lane 6) than in the control (Figure 2, upper panel, lane 2). Similarly an effect was also manifested when EDTA was added to the culture medium during biosynthetic labelling (Figure 2, upper panel, lane 4). Similar results were obtained in two further experiments. Scanning of the fluorogram in all these cases demonstrated that $27.7 \pm 1.7\%$ of ACEsec molecules was released into the medium in the presence of aprotinin and leupeptin, in comparison with $41 \pm 1.5\%$ in the control sample. EDTA also affected the release of ACEsec. Here, approximately $25.6 \pm 1.1\%$ of ACEsec molecules was secreted. As expected, the control integral membrane glycoprotein DPPIV was not released into the medium under either of the conditions employed (Figure 2, lower panel, lanes 2, 4 and 6).

The deglycosylated forms of ACEsec and ACEm have slight differences in their carbohydrate contents

By virtue of the membrane association of ACEm via a trans-membrane domain, proteolytic cleavage of ACEsec from ACEm would be expected to be accompanied by a shift of about 5 kDa in the size of the secreted molecule. This has not been observed for the secreted form in biosynthetically labelled biopsy samples [8,9] (Figure 3, upper panel, lanes 1 and 2) or the form spontaneously released by incubation of biopsy membranes at 37 °C. One possible explanation implicates variations in the carbohydrate contents of secreted and membrane-bound ACE. I therefore examined the N-glycosylation pattern of the two species. Here, ACEm and ACEsec were treated with PN Gase F, which cleaves N-linked oligosaccharides of the complex type [26]. Figure 3 (upper panel) demonstrates that the N-deglycosylated forms of ACEm (lane 3) and ACEsec (lane 4) revealed polypeptides of different apparent molecular masses, whereby the N-deglycosylated form of ACEsec (lane 4) had a slightly faster electrophoretic mobility than the N-deglycosylated form of ACEm (lane 3). Because ACEm (Figure 3, upper panel, lane 1) and ACEsec (Figure 3, upper panel, lane 2) have similar apparent molecular masses and their N-deglycosylated species do not, the results imply that there exist some variations in the degree of glycosylation of ACEm and ACEsec.

To investigate possible structural alterations in the nature and type of the carbohydrate chains, lectin binding studies with purified ACEsec and ACEm were performed. To assess the binding to N-linked carbohydrates, wheat germ agglutinin and *Lens culinaris* lectin were used. The membrane and secretory forms of ACE were first immunoprecipitated from biopsy lysates and the culture media respectively and then subjected to lectin binding.

As shown in Figure 3 (lower panel), ACEsec and ACEm bound to wheat germ agglutinin, which has predominant affinity for N-acetylglucosamine, but also binds terminal sialic acid residues [27] (Figure 3, lower panel, lanes 1 and 2). Likewise, both ACE forms exhibited binding to *Lens culinaris* lectin (lanes 3 and 4), which preferentially binds mannose residues [27]. To examine the presence of O-linked sugars, *Helix pomatia* lectin was used. This lectin interacts specifically and strongly with N-acetylgalactosamine (GalNAc) and galactose- $\beta(1 \rightarrow 3)$ -N-acetylgalactosamine structures (Gal-GalNAc) [28,29] and is

Figure 3 Deglycosylation of ACEm and ACEsec and analysis of ACEm and ACEsec by lectin-Sepharose

Upper panel: deglycosylation of ACEm and ACEsec. Biopsy samples were biosynthetically labelled for 6 h with [35 S]methionine and the biopsy lysates (lane 1) or culture medium (lane 2) were immunoprecipitated with anti-ACE antibodies. Part of the immunoprecipitates was treated with PN Gase F (lanes 3 and 4) or not treated (lanes 1 and 2). The samples were finally analysed by SDS/PAGE on 5% slab gels, followed by fluorography. The positions of the following molecular-mass markers are indicated at the left: myosin, 202 kDa; phosphorylase b, 97.5 kDa; BSA, 68 kDa; ovalbumin, 46 kDa. Lower panel: analysis of ACEm and ACEsec by lectin-Sepharose. ACEm and ACEsec (indicated as ACEs) were purified from biopsy lysates and culture media by immunoprecipitation. The proteins were eluted and subjected to wheat germ agglutinin (WGA) (lanes 1 and 2), *Lens culinaris* lectin (LCL) (lanes 3 and 4) or *Helix pomatia* lectin (HPL) (lanes 5, 6 and 7). The eluates from the lectin-Sepharose beads (lanes 1–4, 6 and 7) as well as the material not bound (N.B.) to *Helix pomatia* lectin (lane 5) were subjected to SDS/PAGE. Lanes 1, 4 and 6, ACEm subjected to and retained by the lectins; lanes 2 and 3, ACEsec subjected to and retained by the lectins; lane 5, ACEsec not bound by *Helix pomatia* lectin; lane 7, eluate of ACEsec subjected to *Helix pomatia* lectin. Lanes 1 and 2 and 3–7 were from two different gels. The exposure time of lanes 1 and 2 was 3 weeks and that of lanes 3–7 was 1 week.

therefore suitable for monitoring the existence of O-linked glycan units in glycoproteins. Because of the binding specificity of this lectin, prior removal of terminal sialic acid residues from the carbohydrate chains is required. Immunoprecipitated ACEsec and ACEm were therefore treated with neuraminidase and then subjected to the lectin. Figure 3 (lower panel) demonstrates that ACEm bound specifically to the lectin because it was recovered in the eluates with GalNAc (lane 6); ACEsec, in contrast, was

not retained by the lectin (lane 7) and was found exclusively in the non-bound material (lane 5). The result therefore indicates that ACEm is O-glycosylated whereas ACEsec does not carry O-linked Gal-GalNAc structures.

Altogether, ACEm is both N- and O-glycosylated whereas ACEsec is only N-glycosylated. Furthermore the PN Gase F data and the lectin binding studies suggest that the two species most probably differ in the content, but not in the nature or composition, of the N-linked sugar chains, with ACEsec being slightly more N-glycosylated.

Secreted ACE is synthesized and processed by crypt cells

The differences in the glycosylation patterns of ACEsec and ACEm are in line with a previous hypothesis that proposed that membrane-bound ACEm consists of a heterogeneous population of differently glycosylated molecules and these cannot be clearly distinguished electrophoretically from each other [9]. Some of these molecules are more susceptible than others to proteolytic cleavage and end up as soluble ACEsec. The rationale for this hypothesis is that the intestinal mucosa harbours a heterogeneous population of cells that could be discriminated according to their state of differentiation. Because differentiation of intestinal cells is accompanied by marked changes in the levels of oligosaccharyltransferases [30–38], it is reasonable to assume that the extent of glycosylation of ACE depends on the differentiation state of the various subpopulations of the intestinal cells.

To examine this possibility I investigated the biosynthesis of ACE in biopsy specimens taken from patients with CD. Specimens from these patients are characterized by a completely flat surface and the absence of villi. Cells in this tissue type are predominantly non-differentiated, non-epithelial, proliferative crypt cells [16].

Three biopsy samples from patients with CD were investigated; essentially similar results were obtained. The results described below were obtained with one individual case and are representative of the other analysed cases. Here, biopsy samples from the CD patient (predominant crypt) and a normal control (epithelial and crypt cells) were labelled for 2 h with [35 S]methionine and chased for 20 h with unlabelled methionine. The supernatants and the detergent extracts of the biopsy homogenates were immunoprecipitated with anti-ACE antibodies and antibodies against ApN as a control, non-secretory glycoprotein. Figure 4 shows that ACE was immunoprecipitated from the culture media of the normal and coeliac biopsy specimens as a 184 kDa polypeptide (lanes 2 and 4). Immunoprecipitation of the membrane-bound ACEm from the detergent extracts of the normal biopsy sample revealed a strongly labelled 184 kDa complex glycosylated species and little of the 174 kDa mannose-rich precursor (Figure 4, lane 1) [8]. The biopsy sample from the coeliac patient, in contrast, contained very little of the 184 kDa ACEm polypeptide (Figure 4, lane 3). The ratios of the secreted form to total synthesized and processed ACE in both mucosa types were assessed by comparing the densitometric scans of ACEm and ACEsec bands. The results of all three cases studied revealed that $80.6 \pm 1.5\%$ of ACE was found as ACEsec in the intestinal mucosa of coeliac patients. Normal intestinal mucosa, in contrast, revealed 2–3-fold more ACEm than ACEsec. Variations were obtained in the levels of ACEsec in different experiments with different intestinal tissues. This could reflect the heterogeneity of the intestinal mucosal cell population (see, for example, the control sample in Figure 3, upper panel, lanes 1 and 2). Nevertheless the level of ACEsec did not exceed 45% of the total synthesized ACE and is therefore markedly lower than ACEsec in CD. In the experiment shown in

Figure 4 Biopsy specimens from patients with CD express predominantly secretory forms of ACE

Biopsy specimens from patients with CD and from normal controls (N) were labelled for 2 h with [35 S]methionine and chased for 20 h with unlabelled methionine. The biopsy homogenates (H) of the normal control (lanes 1 and 5) and the CD patient (lanes 3 and 6) as well as the culture media (M) of the normal control (lanes 2 and 7) and the CD patient (lanes 4 and 8) were immunoprecipitated with anti-ACE antibodies (lanes 1–4) and anti-ApN antibodies (lanes 5–8). The immunoprecipitates were analysed by SDS/PAGE on 5% slab gels, followed by fluorography.

Figure 4, for example, almost 32 % of the ACEsec was released into the culture medium after a chase period of 20 h (lane 2).

The control glycoprotein ApN was found in the normal (Figure 4, lane 5) and in the coeliac (Figure 4, lane 6) biopsy samples exclusively as a membrane-bound form and was not detected in the culture medium of either biopsy sample (Figure 4, lanes 7 and 8).

Altogether, the results suggest that: (1) most of the newly synthesized ACE molecules in crypt cells are secreted within 20 h of the chase, whereas normal intestinal mucosa secretes one-half to one-third of the synthesized molecule in the same period; (2) secretory forms of ACE are synthesized and processed by intestinal crypt cells, whereas membrane-bound ACE is predominantly expressed by differentiated epithelial cells; and (3) the proportion of ACEsec in the normal biopsy samples may depend on the proportion of crypt cells in that tissue.

DISCUSSION

Differentiation of cells in many tissues is a dynamic process that is accompanied by a series of dramatic changes in gene expression as well as in the architecture and macromolecular composition of the cell. The structure and function of many proteins could also be subject to substantial alterations conforming to the new cellular environment that results from the expression of new genes (reviewed in [39]).

The differentiation of intestinal crypt cells to mature polarized epithelial cells seems to have immediate implications on the structural features of ACE. The results presented here propose that membrane-bound ACE is found mainly in mature, mucosal cells, whereas secreted forms of ACE are found predominantly in non-differentiated crypt cells. This observation raises questions regarding the potential mechanisms that lead to the generation of secretory forms of ACE and the relation of these mechanisms to the differentiation state of intestinal cells. Several possibilities could be considered. For example, ACEsec is proteolytically

cleaved from ACEm by cell surface components, which are expressed in non-differentiated intestinal cells at substantially higher levels than in the mature epithelium. Cleavage processing is in many cellular systems responsible for the generation of soluble forms of several membrane-bound proteins, including cell surface growth factors, cytokine receptors and cell adhesion proteins [40]. The cleavage events in all these cases follow a similar model, which includes a proteolytic cleavage of a large ectodomain taking place in the vicinity of the membrane. Nevertheless this general concept does not imply that cleavage processing is accomplished by similar or identical cell surface components; the cleavage process seems to be specific for the individual protein and is mediated by different proteases [41–45].

Recently a secretase activity has been characterized in kidney membranes as the protease responsible for generating secretory ACE from membrane-bound ACE [25]. In this tissue mere incubation of membranes at 37 °C results in the release of a hydrophilic ACE form, the molecular mass of which is smaller than that of the membrane-bound species. A similar mechanism for the generation of secretory intestinal ACE is favoured by our results, because at 37 °C spontaneous release of ACE molecules from intestinal membranes into the incubation medium also occurs. Furthermore, a reduction in the size of the N-deglycosylated form of ACEsec from that of ACEm strongly supports the idea that ACEsec is derived from ACEm, for example by truncation of the transmembrane domain. That the release of intestinal ACE is genuine and not, for example, due to leaky membranes, is corroborated by the finding that the non-secretory integral brush border membrane protein SI [2] is not released into the medium under similar conditions. Although the nature of the components implicated in this process has not yet been elucidated, cleavage processing involving a cell surface component is potentially the most likely mechanism. Our current knowledge of the biosynthesis and structure of human ACE does not favour an early cleavage step of the membrane-bound form, for example in the endoplasmic reticulum, being the underlying mechanism for the generation of secretory ACEsec. In fact, ACEm spans the membrane at least once [8] and therefore an early cleavage step of the transmembrane domain would be compatible with a reduction in the size of ACE by almost 5 kDa (the possible size of the transmembrane domain). Consequently two mannose-rich precursors (and their corresponding unglycosylated forms) with a molecular mass difference of about 5 kDa should be revealed; this does not happen, as has been shown previously [9].

It is not clear yet whether in the small intestine, as in the kidney, secretase is responsible for cleavage [25]. Similarities in the structural features of all ACE molecules in various species favour similar mechanisms implicating similar components. Consistent with the observed effects of EDTA on secretase activity in the kidney, the secretion of ACEsec in biosynthetically labelled intestinal biopsy samples is also affected by EDTA. Nevertheless, in contrast with ACE in the kidney, serine protease inhibitors, such as leupeptin and aprotinin, also affect the generation of ACEsec. It therefore seems that other components, in addition to or in conjunction with secretase, may be implicated in the secretion of human ACEsec. Secretase alone cannot be responsible for the release of ACEsec from ACEm because the activities of secretase in the intestinal microvillar membrane are very low [25].

The proteolytic cleavage process may very well depend on the glycosylation state of ACE. Consistent with this are the observed differences in the glycosylation patterns of ACEsec and ACEm. Whereas ACEm is both N- and O-glycosylated, ACEsec is exclusively N-glycosylated. Moreover N-linked carbohydrate

contents of ACEsec and ACEm are slightly different. The different glycosylation pattern strongly suggests that ACE processed by the intestinal mucosa comprises several populations of differently glycosylated forms. ACE is not the only intestinal protein to show these characteristics. Several glycoforms of SI [20] and lactase-phlorizin hydrolase [21] have been identified that vary in their content of O-linked carbohydrates. Differentiation of intestinal crypt cells to mature columnar epithelial cells is associated with marked variations in the expression levels of several oligosaccharyltransferases [30–38]. This in turn could be responsible for eliciting different glycosylation patterns of intestinal glycoproteins. Along these lines it is conceivable that the glycosylation pattern of ACE in immature coeliac biopsy samples, for example, differs from that in the more differentiated normal tissue. For ACE this variation could elicit variable susceptibilities of ACE glycoforms towards the putative cleaving protease. Interestingly, O-glycosidically linked carbohydrates have been shown to be implicated in conferring glycoprotein stability and protection against proteolytic cleavage [46–48]. It is therefore possible that ACEsec predominantly found in non-differentiated crypt cells is generated from N-glycosylated ACE, which is a better substrate for the putative cleaving protease than N- and O-glycosylated ACE predominantly found in well differentiated cells.

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