Human small intestinal angiotensin-converting enzyme: intracellular transport, secretion and glycosylation

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Human intestinal angiotensin-converting enzyme (ACE) exists in the brush-border membrane as a monomeric protein of apparent molecular mass 184 kDa. It is associated with the membrane via a hydrophobic segment and has a transmembrane orientation [Naim (1992) *Biochem. J.* **286**, 451–457]. In addition to the membrane-bound form (ACE_m), hydrophilic forms of ACE (ACE_{sec}) can be identified in biosynthetically labelled intestinal cells. Thus the culture medium of biosynthetically labelled human biopsy samples contains an ACE molecule which has an apparent molecular mass similar to that of its membrane-bound counterpart. The secreted ACE_{sec} forms follow a precursor/product relationship with the mature ACE molecule. The effect of the monomeric structure of ACE in its intracellular transport and secretion was investigated by pulse-chase experiments on human biopsy samples labelled with [³⁵S]methionine. The results reveal 2–3-fold slower transport of ACE from the endoplasmic reticulum (ER) to the Golgi as compared with the homodimeric

proteins dipeptidylpeptidase IV and aminopeptidase N. Further, the transport kinetics of ACE are comparable with those of human sucrase–isomaltase and human maltase–glucoamylase, two brush-border disaccharidases that do not form homodimers in the ER of human small-intestinal cells. These findings strongly suggest that homodimerization of brush-border proteins may influence the rate of transport of these proteins from the ER to the Golgi. The effect of glycosylation on the transport and secretion of ACE was investigated by utilizing several inhibitors of glycan processing. Here, secretion of ACE molecules continued to take place, albeit to a considerably lesser extent. In fact, approx. 2-fold less ACE molecules were secreted in the presence of inhibitors of ER glucosidases I and II and *cis*-Golgi mannosidase-I, suggesting that carbohydrate processing is important in the attainment of a transport-competent conformation.

INTRODUCTION

Angiotensin I-converting enzyme (ACE) (kininase II, EC 3.4.15.1) plays an important role in the regulation of blood pressure; it hydrolyses angiotensin I to the potent vasoconstrictor angiotensin II and converts the vasodilator bradykinin into an inactive peptide (Yang et al., 1970; Erdös, 1975; Skeggs et al., 1976; Soffer, 1981; Patchett and Cordes, 1985). ACE is a glycoprotein that has been identified in many tissues, e.g. lung, kidney, areas of the brain, and gut epithelium. It consists of a single subunit of apparent molecular mass in the range 130–180 kDa, depending on the tissue from which the enzyme has been purified (Erdös, 1975; Soffer, 1981). Striking similarities in the physicochemical, immunological and catalytic properties of the enzyme were discerned in ACE purified from different species and tissues (Ehlers and Riordan, 1989). cDNA cloning provided unequivocal support for these findings (Soubrier et al., 1988; Bernstein et al., 1989; Kumar et al., 1989).

ACE is found as a membrane-bound enzyme and as a circulating molecule in body fluids (Das et al., 1977). The membrane-bound forms, e.g. in endothelial cells, testis, kidney or intestinal epithelial cells, are thought to be anchored in the membrane via a hydrophobic segment near the C-terminus (Hooper et al., 1987; Soubrier et al., 1988; Bernstein et al., 1989; Kumar et al., 1989; Naim, 1992).

ACE has also been found associated with the microvilli of intestinal cells (Ward et al., 1980; Defendini et al., 1983), and I have recently investigated some aspects of its structure, membrane association and biosynthesis (Naim, 1992). Thus intestinal

ACE is synthesized as a single polypeptide precursor that matures in the Golgi apparatus by acquisition of complex-type oligosaccharides and is finally inserted via a membrane-spanning segment in the brush-border membrane. Cross-linking experiments and density-gradient centrifugations have provided unequivocal evidence for the existence of human intestinal ACE as a monomer in the brush-border membrane (Naim, 1992). Analysis of the quaternary structure of several membrane and secretory proteins has demonstrated that dimer or oligomer formation is an important event that is required for efficient intracellular transport of these proteins (Gething et al., 1986; Kreis and Lodish, 1986; Rose and Doms, 1988; Hurtley and Helenius, 1989). The findings that most of the brush-border enzymes so far identified are assembled into homo- or homohetero-dimers (Cowell et al., 1986; Naim et al., 1988b; Danielsen, 1990; Jascur et al., 1991; Naim, 1992; for reviews see Kenny and Maroux, 1982; Semenza, 1986) and that this assembly occurs before insertion into the brush-border membrane (Danielsen, 1990; Jascur et al., 1991) are compatible with an essential role of dimerization/oligomerization of brush-border proteins along their secretory pathway. It is therefore important to determine whether the intracellular transport of ACE is hampered by its monomeric structure, or whether ACE folds into a transport-competent dimer-like molecule, since it is composed of two similar domains (Soubrier et al., 1988; Bernstein et al., 1989).

In addition to dimer/oligomer formation, carbohydrate processing of membrane and secretory proteins in the endoplasmic reticulum (ER) and in the Golgi apparatus is an important post-translational modification that could play a role in the protein

Abbreviations used: ACE, angiotensin-converting enzyme; ER, endoplasmic reticulum; LPH, lactose–phlorrhizin hydrolase; ApN, aminopeptidase N; SI, sucrase–isomaltase; DPPIV, dipeptidylpeptidase IV; MGA, maltase–glucoamylase; endo H, endo- β -N-acetylglucosaminidase H; endo F/GF, endo- β -N-acetylglucosaminidase F/N-glycopeptidase F; DSP, dithiobis(succinimidylpropionate); TX-100, Triton X-100; DOC, sodium deoxycholate; DTT, dithiothreitol; PMSF, phenylmethanesulphonyl fluoride; dNM, deoxynojirimycin; dMM, deoxymannojirimycin.

transport to the cell surface. Several studies have demonstrated that glucose trimming in the ER is a prerequisite for an efficient exit of secretory or membrane glycoproteins from the ER (Peyrieras et al., 1983; Lodish and Kong, 1984; Lemansky et al., 1984; Repp et al., 1985; Arakaki et al., 1987; McDowell et al., 1987; Duronio et al., 1988; Danielsen, 1992), suggesting that the ER constitutes the rate-limiting step for efficient transport. Nevertheless, it seems that a second rate-limiting step, perhaps in the Golgi apparatus, may exist along the exocytotic pathway of proteins. This is supported by observations that the synchronous protein transport of sucrase-isomaltase (SI) and dipeptidyl-peptidase IV (DPPIV) in Caco-2 cells is due to two rate-limiting steps, a pre-Golgi and an intra-Golgi step (Stieger et al., 1988). Moreover, in one case of congenital SI deficiency ('CSID'), we demonstrated that SI exits from the ER efficiently, but its transport is blocked in the medial or *trans*-Golgi (Naim et al., 1988c), suggesting that a rate-limiting step in the Golgi exists. This rate-limiting step could be associated with conformational/structural alterations in the secretory protein induced by carbohydrate modifications in the Golgi apparatus.

In the present paper I therefore investigated the biosynthesis, transport and secretion of ACE, with particular emphasis on the implication of the quaternary structure and the glycosylation pattern of ACE in these processes.

EXPERIMENTAL

Materials

Organ-tissue-culture dishes (60 mm × 15 mm style with centre well) were obtained from Falcon Division, Becton, Dickinson and Co. RPMI 1640, methionine-free RPMI 1640, streptomycin, penicillin and foetal-calf serum were purchased from Gibco. Trans-[³⁵S]methionine (> 1000 Ci/mmol, translation grade) was purchased from ICN. Amplify fluorographic reagent was obtained from Amersham. Mouse immunoglobulins, pepstatin, leupeptin, benzamidin, aprotinin, antipain and molecular-mass standards for SDS/PAGE were purchased from Sigma. Acrylamide, *NN'*-methylenebisacrylamide, SDS, Tris, *NNN'*-tetramethylenediamine, ammonium persulphate, 2-mercaptoethanol, dithiothreitol (DTT) and Triton X-100 (TX-100) were obtained from Bio-Rad. Endo- β -*N*-acetylglucosaminidase H (endo H) (from a recombinant *Streptomyces lividans* strain carrying the *Streptomyces plicatus* gene), endo- β -*N*-acetylglucosaminidase F (containing *N*-glycopeptidase F or *N*-glycanase; endo F/GF), deoxynojirimycin (dNM), deoxymannojirimycin (dMM) and phenylmethanesulphonyl fluoride (PMSF) were obtained from Boehringer-Mannheim. Protein A-Sepharose was purchased from Pharmacia. The Protein A-Sepharose beads were washed alternately with 0.1 M sodium phosphate, pH 8.1, and 0.1 M sodium citrate, pH 4.0, three times with each buffer, and three times with PBS, pH 7.4, and finally suspended in an equal volume of PBS (denoted Protein A-Sepharose suspension). All other reagents were of superior analytical grade.

Biological and immunological materials

Human small-intestinal mucosa (approx. 5–10 mg wet wt.) were obtained from patients biopsied for diagnostic purposes. They were examined by light microscopy and showed normal intestinal morphology with villi structures, and expressed normal levels of sucrase and lactase [sucrase 28–80 units/g and lactase 16–49 units/g, determined as described by Asp et al. (1975)].

Monoclonal antibodies against ACE (product of hybridoma HBB 3/264/18), lactase-phlorrhizin hydrolase (LPH) (HBB 1/909/34/74), SI (HBB 2/219/88), DPPIV (HBB 3/775/42),

aminopeptidase N (ApN) (HBB 3/153/63) and maltase-glucoamylase (MGA) (HBB 2/143/17) were generously given by Dr. Hans-Peter Hauri (Biocenter, Basel, Switzerland) (Hauri et al., 1985) and used in the form of ascites prepared from hybridoma-bearing pristane-primed Balb/c mice. The specificities of the antibodies were assessed by enzyme-activity measurements on immunoisolated antigens (Hauri et al., 1985) and confirmed in our laboratory by following the same experimental protocol (Naim, 1992). For immunoprecipitation experiments, 2–3 μ l of the anti-ACE-antibody (for control experiments, anti-LPH, anti-SI, anti-ApN, anti-DPPIV or anti-MGA) was added to 60 μ l of Protein A-Sepharose suspension and 1 ml 0.1 M phosphate buffer, pH 8.0, and incubated for 1 h at 4 °C. The beads (denoted 'immunobeads') were washed with phosphate buffer and further used for immunoisolation of the corresponding antigen.

The negative controls of the immunoprecipitations employed non-immune mouse immunoglobulins that were coupled to Protein A-Sepharose. Here, 10 μ l of mouse immunoglobulins (1 mg/ml in PBS) was added to 60 μ l of Protein A-Sepharose and further prepared as above (denoted 'control beads').

Biosynthetic labelling of biopsy specimens

Human intestinal biopsy specimens were biosynthetically labelled essentially as described by Naim et al. (1987). In brief, each biopsy specimen was placed on stainless-steel grids in organ-culture dishes (Browning and Trier, 1969) and labelled with 1 ml of an incubation medium containing methionine-free RPMI 1640, streptomycin (100 μ g/ml), penicillin (100 units/ml), 10 % dialysed foetal-calf serum and 150 μ Ci of [³⁵S]methionine. Labelling was performed for 6 h. In some experiments, the biopsy samples were labelled for 15 min with [³⁵S]methionine, followed by chase with 2.5 mM unlabelled methionine for different periods of time. 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 benzamidin, 5 μ g of antipain and 1 μ g of aprotinin) were immediately added. The media were then centrifuged at 100 000 *g* for 30 min, and 800 μ l (instead of 1 ml) portions of the supernatants were recovered to avoid contamination with possibly existing membrane pellet. 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 inhibitors of glycosidases. Here, 5 mM dNM, an inhibitor of the ER α -glucosidases I and II (Romero et al., 1983), and 5 mM dMM, an inhibitor of the *cis*-Golgi α -mannosidase I (Fuhrmann et al., 1984) were used. The inhibitors were present in the preincubation and labelling media. Each biopsy sample was labelled with 150 μ Ci of [³⁵S]methionine for 30 min and chased for 4 h with the incubation medium supplemented with 2.5 mM unlabelled methionine and the glycosidase inhibitors. After labelling, the media and the biopsy samples were further processed as described above.

Immunoprecipitations

Immunoprecipitations were performed essentially as described by Naim et al. (1987). In brief, biopsy homogenates were solubilized on ice with Triton X-100 (TX-100) and sodium deoxycholate (DOC) (final concns. 0.5 %). The solubilized ma-

terial was centrifuged at 100 000 *g* for 1 h at 4 °C. The supernatant was pre-cleared once with mouse immunoglobulins bound to Protein A-Sepharose (control beads), followed by two pre-clearings with Protein A-Sepharose alone (35 μ l of packed beads). The pre-cleared lysates were transferred to tubes containing 40 μ l of anti-ACE immunobeads (or anti-LPH, anti-ApN, anti-SI, anti-MGA or anti-DPPIV immunobeads). After 2 h at 4 °C the beads were washed four times with PBS containing 0.5% TX-100, 0.05% DOC and 0.01% SDS, and then twice with 125 mM Tris/HCl/500 mM NaCl/1 mM EDTA/0.5% TX-100, pH 8.1.

Immunoprecipitation of the culture media with anti-ACE (or anti-ApN, anti-LPH, anti-SI, anti-MGA, anti-DPPIV) was performed as described for the biopsy samples. Before the immunoprecipitations, the culture media were centrifuged at 100 000 *g* for 1 h at 4 °C and the supernatants were further used.

SDS/PAGE

One-dimensional electrophoresis was conducted in 5%- or 6%-polyacrylamide slab gels containing 0.1% SDS as described by Laemmli (1970). Immunoprecipitates were dissolved in 80 mM Tris/HCl (pH 6.8)/0.1 M DTT/4% SDS/10% glycerol/0.01% Bromophenol Blue (sample buffer) at 100 °C for 5 min before layering on to the gel. The following molecular-mass standards were used: myosin, 202 kDa; β -galactosidase, 116 kDa; phosphorylase *b*, 97.5 kDa; BSA, 68 000; ovalbumin, 46 000. The gels were stained with Coomassie Blue, destained, soaked in Amplify, dried, and kept in contact with Kodak SO-282 films. The films were scanned in a Molecular Dynamics Computing Densitometer.

Other procedures

Digestions of ³⁵S-labelled immunoprecipitates with endo H and endo-*N*- β -acetylglucosaminidase F/*N*-glycopeptidase F (endo F/GF) were performed as previously described (Naim et al., 1987), by using 3 m-units of endo H and 0.2 unit of endo F/GF for each sample. Digested proteins were recovered by precipitation with trichloroacetic acid (final concn. 15%, w/v) and the pellets were washed twice with ice-cold acetone and kept at -20 °C. Cross-linking of biopsy homogenates with dithiobis-(succinimidylpropionate) (DSP) was performed as described by Naim et al. (1988b).

RESULTS

Identification of hydrophilic forms of ACE in the culture medium of intestinal biopsy samples

ACE has been shown to exist in both membrane-bound and circulating, hydrophilic, forms (Das et al., 1977; Hooper et al., 1987; Ehlers and Riordan, 1989). I recently identified ACE in the intestinal brush-border membrane and demonstrated that it has a transmembrane orientation (Naim, 1992). It was therefore decided to examine the presence of hydrophilic or secreted forms of ACE in human intestinal biopsy samples and to identify these molecular forms.

For this purpose, human biopsy specimens were labelled for 4 h with [³⁵S]methionine. The culture medium and the biopsy homogenates were further processed for immunoprecipitation with anti-ACE antibodies. Figure 1(a) shows that the culture medium contained immunoprecipitable ACE molecules (lane 3), which had an apparent molecular mass similar to that of the brush-border membrane form (184 kDa) (Figure 1a, lane 1) (both forms are compared in lanes 5 and 6). Molecular species

corresponding to the high-mannose precursor of ACE (174 kDa) (Figure 1a, lane 1) were not discerned in the medium (Figure 1a, lane 3). The 184 kDa species found in the biopsy sample, as well as the 184 kDa species found in the culture medium, were only slightly sensitive to treatment with endo H, which cleaves mannose-rich carbohydrates (Tarentino and Maley, 1974) (compare lanes 2 and 4 in Figure 1a; see also Figure 5, lanes 1 and 2), indicating that both forms are complex glycosylated species and have passed the Golgi apparatus. By contrast, the 174 kDa form was completely sensitive to endo H and was converted into 150 kDa form (Figure 1a, lane 2), demonstrating that it is the mannose-rich precursor of ACE.

The control employed two integral membrane and non-secretory brush-border glycoproteins, LPH and ApN (for reviews see Kenny and Maroux, 1982; Semenza, 1986). As shown in Figure 1(b), neither LPH nor ApN was precipitated from the culture medium (lanes 2 and 4 respectively), whereas the membranes from the biopsy samples contained the mannose-rich and complex-glycosylated forms of these two proteins (Figure 1b, lanes 1 and 3) (Naim et al., 1987, 1988b).

The following lines of evidence demonstrate that ACE precipitated from the culture medium is not an artifact due to ACE present in desquamated epithelial cells and that it represents a soluble form of the enzyme. (i) It was immunoprecipitated from the supernatant of the culture medium that had been centrifuged at 100 000 *g* for 1 h. (ii) Purification of ACE from the culture medium did not require prior treatment of the latter with detergents, such as TX-100 or DOC, thus emphasizing the soluble nature of ACE found in the culture medium. (iii) Intracellular mannose-rich forms of ACE were not recovered in

Figure 1 (a) Secretion of ACE in intestinal biopsy specimens, (b) LPH and ApN are not secreted into the culture medium of intestinal biopsy samples

(a) Biopsy samples were biosynthetically labelled for 6 h with [³⁵S]methionine. The biopsy homogenates (H) (lanes 1, 2 and 5) and the culture medium (M) (lanes 3, 4 and 6) were processed for immunoprecipitation with anti-ACE antibodies. The immunoprecipitates were analysed by SDS-PAGE on 5% gels after endo H treatment (lanes 2 and 4) or without treatment (lanes 1, 3, 5 and 6). A fluorogram of the gel is shown. For better comparison of the molecular forms of membrane-bound ACE (lane 1) and secreted ACE (lane 3), these two lanes were placed next to each other in lanes 5 and 6. The migration positions of molecular-mass standards are indicated on the right. (b) In the same experiment as in (a), the biopsy homogenates (H) (lanes 1 and 3) and the culture medium (M) (lanes 2 and 4) were immunoprecipitated with antibodies directed against two membrane-associated brush-border glycoproteins, LPH (lanes 1 and 2) and ApN (lanes 3 and 4). Note that LPH (lanes 1 and 2) and ApN (lanes 3 and 4) were run on two different gels.

Figure 2 Pulse-chase analysis of ACE

(a) Biopsy samples were pulse-labelled for 15 min with [35 S]methionine and chased for the indicated times with unlabelled methionine, and then homogenized, solubilized and immunoprecipitated with anti-ACE antibodies. Each immunoprecipitate was divided into two equal parts, one of which was treated with endo H (lanes 2, 4, 6, 8, 10, and 12). The samples were analysed by SDS/PAGE on 5% slab gels. The migration positions of molecular-mass standards are indicated on the left. (b) The culture media of the biopsy samples in (a) were pre-cleared with mouse immunoglobulins to identify non-specific protein bands (lanes 13–16) and then immunoprecipitated with anti-ACE (lanes 1–6) or anti-SI (lanes 7–12). The arrowheads mark the non-specific protein band. The migration positions of molecular-mass standards are indicated on the right. (c) The labelling intensities of mannose-rich ACE (ACEman), complex-glycosylated ACE (ACEcplx) and secreted ACE (ACEsec) were evaluated by densitometric scanning of the fluorograms in (a) (lanes 1, 3, 5, 7, 9 and 11) and (b) (lanes 1–6). The proportion of each molecular form in total ACE (i.e. ACEman, ACEcplx and ACEsec) was calculated and plotted versus chase time.

the culture medium, indicating that the mucosal cells were not leaky and remained viable during the labelling experiments. Finally, (iv), the two integral membrane glycoproteins LPH and ApN could not be isolated from the same culture medium.

In the following text, hydrophilic or secreted ACE will be referred to as ACEsec and membrane-bound ACE as ACEm.

Biosynthesis, transport and secretion of ACE

I have recently demonstrated by cross-linking and density-gradient centrifugation that membrane-bound ACE is a monomeric molecule. An increasing body of information assumes that glycoproteins dimerize or oligomerize before their exit from the ER (Gething et al., 1986; Kreis and Lodish, 1986). I therefore

sought to determine whether the monomeric structure of ACE affects its transport efficiency and secretion. For this purpose, the transport kinetics of newly synthesized ACE were investigated by employing a pulse-chase protocol. For this, human intestinal explants were pulse-labelled with [35 S]methionine for 15 min, followed by chases with unlabelled methionine for different intervals of time. ACE was immunoprecipitated and subjected to SDS/PAGE with or without prior treatment with endo H. Endo H sensitivity is indicative of early forms of glycoproteins localized in the ER. Figure 2(a) shows a typical maturation pattern of ACE. In these experiments two main forms were detected that could be discriminated on the basis of their sensitivity towards endo H: the mannose-rich precursor (174 kDa) and the brush-border, mature and complex-

Figure 3 Pulse-chase analysis of DPPIV

(a) Biopsy samples were pulse-labelled for 15 min with [35 S]methionine and chased for the indicated times with unlabelled methionine, and then homogenized, solubilized and immunoprecipitated with anti-DPPIV antibodies. Each immunoprecipitate was divided into two equal parts, one of which was treated with endo H (lanes 2, 4, 6, 8, and 10). The samples were analysed by SDS/PAGE on 6% slab gels. The migration positions of molecular-mass standards are indicated on the right. (b) The labelling intensities of mannose-rich DPPIV (DPPIVman) and complex-glycosylated DPPIV (DPPIVcplx) were evaluated by densitometric scanning of the fluorograms in (a) (lanes 1, 3, 5, 7 and 9). The proportion of each molecular form in total DPPIV (i.e. DPPIVman and DPPIVcplx) was calculated and plotted versus chase time.

glycosylated form (184 kDa) (see Naim, 1992). The mannose-rich ACE precursor was the earliest detectable form after 0 min of chase (Figure 2a, lane 1) and was converted by endo H into the 150 kDa non-glycosylated polypeptide (Figure 2a, lane 2). At 30 min into the chase, the brush-border-membrane form of ACE (184 kDa) started to appear (Figure 2a, lane 3). With prolonged chase periods the intensity of this form increased, concomitantly with decrease in that of mannose-rich ACE (lanes 5, 7, 9 and 11). The partial sensitivity of this form to endo H was maintained throughout the chase time up to 240 min (lanes 6, 8, 10 and 12) and for longer chase periods (results not shown). This indicates that no further processing of the carbohydrate chains occurred after the initial detection of the 184 kDa glycoprotein. The transport kinetics of ACE from the ER to the Golgi apparatus were evaluated in terms of the rate of conversion of the mannose-rich precursor into the mature form, as assessed by comparing the labelling intensities of the two species by densitometric scannings

Figure 4 Cross-linking of (a) DPPIV, (b) SI and (c) MGA

(a) Biopsy samples were labelled for 10 min with [35 S]methionine, homogenized and divided into two equal parts; one part was cross-linked with DSP (lane 2) and the other was not (lane 1). Cross-linked and non-cross-linked samples were solubilized and immunoprecipitated with anti-DPPIV antibodies. The immunoprecipitates were analysed by SDS-PAGE on 5% slab gels under reducing (lane 1) or non-reducing (lane 2) conditions. The migration positions of molecular-mass standards are indicated on the right. (b) As in (a), but samples were immunoprecipitated with anti-SI antibodies. Lane 1 is non-cross-linked sample; lane 2 is cross-linked sample. (c) As in (a), but samples were immunoprecipitated with anti-MGA antibodies. Lane 1 is non-cross-linked sample; lane 2 is cross-linked sample.

(Figure 2c). The finding that ACE could be secreted into the medium provides a useful tool to study the post-Golgi transport of this molecule. Therefore, I studied the secretion of ACE by immunoprecipitation from the culture medium with anti-ACE antibodies. The negative control employed immunoprecipitations of the culture medium with antibodies directed against SI (a non-secretory brush-border membrane component) (Semenza, 1986). Moreover, the media were pre-cleared before the immunoprecipitations with mouse immunoglobulins. Figure 2(b) shows that ACEsec was immunoprecipitated from the culture medium after 90 min of chase (lane 4). The intensity of this band increased considerably after 240 min of chase (Figure 2b, lane 6). The increase in the labelling intensity of ACEsec paralleled the decrease in the intensity of complex-glycosylated ACE (ACEm) during the chase (Figure 2c; and compare lanes 9 and 11 in Figure 2a).

Immunoprecipitations of the media with anti-SI (Figure 2b, lanes 7–12) did not reveal polypeptides corresponding to SI, confirming that this molecule is not secreted. It should be noted that these immunoprecipitations (with anti-ACE and anti-SI) revealed a sharp band of slightly higher molecular mass than that of ACE (indicated by the arrowheads). This band is non-specific, since it was also found in the pre-clearings with non-immune mouse serum (Figure 2b, lanes 13–16, indicated by the arrowhead). Moreover, this band could be eliminated by subjecting the medium to several cycles of pre-clearings with non-immune immunoglobulins before isolation of ACE (Figures 1a and 1b represent immunoprecipitation of the culture medium with anti-ACE, anti-SI and anti-ApN antibodies after pre-clearings with non-immune serum). The proportions of mannose-rich, complex-glycosylated and secreted ACE in total synthesized and processed ACE were quantified by scanning densitometry. The results depicted in Figure 2(c) revealed a precursor/product(s) relationship between the mannose-rich ACE, on one hand, and the complex-glycosylated molecule and secreted forms on the other. In fact, the proportion of mannose-rich ACE (ACEman) de-

Figure 5 Processing of ACE in the presence of inhibitors of oligosaccharide processing

Biopsy samples were biosynthetically labelled with [35 S]methionine in the presence of dNM (lanes 4–6 and 11), dMM (lanes 7–9 and 12) or in the absence of the inhibitors (c) (lanes 1–3 and 10) for 30 min and chased with unlabelled methionine for 4 h. Detergent extracts of the biopsy samples were immunoprecipitated with anti-ACE, and each immunoprecipitate was divided into three equal parts; one was treated with endo H (lanes 2, 5 and 8), another with endo F/GF (lanes 3, 6 and 9) and one was not treated with either endoglycosidase (lanes 1, 4 and 7). The culture media were also immunoprecipitated with anti-ACE antibodies (lanes 10–12). Samples were analysed by SDS/PAGE on 5% slab gels, followed by fluorography. The arrowhead marks the non-specific protein band obtained also by pre-clearing of the culture media with non-immune mouse immunoglobulins (see also Figure 2b). The migration positions of molecular-mass standards are indicated on the left.

creased with increasing chase times. By contrast, the proportion of complex-glycosylated ACE (ACE_{cplx}) increased and reached a maximum at 2 h of chase, followed by a decrease after 4 h of chase. This is concomitant with an increase in the proportion of secreted ACE (ACE_{sec}).

The processing and transport kinetics of ACE were compared with those of another brush border glycoprotein, namely DPPIV. Figure 3(a) shows that the most predominantly labelled form of DPPIV after 0 min of chase was a single polypeptide of 105 kDa (lane 1). This band represents the mannose-rich form of DPPIV, since it was sensitive to treatment with endo H and was converted into a 82 kDa form (Figure 3a, lane 2). A faint diffuse band of 120 kDa was also detected, which became strongly labelled after 30 min of chase (Figure 3a, lane 3). This band was resistant to endo H treatment (Figure 3a, lane 4), indicating that it is the complex-glycosylated mature form of DPPIV. The fact that the complex-glycosylated DPPIV molecule had already appeared after 0 min of chase is compatible with a rapid transport of mannose-rich DPPIV to the Golgi, where processing to the complex-glycosylated form takes place. Almost 90% of the mannose-rich DPPIV was converted into the complex-glycosylated molecule after 60 min of chase (Figure 3a, lane 5). Analysis of the fluorogram by densitometric scanning demonstrated that approx. 24 min was required for the conversion of 50% of mannose-rich DPPIV into the complex-glycosylated form (Figure 3b). This is consistent with data obtained in Caco-2 cells (Hauri et al., 1985). Therefore the transport of DPPIV from the ER to the Golgi, the site of complex glycosylation, is almost 3 times faster than that of ACE. Since ACE is a monomer,

I sought to determine whether the more rapid transport kinetics to the Golgi are due to dimer formation of mannose-rich forms of DPPIV in the ER. Here, biopsy samples were labelled for 10 min with [35 S]methionine and the biopsy homogenates were chemically cross-linked with DSP as described previously for brush-border membranes (Naim et al., 1988b; Naim, 1992). Figure 4(a) demonstrates that cross-linking has completely converted the mannose-rich DPPIV (105 kDa) (lane 1) into a high-molecular-mass polypeptide of 200–220 kDa (lane 2). By contrast, the mannose-rich forms of human SI (210 kDa) (Figure 4b, lanes 1 and 2) and human MGA (285 kDa) (Figure 4c, lanes 1 and 2) obtained after 10 min of labelling (Naim et al., 1988a,b) were not cross-linked. The results therefore demonstrate that the mannose-rich form of DPPIV forms dimers (2×105 kDa), whereas those of SI and MGA do not. This indicates that this step is important before complex glycosylation of DPPIV.

The transport kinetics of ACE from the ER to the Golgi occur at a significantly slower rate than those estimated for another brush-border peptidase, namely ApN (30 min) (Hauri et al., 1985; Naim et al., 1988b) and at a rate comparable with those of the human disaccharidases SI (85 min) (Hauri et al., 1985; Naim et al., 1988a) and MGA (75–80 min) (Naim et al., 1988b). ApN, like DPPIV, has been shown to require homodimer formation in the ER for efficient transport (Danielsen, 1990). By contrast, neither human SI (Jascur et al., 1991; the present paper) nor human MGA (Naim et al., 1988b; the present paper) is thought to form dimers in the ER or Golgi. It appears therefore that the quaternary structure of brush-border enzymes could be directly involved in affecting their transport rates. It should be noted that homodimers of pig intestinal SI and MGA have been identified (Norén et al., 1986; Cowell et al., 1986). However, the site of dimerization of these molecules has not been characterized.

Transport and secretion of ACE are affected by carbohydrate modification

Carbohydrate processing of membrane and secretory proteins in the ER and in the Golgi apparatus is an important post-translational modification that could play a role in protein transport to the cell surface (Peyrieras et al., 1983; Lodish and Kong, 1984; Lemansky et al., 1984; Repp et al., 1985; Arakaki et al., 1987; McDowell et al., 1987; Duronio et al., 1988). To determine whether processing and modification of the sugar residues in the ER and in the Golgi are important in the intracellular transport and secretion of ACE, glycosidase inhibitors were utilized in biosynthetic labelling experiments. Human intestinal biopsy samples were labelled for 30 min with [35 S]methionine and chased for 4 h with non-radioactive methionine. The culture media and detergent extracts of the biopsy samples were immunoprecipitated with anti-ACE antibodies (Hauri et al., 1985) and analysed by SDS/PAGE.

After 4 h of chase the control biopsy revealed a single band with an apparent molecular mass of 184 kDa (Figure 5, lane 1). This polypeptide was only slightly sensitive to treatment with endo H, which cleaves mannose-rich oligosaccharides (Tarentino and Maley, 1974) (Figure 5, lane 2), whereas endo F/GF, which cleaves mannose-rich as well as complex-glycosylated N-linked glycans (Plummer et al., 1984) converted it into the 150 kDa species (Figure 5, lane 3).

To examine the influence of the initial steps of glucose trimming in the ER (Kornfeld and Kornfeld, 1985) on the transport and further secretion of ACE, I treated human intestinal biopsy samples with dNM. dNM inhibits the removal of the first glucose residue from the N-linked oligosaccharide chain, Glc₃Man₉GlcNAc₂, by α -glucosidase I, as well as the second and

Table 1 Effect of glycosidase inhibitors on the secretion of ACE

The values are means ($\pm$ S.E.M.) of duplicate determinations obtained in two experiments.

Inhibitor	Percentage in total ACE		ACEm/ACEsec	ACEm/ACEsec (inhibitor) ACEm/ACEsec (control)
	ACEm	ACEsec		
No inhibitor	50.5 $\pm$ 2.6	49.5 $\pm$ 1.4	1.0225 $\pm$ 0.08	—
dNM	66.0 $\pm$ 3.0	34.0 $\pm$ 0.7	1.94 $\pm$ 0.128	1.92 $\pm$ 0.275
dMM	70.0 $\pm$ 1.8	30.0 $\pm$ 2.3	2.370 $\pm$ 0.26	2.35 $\pm$ 0.44

third glucose residues by α -glucosidase II (Romero et al., 1983). Figure 5 demonstrates that ACE immunoprecipitated from the dNM-treated biopsy specimen revealed a polypeptide of a slightly higher apparent molecular mass (lane 7) than ACE obtained in the control (lane 1). This polypeptide revealed marked sensitivity towards endo H (Figure 5, lane 8), indicating that some glycan units are still not processed to complex-type. Endo F/GF converted this form into the 150 kDa species (Figure 5, lane 9). The most significant effect on the oligosaccharide processing of ACE was observed with dMM. dMM inhibits the first steps in the processing of mannose-rich oligosaccharides by blocking the removal of four α -1,2-linked mannose-residues by the *cis*-Golgi α -mannosidase I (Fuhrmann et al., 1984). In the presence of dMM the apparent molecular mass of ACE was substantially decreased (Figure 5, lane 4). This glycoprotein showed considerable sensitivity towards treatment with endo H, indicating that it contains a significant proportion of unprocessed mannose-rich glycans. Treatment with endo F/GF (Figure 5, lane 6) generated a glycoprotein similar in its apparent molecular mass to those obtained in the control and dNM-treated samples (Figure 5, lanes 3 and 9 respectively). The culture media were immunoprecipitated with anti-ACE to determine whether the observed alterations in the glycosylation pattern have effects on the secretion of ACE (ACEsec). Figure 5 shows that modified forms of ACEsec could be detected in the medium derived from a dMM-treated biopsy (lane 11), and in the medium of a dNM-treated sample (lane 12), whereas the control medium contained the 184 kDa species (lane 10).

The labelling intensities of these forms and of ACEm in the biopsy lysates were compared with each other by densitometric scannings (Table 1). The results revealed a marked decrease in the proportion of ACEsec in total ACE in the presence of dNM and dMM. Thus after 4 h of chase 30% and 34% of ACE was secreted in the presence of dMM and dNM respectively, whereas almost 50% was secreted in the control sample. The resulting ratios of membrane-bound ACE (ACEm) to ACEsec were considerably higher in the presence of the glycosidase inhibitors than in the control (more than 2-fold), indicating that a significant delay in the secretion of ACE has occurred.

Although inhibition of oligosaccharide modification did not dramatically affect the transport of ACE, the observed effects suggest that the glycosylation pattern of ACE co-operates with some other structural determinants in giving the ACE molecule a transport-competent conformation.

DISCUSSION

An interesting feature of the ACE molecule is its existence in both circulating hydrophilic forms and membrane-bound forms (Ehlers and Riordan, 1989). Consistent with this is the identifi-

cation of secreted forms of human intestinal ACE in the culture medium of biosynthetically labelled biopsy samples. By contrast, LPH, SI and ApN, used in this study as controls of integral membrane-associated brush-border enzymes, are not secreted into the culture medium. The generation of secreted ACE forms may involve an EDTA-dependent proteolytic cleavage of membrane-bound ACE at the membrane, similar to that demonstrated for the pig kidney molecule (Hooper et al., 1987). More recently, Oppong and Hooper (1993) have characterized a secretase activity in the brush-border membranes of pig kidney cells that is responsible for the generation of secretory ACE forms. However, the secretase activity was found to be very low or even negligible in the duodenum or jejunum of pig or human small intestine, suggesting that other cellular mechanisms might be implicated in the generation of ACEsec in intestinal cells. A proteolytic cleavage of ACEsec from ACEm would be compatible with a shift in the size of the hydrophilic form of about 5–10 kDa, since ACEm is a transmembrane protein, i.e. contains a membrane anchor and a cytoplasmic tail (Soubrier et al., 1988; Bernstein et al., 1989; Kumar et al., 1989; Naim, 1992). However, such an apparent molecular-mass difference could not be discerned in our experiments, suggesting that mechanisms other than proteolytic cleavage should be considered to be involved in the generation of ACEsec. Another possibility is that intestinal cells express a heterogeneous population of differently glycosylated membrane-bound ACE molecules (ACEm), as has been shown for SI and LPH (Naim et al., 1988a; Naim and Lentze, 1992). Due to variations in the glycosylation pattern, some of these ACEm molecules are more susceptible to proteases in the membrane than are others, and are ultimately cleaved to ACEsec. According to this mechanism, it is possible that ACEsec is generated from a more extensively glycosylated population of ACEm, whereas the remainder of ACEm is less glycosylated. The increased glycosylation of ACEsec would then explain why no molecular-mass shift between ACEm and ACEsec could be detected. Analysis of the glycosylation patterns of ACEsec and ACEm will be required to examine this possibility. It is therefore important to unravel the mechanism of secretion of ACE in intestinal cells and compare it with the mechanisms prevailing in other systems.

Analysis of the biosynthesis and intracellular transport of ACE demonstrated that the monomeric structure of ACE has substantial effects on its transport to the Golgi. In fact, the transport kinetics of ACE are 2–3-fold slower than for the dimeric proteins ApN (Hauri et al., 1985; Naim et al., 1988b) and DPPIV. Current views suggest that dimerization or oligomerization of secretory and membrane glycoproteins is an essential event that precedes protein transport from the ER to the Golgi membranes (Gething et al., 1986; Kreis and Lodish, 1986). Analysis of the quaternary structure of several brush-

border enzymes provides only limited support to this view. Although human SI in Caco-2 cells (Jascur et al., 1991) and, in intestinal mucosa, human MGA and human ACE (the present paper) do not form homodimers in the ER, homodimerization of the mannose-rich precursors of pig intestinal ApN (Danielsen, 1990) and human intestinal DPPIV (the present paper) takes place, suggesting that this event may be important before exit of these proteins from the ER. A correlation between the quaternary state of these proteins and their transport from the ER to the Golgi is suggested by the findings that the dimeric proteins ApN and DPPIV are transported to the Golgi at a rate 2–3-fold faster than for the monomeric proteins ACE, human SI and human MGA. It seems therefore that homodimerization may affect the ER-to-Golgi transport rates, but does not constitute an absolute requirement for proteins to leave the ER.

On the other hand, by virtue of its being composed of two virtually similar domains (Soubrier et al., 1988; Bernstein et al., 1989; Ehlers and Riordan, 1989), ACE may fold into pseudodimers that are transport-competent and could leave the ER. A similar type of dimer formation has also been suggested to be responsible for the transport of SI to the Golgi (Jascur et al., 1991). Analysis of the individual domains of ACE could provide important clues as to the implication of these domains in endowing ACE with a transport-competent conformation.

Another post-translational modification that has marked effects on the transport and secretion of ACE is carbohydrate modification in the ER and Golgi. Inhibition of these modifications leads to a delayed transport of ACE to the cell surface and its secretion into the exterior milieu. The results suggest therefore that oligosaccharide modifications in the ER and the Golgi apparatus induce alterations in the three-dimensional structure or folding of the ACE molecule, thus affecting its transport competency. Previous studies have shown that glucose trimming in the ER is necessary for an efficient transport of many secretory, membrane and lysosomal glycoproteins (Peyrieras et al., 1983; Lodish and Kong, 1984; Lemansky et al., 1984; Repp et al., 1985; Arakaki et al., 1987; McDowell et al., 1987; Duronio et al., 1988; Danielsen, 1992). I have also shown that trimming of the core sugars in the ER is implicated in the acquisition of transport-competent conformation of human intestinal LPH (H. Y. Naim, unpublished work). Most of these studies, if not all, are consistent with the notion that the exit from the ER of secretory and membrane proteins constitutes the rate-limiting step along their secretory pathway. A comparable influence of carbohydrate modifications in the Golgi on the intracellular transport of secretory proteins has not been well documented. Our finding, therefore, that inhibition of glycan processing in the *cis*-Golgi has substantially affected the rate of transport and secretion of ACE strongly suggests that a rate-limiting step in the Golgi exists along the secretory pathway of ACE. This interpretation is supported by the observations that the asynchronous protein transport of SI and DPPIV in Caco-2 cells is due to two rate-limiting steps, a pre-Golgi and an intra-Golgi step (Stieger et al., 1988). Moreover, in one case of congenital sucrase-isomaltase deficiency ('CSID'), we demonstrated that SI exits from the ER efficiently, but its transport is blocked in the medial or *trans*-Golgi (Naim et al., 1988c), suggesting that a rate-limiting step in the Golgi exists.

It seems that conformational/structural alterations in secretory and membrane proteins induced by carbohydrate modifications in the ER or in the Golgi apparatus may affect their transport rates. Nevertheless, an effect may only be detected if the alterations take place in the vicinity of, or in structural motifs of, the protein that are implicated in intercompartmental transport. This may explain why in some cases oligosaccharide processing

does not have substantial effects on protein transport (Burke et al., 1984; Schlesinger et al., 1985).

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