Role of Glycosylation in the Biosynthesis and Activity of Rabbit Testicular Angiotensin-Converting Enzyme†

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ABSTRACT: Angiotensin-converting enzyme (ACE) is a type I glycoprotein anchored in the plasma membrane by a hydrophobic domain near its carboxyl terminus. The enzymatically active extracellular domain of ACE is slowly released from the cell by cleavage–removal of its membrane-anchoring carboxyl-terminal region. In the present study, we investigated the role of N- and O-glycosylation in intracellular transport and extracellular cleavage–secretion of rabbit testicular ACE. For ACE expression, we used an *in vitro* translation system, a permanently transfected mouse cell line, and human and Chinese hamster cells transiently transfected with vaccinia virus–T7 RNA polymerase-driven expression vectors. Sugar modifications of ACE were analyzed by testing its sensitivity to specific glycosidases. Cellular protein glycosylation was inhibited by using chemical inhibitors and a mutant cell line defective in protein glycosylation. Our experiments demonstrated that newly synthesized ACE acquires both N- and O-linked sugars before its cleavage–secretion and complete blockage of glycosylation results in rapid intracellular turnover of underglycosylated ACE. However, ACE synthesized without N-linked complex sugars and O-linked sugars can undergo normal transport and cleavage–secretion, and the underglycosylated protein is enzymatically active.

Angiotensin-converting enzyme (EC 3.4.15.1), a peptidyl dipeptidase, plays an important role in maintaining cardiovascular homeostasis by generating the vasopressor angiotensin II from its inactive precursor angiotensin I and by inactivating the vasodepressor peptide bradykinin (Skeggs et al., 1981; Erdos & Yang, 1967; Cushman & Ondetti, 1980). Angiotensin-converting enzyme (ACE)[1] has two isozymic forms, pulmonary (P) and testicular (T), which are expressed in a tissue-specific fashion (Soffer, 1981; El-Dorry et al., 1982a,b). The two isozymes are structurally related and encoded by the same gene (Kumar et al., 1989, 1991; Thekkumkara et al., 1992; Soubrier et al., 1988; Bernstein et al., 1989; Howard et al., 1990; Hubert et al., 1991). Rabbit ACE_T has 737 amino acid residues, and ACE_P has 1309 residues (Kumar et al., 1989; Thekkumkara et al., 1992). The C-terminal 665 residues of the 2 isozymes are identical, whereas their N-termini are unique. Both isozymes are cell-surface type I ectoproteins anchored in the plasma membrane via a 17-residue-long hydrophobic transmembrane domain located 30 residues away from their C-termini. They carry signal sequences at their N-termini that are removed during their biosynthesis. This process is accompanied by extensive glycosylation of the proteins. In tissue culture, the extracellular domain of ACE is released into the culture medium by regulated proteolytic cleavage of the anchoring C-terminal region (Sen et al., 1991; Ehlers et al., 1991; Wei et al., 1991; Ramchandran et al., 1994). A similar C-terminally truncated, soluble form of enzymatically active ACE is also found in many body fluids, including serum (Wei et al., 1991). The ACE biosynthetic pathway, therefore, includes several important posttranslational steps.

The primary structures indicate that rabbit ACE_P has 11 potential N-glycosylation sites and ACE_T has 5 (Kumar et al., 1989; Thekkumkara et al., 1992). In addition, ACE_T has a cluster of threonine residues near the amino terminus that are potential O-glycosylation sites (Kumar et al., 1989). Both isozymes, as isolated from tissues, are heavily glycosylated. Depending on the source, sugars contribute 18–30% of the molecular mass of ACE (Ehlers et al., 1992). It has been shown that removal of sialic acid residues from mature ACE_P does not alter its enzymatic activity (Conroy et al., 1978). Similarly, a mutant ACE_T devoid of most if its O-linked sugars has enzyme activity (Ehlers et al., 1992). However, the biosynthetic roles of N- and O-linked sugars in the proper intracellular transport and cleavage secretion of enzymatically active ACE have not been studied systematically.

Biosynthesis of different glycoproteins is affected differently if glycosylation is blocked. For example, synthesis and secretion of IgM, IgA, and several other proteins are affected by the glycosylation inhibitor tunicamycin. In contrast, the same inhibitor has no effect on the biosynthesis of several other glycoproteins, including IgG (Elbein, 1987). Similarly, tunicamycin-mediated inhibition of glycosylation has variable effects on the enzyme activity of different glycoproteins (Volpe & Goldberg, 1985; Onishi et al., 1979). O-Glycosylation of cell-surface proteins has been suggested to play an important role in their stability, since several such proteins, when synthesized in a mutant CHO cell line that does not allow O-linked glycosylation, are rapidly degraded in the cell (Krieger, 1989).

The current study was undertaken to examine the nature of sugar modifications of rabbit ACE_T synthesized in tissue

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[1] Abbreviations: ACE, angiotensin-converting enzyme; CHO, Chinese hamster ovary; ACE_T, testicular angiotensin-converting enzyme; ACE_P, pulmonary angiotensin-converting enzyme; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Endo H, endo-β-*N*-acetylglucosaminidase H; ER, endoplasmic reticulum.

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culture and to evaluate the roles of specific sugar modifications in its intracellular transport, cleavage–secretion, and enzymatic activity. Our results demonstrate that ACE_T is both N- and O-glycoslyated. They also indicate that some, but not all, sugar modifications are necessary for the proper transport and processing of active ACE.

EXPERIMENTAL PROCEDURES

Transient Expression of ACE Proteins in HeLa Cells and CHO Cells Using the Vaccinia Virus–T7 RNA Polymerase System. Rabbit testicular ACE was expressed in HeLa cells, CHO cells (American Type Culture Collection, CHO-K1), and the mutant CHO cells (*ldl*D, kindly provided by Dr. Monty Krieger, Massachusetts Institute of Technology) using the recombinant vaccinia virus–T7 RNA polymerase transient expression system (Fuerst *et al.*, 1987). cDNA encoding the full-length ACE_T (Sen et al., 1991) was cloned behind the T7 promoter in pGEM7Zf vector (Promega, Madison, WI) and transfected by lipofectin-mediated transfection (Felgner et al., 1987) into HeLa cells that had been infected with the recombinant vaccinia virus, vTF7-3, that carries the T7 RNA polymerase gene. For expressing EACE, which is missing the C-terminal anchoring domain of ACE_T and contains only its first 673 residues, ACE 8 cDNA was digested with *Eag*I, blunted, and then digested with *Eco*RI. The released cDNA was cloned in the pGEM9T vectors (Patel & Sen, 1992) that had been linearized by digestion with *Eco*RI and *Sma*I. The pGEM9T vector provides a T7 promotor on the 5′ side and an in-frame translational termination signal on the 3′ side of the cloned cDNA. EACE in pGEM9T was expressed in HeLa cells using the vaccinia virus–T7 RNA polymerase system; 15–20 h after transfection, the cells were processed for expression analysis by labeling with [^{35}S]methionine.

Cell Labeling and Immunoprecipitation. Cells were pulse-labeled with [^{35}S]methionine and chased for different lengths of time. Cell lysates and medium were immunoprecipitated using an antiserum against pure rabbit ACE_P (kindly provided by Dr. Richard L. Soffer) and analyzed by SDS–PAGE and autoradiography. For experiments with the permanently transfected ACE 89 cells, labeling with [^{35}S]methionine and immunoprecipitation were done according to the procedure described earlier (Sen et al., 1991).

For transiently transfected cells, a similar procedure was followed with some modifications. Before expression analysis by metabolic labeling, the CHO and the *ldl*D cells were grown in serum-free Dulbecco's modified Eagle's medium containing F-12 and ITS mixture (GIBCO) for 24 h. The same medium was used for pulse–chase experiments for these cells. All transfected cells (60-mm plates) were starved in methionine-free medium for 1 h and pulse-labeled for 30 min, and the label was chased in the appropriate medium. Plates were removed at various times, the supernatant medium was aspirated, and the cell lystates were prepared as described earlier. Cell lysates and the medium were precleared by incubation for 90 min with 0.6 μL of normal goat serum and protein A–Sepharose. After centrifugation, immunoprecipitation was carried out using anti-ACE antibody as described for ACE 89 cells. The immunoprecipitates were washed 3 times with 1% Triton X-100 in 50 mM Tris-HCl buffer, pH 7.6, containing 0.1 M NaCl, 1 mM EDTA, 0.1% SDS, and 2 mg/mL BSA, and analyzed by SDS–PAGE. Apparent molecular masses of radioactive bands were determined by reference to the mobilities of standard proteins: phosphorylase *b*, 97 kDa; and bovine serum albumin, 66 kDa.

Treatment with Deglycosylating Enzymes. For endo-β-*N*-acetylglucosaminidase H (Endo H) treatment, cell lysates and medium were immunoprecipitated as described above. The immune complex with protein A–Sepharose was suspended in 40 μL of 1% SDS and heated at 100 °C for 3 min. Water (160 μL) was added, and the sample was heated again for 1 min. The Sepharose beads were removed by centrifugation, and the supernatant was adjusted to 50 mM sodium acetate, pH 5.5, containing 1 mM phenylmethanesulfonyl fluoride, divided into equal aliquots, and incubated with or without 10 milliunits of Endo H (Boehringer Mannheim) for 18 h at 37 °C. The samples were dried, dissolved in SDS–sample buffer, and analyzed by SDS–PAGE.

For *O*-glycosidase treatment, immunoprecipitated protein was eluted as described for Endo H digestion and was adjusted to 50 mM sodium phosphate, pH 7.5, containing 1% Triton X-100, divided into equal aliquots, and incubated with or without 2.5 milliunits of *O*-glycosidase (Boehringer Mannheim) for 18 h at 37 °C. The samples were dried, dissolved in SDS sample buffer, and analyzed by SDS–PAGE.

In Vitro Translation of ACE mRNA. For expression of ACE *in vitro*, cDNA encoding the testicular ACE was linearized by digestion with *Hin*dIII, and transcription was performed using T7 RNA polymerase. The *in vitro* synthesized ACE mRNA was programmed for translation using rabbit reticulocyte lysates and [^{35}S]methionine. The translation was performed in the presence or absence of dog pancreas microsomes. All *in vitro* procedures were performed according to manufacturers' instructions (Promega). The syntheiszed proteins were precipitated with acetone, resuspended in 50 mM Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl, 1 mM EDTA, 0.1% SDS, and 1% Triton X-100, immunoprecipitated, and analyzed by SDS–PAGE and autoradiography before or after digestion with Endo H.

Enzyme Activity Measurements. Sixty-millimeter plates of wild-type CHO or *ldl*D cells transfected with ACE cDNAs were extracted 20-h posttransfection with 200 μL of 50 mM sodium borate buffer, pH 8.3, containing 0.1% Triton X-100 and 112 mM NaCl, in a glass–glass homogenizer. The extract was centrifuged at 50000*g* for 20 min, and the supernatant containing the solubilized ACE was assayed for enzyme activity by using hippuryl-L-histidyl-L-leucine (Hip-His-Leu) as substrate and measuring fluorometrically the His-Leu liberated at varying concentrations of Hip-His-Leu (0.4–5 mM) in 0.4 M sodium borate buffer, pH 8.3, containing 0.3 M NaCl in a volume of 0.5 mL for 15–30 min at 37 °C. The dissociation constant (K_m) was calculated by nonlinear regression using the equation $V = V_{max}[S]/K_m[S]$ where V is the initial velocity and [S] is the concentration of substrate.

RESULTS

Posttranslational Modifications of ACE. For examining the nature of posttranslational processing of ACE_T, we used both *in vitro* and *in vivo* expression systems. We have previously reported *in vitro* translation of rabbit ACE_T mRNA using rabbit reticulocyte lysate (Kumar et al., 1989). Since ACE is a glycoprotein, we examined further processing of the primary translation product in the presence of added microsomal membrane (Figure 1A). ACE mRNA was translated *in vitro* in the presence or absence of added membrane, and the products were immunoprecipitated and digested with Endo H for testing their glycosylation status. Without added membrane, an ACE protein of 79 kDa was synthesized (lane 1). As expected, this protein was resistant to the action of Endo H (lane 2), an enzyme that removes simple sugar residues that are attached to proteins during their synthesis in the endoplasmic reticulum (ER). In the presence of added membrane, an 86-kDa protein was synthesized (lane 3) which

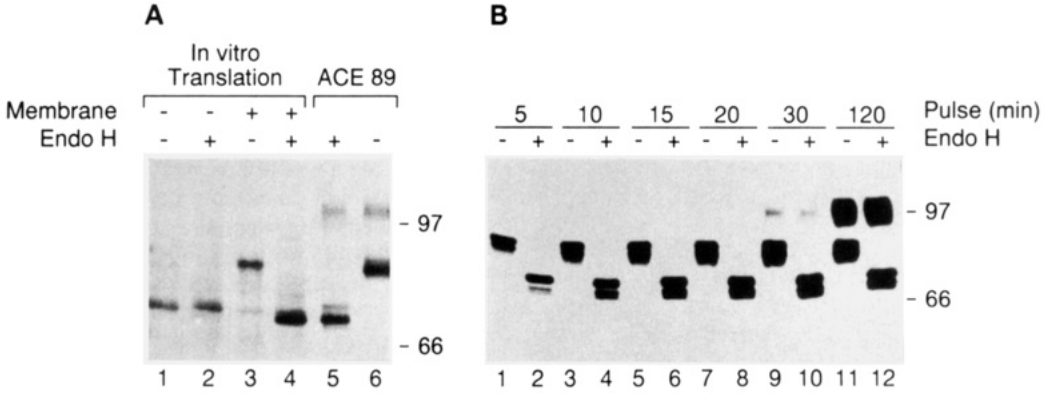

FIGURE 1: (Panel A) *In vitro* translation of ACE mRNA. mRNA was transcribed from the ACE cDNA and translated *in vitro* in the presence of [^{35}S]methionine in the presence (lanes 3, 4) or absence (lanes 1, 2) of microsomal membranes. The translation products were immunoprecipitated and analyzed, before (lanes 1, 3) or after (lanes 2, 4) treatment with Endo H, by gel electrophoresis and fluorography. Lanes 5 and 6 represent Endo H-treated (+) or untreated (–) immunoprecipitates from ACE 89 cells pulse-labeled with [^{35}S]methionine for 30 min. (Panel B) Endo H digestion of ACE labeled with [^{35}S]methionine for different periods of time. ACE 89 cells were pulse-labeled with [^{35}S]methionine for 5–120 min (as indicated). The cell extracts were immunoprecipitated and incubated with (+) or without (–) Endo H and analyzed by electrophoresis and fluorography. Numbers on the right of each panel the sizes of standard proteins in kilodaltons.

was converted to a 76-kDa species upon Endo H digestion (lane 4), indicating that the 86-kDa protein contains simple sugar modifications. The deglycosylated protein (lane 4) had a mobility slightly faster than that of the unglycosylated protein synthesized in the absence of membrane (lane 1). This higher mobility was possibly the result of the cotranslational removal of the signal peptide from the protein synthesized in the presence of microsomal membrane. These results indicate that ACE synthesized *in vitro* in the presence of microsomal membrane undergoes both glycosidic modifications and signal peptide cleavage.

For investigating the nature of posttranslational modifications of ACE as it travels from the ER to the Golgi compartments, we examined the kinetics and the nature of glycosylation of different species of ACE proteins synthesized *in vivo*. ACE_T-producing ACE-89 cells (Sen et al., 1991) were pulse-labeled with [^{35}S]methionine for different lengths of time, and ACE-related proteins were immunoprecipitated and analyzed before and after digestion with Endo H (Figure 1B). Short pulses (5–20 min) produced a closely migrating triplet of about 86 kDa (Figure 1B, lanes 1, 3, 5, 7), which comigrates with the 86-kDa species synthesized *in vitro* in the presence of membranes (Figure 1A, lanes 3 and 6). The 86-kDa species of ACE protein synthesized *in vivo* was completely sensitive to Endo H (Figure 1B, lanes 2, 4, 6, 8, 10, and 12), suggesting that these proteins did not contain any complex sugar modifications and therefore represent the ER form of ACE. Endo H digestion of the *in vivo* synthesized 86-kDa species produced a triplet of about 76 kDa (Figure 1B). The relative proportions of the members of this triplet changed with pulsing time (Figure 1B). It also varied from experiment to experiment (compare Figure 1A, lane 5, Figure 1B, lane 10, and Figure 2, upper panel, second lane). Although the reason for the observed heterogeneity is not clear, it may reflect nonuniform posttranslational modifications of the primary translation products. The mobility of the middle band corresponds most closely to that of the Endo H-treated *in vitro* product (Figure 1A, lanes 4 and 5). The upper band may represent a protein from which the signal peptide has not been removed, because its migration is similar to the primary translation product (compare the upper faint band in lane 5 to the band in lane 2 of Figure 1A). A longer labeling period of ACE89 cells produced another species of ACE-related protein of about 116 kDa (Figure 1B, lanes 9–12). This protein was resistant to Endo H treatment, indicating that it contains complex sugar modifications that occur in the Golgi compartment (Figure 1B, lanes 10 and 12). The transit of newly

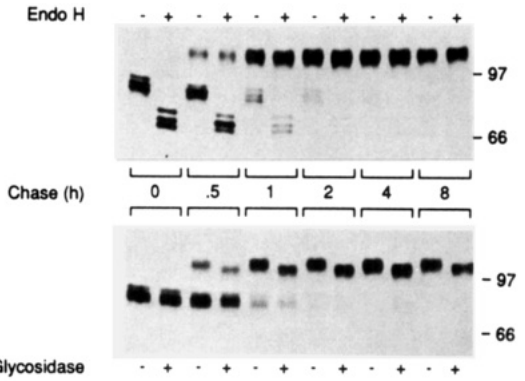

FIGURE 2: Endo H and *O*-glycosidase digestion of ACE labeled with [^{35}S]methionine in ACE 89 cells. The fluorograph shows the results of Endo H digestion (upper panel) or *O*-glycosidase digestion (lower panel) of ACE immunoprecipitates of cell extracts obtained after pulse-labeling of ACE 89 cells with [^{35}S]methionine for 30 min, and then chasing for the indicated periods of time. The absence or presence of Endo H or *O*-glycosidase digestion is indicated by (–) or (+), respectively.

synthesized ACE protein from the ER to the Golgi compartment was confirmed by the pulse–chase experiment shown in Figure 2. The Endo H-sensitive 86-kDa species was chased into the Endo H-resistant 116-kDa species (Figure 2, upper panel). The transit time of newly synthesized ACE from the ER to the Golgi compartment of ACE 89 cells is about 2 h, because that was the time required for the complete conversion of the 86-kDa species to the 116-kDa species. The 116-kDa protein also contained O-linked sugars, because treatment with *O*-glycosidase reduced its apparent molecular mass to 110 kDa (Figure 2, lower panel). Taken together, the observations presented in Figures 1 and 2 indicate that the ACE protein undergoes sequential cotranslational and posttranslational modifications as it transits from the ER to the Golgi compartment. Such modifications include signal peptide cleavage, N-glycosylation, and O-glycosylation. The observed heterogeneity of ACE proteins from which sugars have been removed suggests that the protein may undergo additional posttranslational modifications, such as phosphorylation.

Effects of Inhibition of Glycosylation. In the next series of experiments, we investigated the effects of different inhibitors of glycosylation on posttranslational processing and cleavage–secretion of ACE. Treatment of ACE 89 cells with the ionophore monensin, which impairs modifications in the Golgi compartments, reduced the apparent molecular mass of the 116-kDa species to about 110 kDa (Figure 3). Like

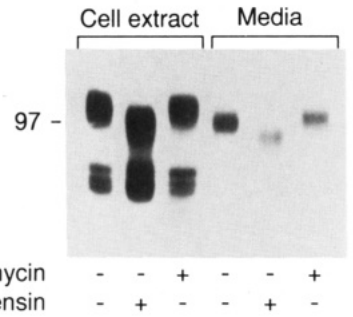

FIGURE 3: Effect of tunicamycin and monensin on synthesis and secretion of ACE by ACE 89 cells. ACE 89 cells were pulse-labeled with [^{35}S]methionine for 30 min and chased in the absence (lanes 1, 4) or in the presence of 5 μg/mL tunicamycin (lanes 3, 6) or 1 μM monensin (lanes 2, 5) for 2 h. Cell extracts (lanes 1–3) and media (lanes 4–6) were immunoprecipitated before analysis.

the 116-kDa protein, the 110-kDa protein was released into the medium at a slow rate, thereby suggesting that the modifications blocked by monensin may not be necessary for cleavage–secretion of ACE. Surprisingly, treatment with tunicamycin, which is known to block the addition of N-linked sugars to nascent polypeptide chains synthesized in the ER, did not affect the degree of glycosylation or the cleavage–secretion of ACE in ACE 89 cells. This lack of effect was the result of a global ineffectiveness of this inhibitor in these cells as judged by its failure to block the glycosylation of vesicular stomatitis virus G protein (data not shown). To test the effects of tunicamycin, we had to resort to a different cell line, human HeLa cells.

The next series of ACE expression analysis was done in human HeLa cells. For these and subsequent experiments, we used the vaccinia virus–T7 RNA polymerase transient expression system. Because protein glycosylation is blocked by tunicamycin in HeLa cells (Cox, 1981), we used this expression system for studying the effects of inhibiting glycosylation on ACE synthesis and processing. The ACE cDNA to be expressed was cloned behind a T7 promoter. HeLa cells were infected with the recombinant vaccinia virus, vTF7-3, that carries the T7 RNA polymerase gene, and the cDNA was transfected with those cells. ACE synthesis, processing, and cleavage–secretion were studied 14–24 h after transfection. The synthesis processing, and cleavage–secretion of ACE in this system were very similar to those in the ACE 89 cells, with only minor differences in the molecular weights of the expressed ACE species but with a considerable difference in the rate of conversion to the mature glycosylated form and consequently cleavage–secretion. As shown in Figure 4A, a 90-kDa ACE was produced by 30 min of labeling (lane 3) and was slowly converted to a species of about 110 kDa with increasing lengths of chase (lanes 4–7). The 110-kDa form of mature glycosylated ACE was slowly released into the medium (lanes 9–12) by a cleavage–secretion process. After 24 h of chase, about half of the glycosylated ACE was in the medium (lanes 7 and 12). In tunicamycin-treated cells, in contrast, a 76-kDa form of ACE protein was synthesized (Figure 4B, lane 3). This protein was probably totally unglycosylated because its mobility did not change when treated with Endo H (Figure 5, lanes 3 and 4). Moreover, the 90-kDa form synthesized in the absence of tunicamycin was converted to the 76-kDa form upon Endo H treatment (Figure 5, lanes 1 and 2), suggesting that the 76-kDa form is unglycoslyated. In tunicamycin-treated cells, neither the electrophoretic mobility (Figure 4B, lanes 4–7) nor the Endo H-sensitivity (Figure 5, lanes 7 and 8) of the 76-kDa form changed with increasing lengths of chase. However, the newly synthesized protein was degraded in the tunicamycin-treated

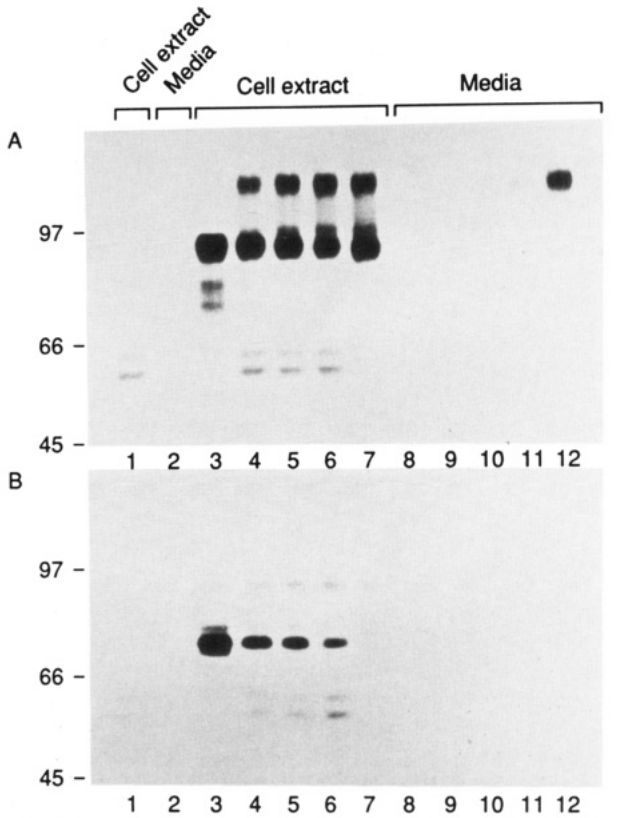

FIGURE 4: (Panel A) Transient expression of ACE proteins using the vaccinia virus–T7 RNA polymerase system in HeLa cells. HeLa cells, infected with the recombinant vaccinia virus expressing the T7 RNA polymerase, were transfected with ACE cDNA. The cells were pulse-labeled with [^{35}S]methionine for 30 min followed by incubation without the label for different periods of time. Lanes 1, 3–7, immunoprecipitation of cell extracts; lanes 2, 8–12, immunoprecipitation of culture media. Lanes 3, 8, 0-min chase; lanes 4, 9, 2-h chase; lanes 5, 10, 4-h chase; lanes 1, 2, 6, 11, 8-h chase; lanes 7, 12, 24-h chase; lanes 1, 2, without ACE cDNA. (Panel B) Effect of tunicamycin on synthesis and secretion of ACE in HeLa cells. Experimental conditions were the same as described for panel A except that tunicamycin (5 μg/mL) was present throughout the experiment in the culture media. Lanes 1, 3–7, immunoprecipitation of cell extracts; lanes 2, 8–12, immunoprecipitation of culture media. Lanes 3, 8, 0-min chase; lanes 4, 9, 2-h chase; lanes 5, 10, 4-h chase; lanes 1, 2, 6, 11, 8-h chase; lanes 7, 12, 24-h chase; lanes 1, 2, without ACE cDNA.

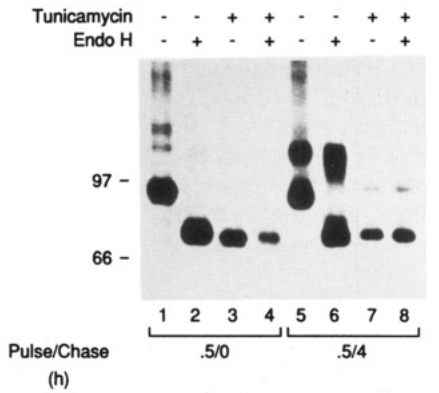

FIGURE 5: Endo H digestion of ACE synthesized in the presence of tunicamycin. HeLa cells transfected with ACE cDNA were labeled for 30 min and chased for 0 h (lanes 1–4) or 4 h (lanes 5–8) in the presence (lanes, 3, 4, 7, 8) or in the absence (lanes 1, 2, 5, 6) of 5 μg/mL tunicamycin. Cell extracts were immunoprecipitated, treated with (lanes 2, 4, 6, 8) or without (lanes 1, 3, 5, 7) Endo H, and analyzed.

cells (Figure 4B, lanes 3–7) with an approximate half-life of 1.5 h. Whereas a large amount of the newly synthesized ACE was present after 24 h of chase in untreated HeLa cells (Figure

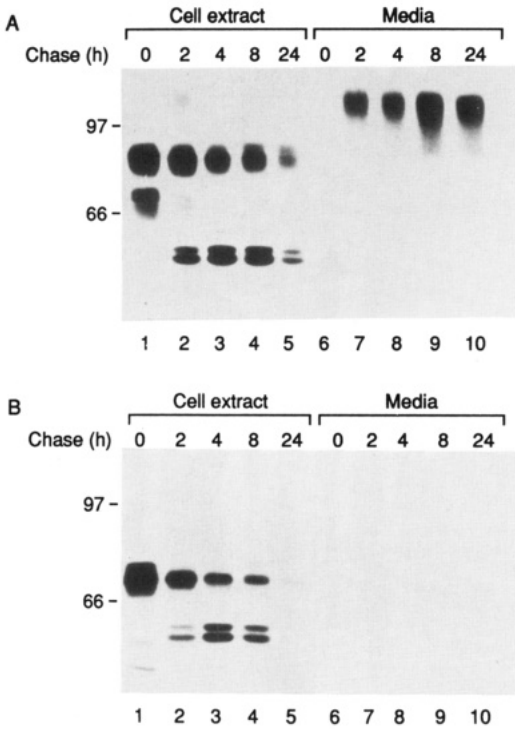

FIGURE 6: Effect of tunicamycin on the synthesis and secretion of truncated ACE in HeLa cells. The figure shows the pulse–chase kinetics of synthesis, intracellular processing, and secretion of EACE in HeLa cells in the absence (panel A) or in the presence (panel B) of 5 μg/mL tunicamycin in the culture media. HeLa cells infected with vaccinia virus expressing the T7 RNA polymerase were transfected with EACE cDNA. The cells were pulse-labeled with [^{35}S]methionine for 30 min followed by incubation without the label for different periods of time, as indicated. Lanes 1–5, immunoprecipitation of cell extracts; lanes 6–10, immunoprecipitation of culture media. Lanes 1, 6, 0-min chase; lanes 2, 7, 2-h chase; lanes 3, 8, 4-h chase; lanes 4, 9, 8-h chase; lanes 5, 10, 24-h chase.

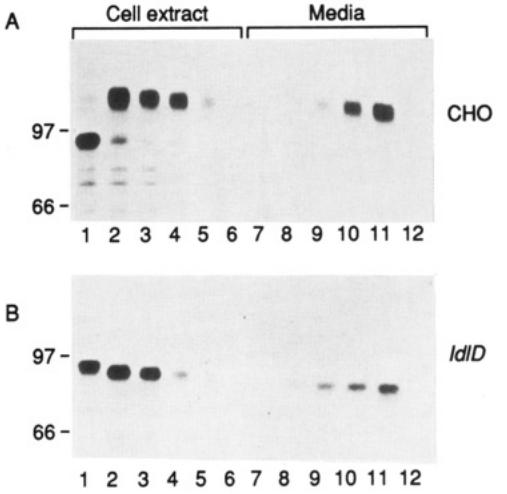

FIGURE 7: Synthesis and secretion of ACE in wild-type CHO and mutant CHO (*ldl*D) cells. CHO (panel A) and *ldl*D (panel B) cells infected with vaccinia virus expressing the T7 RNA polymerase were tranfected with ACE cDNA. 14–16-h posttransfection, the cells were transferred to serum-free Dulbecco's modified Eagle's medium containing F-12 and ITS mixture for 24 h. The cells were then pulse-labeled with [^{35}S]methionine for 30 min and chased for different periods of time. Lanes 1–6, immunoprecipitation of cell extracts; lanes 7–12, immunoprecipitation of culture media. Lanes 1, 7, 0-min chase; lanes 2, 8, 2-h chase; lanes 3, 9, 4-h chase; lanes 4, 10, 6, 12, 6-h chase; lanes 5, 11, 24-h chase; lanes 6, 12, without ACE cDNA.

4A, lane 7), none could be detected in the corresponding tunicamycin-treated cells (Figure 4B, lane 7). No ACE was secreted from the tunicamycin-treated cells (Figure 4B, lanes 8–12), suggesting that glycosylation is essential for its cleavage–secretion. We have previously shown that a truncated form of ACE (EACE), which lacks the membrane-anchoring domain near its carboxyl terminus, is rapidly secreted out of EACE 82 cells soon after its synthesis (Sen et al., 1991). The same was true for the HeLa cell system (Figure 6A). However, when glycosylation was blocked by tunicamycin treatment, no EACE protein was secreted, and the cell-bound protein was rapidly degraded (Figure 6B). These results show that unglycosylated ACE cannot be released from the cells, even when it is not anchored in the membrane by its hydrophobic domain, indicating that tunicamycin treatment does not directly affect cleavage secretion but the block in these cells is somewhere upstream. In Figures 4 and 6, in addition to the immunoprecipitated ACE-related proteins discussed above, a 55-kDa doublet is also visible. These are probably viral proteins that cross-react with the polyclonal ACE antibody used for immunoprecipitation, as the doublet can be seen in cells which are only infected with virus but has not been transfected with ACE cDNA (Figure 4, lanes 1 and 2; data not shown for the EACE in Figure 6).

Processing and Secretion of ACE in a Mutant CHO Cell Line. Because totally unglycosylated ACE is rapidly degraded and not at all secreted, we wondered whether the same is true for partially glycosylated ACE. To answer this question, we took advantage of a mutant Chinese hamster ovary (CHO) cell line (*ldl*D) in which the addition of N-linked complex sugars and O-linked glycosylation are impaired. The *ldl*D cells are deficient in the enzyme 4-epimerase that catalyzes the synthesis of UDP-galactose of UDP-*N*-acetylgalactosamine from glucose. Hence, under normal culture conditions, with glucose as the only source of sugar, these cells cannot synthesize O-linked glycan chains, whose synthesis is initiated by the linkage of *N*-acetylgalactosamine to a serine or threonine. The synthesis of N-linked sugars is also impaired, and the most mature form of complex N-linked glycoproteins made in these cells contains truncated Endo H-resistant oligosaccharides. ACE was expressed in the parental CHO cells and the mutant *ldl*D cells using the vaccinia virus–T7 polymerase system (Figure 7). As in HeLa cells, in CHO cells, a 90-kDa form of ACE was completely glycosylated to a 110-kDa form which was secreted into the medium (Figure 7A). However, in CHO cells, the conversion to the 110-kDa form was faster, and it was complete in 4 h after the synthesis of the 90-kDa form. Similarly, there was almost total cleavage–secretion of ACE in 24 h (Figure 7A, lanes 5 and 11). As expected, Endo H digestion of the 90-kDa form produced the 76-kDa unglycosylated species (Figure 8A, lanes 1 and 2), whereas the higher molecular mass forms of secreted and cell-bound ACE were totally insensitive to Endo H (Figure 8A, lanes 3–6). The 110-kDa form was, however converted to a 106-kDa form after digestion with *O*-glycosidase (Figure 8B). The molecular masses of the different forms of ACE produced in *ldl*D cells were quite different. Again, the initial product was a protein of 90 kDa (Figure 7B, lane 1), which was Endo H-sensitive (Figure 8, lanes 7 and 8). However, in these cells, the 90-kDa protein was not glycosylated further to produce the 110-kDa form. Instead, it was rapidly converted to an 88-kDa form (Figure 7B, lane 2). Unlike the 90-kDa protein, the 88-kDa protein was insensitive to Endo H (Figure 8A, lanes 9 and 10), suggesting that, in *ldl*D cells, the trimming of initially attached sugars occurs but the subsequent addition of sugars or sialic acid to form a typical complex-type N-linked oligosaccharide does not take place. Moreover, the 88-kDa protein is presumably not O-glycosylated because it was insensitive to *O*-glycosidase treatment (Figure 8B, lanes 3 and 4). This severely underglycosylated form of ACE, however, underwent efficient cleavage–secretion (Figure 7B,

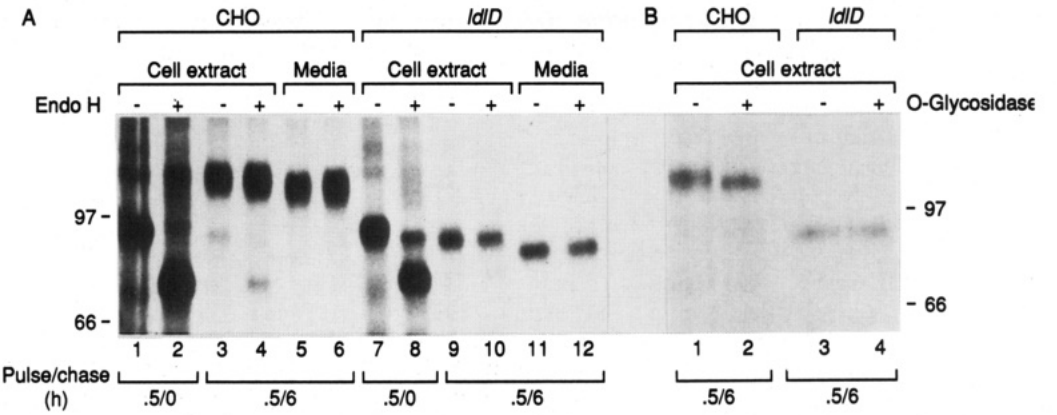

FIGURE 8: (Panel A) Endo H digestion of ACE synthesized in CHO and *ldl*D cells. CHO (lanes 1–6) and *ldl*D (lanes 7–12) cells infected with vaccinia virus expressing the T7 RNA polymerase were transfected with ACE cDNA, pulse-labeled for 30 min, and chased for 0 h (lanes 1, 2, 7, and 8) or 6 h (lanes 3–6 and 9–12). Cell extracts (lanes 1–4 and 7–10) and media (lanes 5, 6, 11, and 12) were immunoprecipitated and treated with (lanes 2, 4, 6, 8, 10, and 12) or without (lanes 1, 3, 5, 7, 9, and 11) Endo H and analyzed. (Panel B) *O*-Glycosidase digestion of ACE synthesized in CHO and *ldl*D cells. CHO and *ldl*D cells were infected and transfected with ACE cDNA as described above. Cells were pulse-labeled for 30 min and chased for 6 h. Cell extracts were immunoprecipitated and treated with (lanes 2, 4) or without (lanes 1, 3) *O*-glycosidase and analyzed.

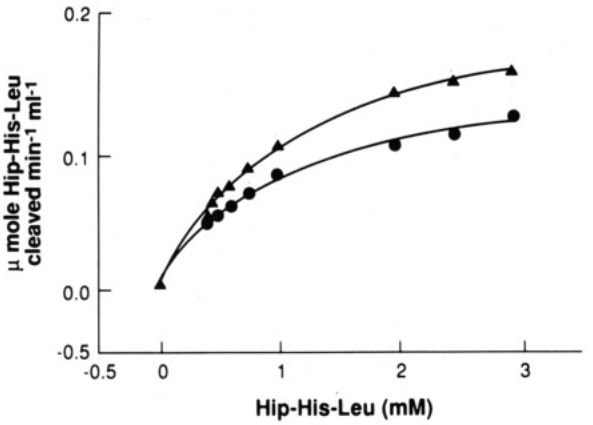

FIGURE 9: ACE enzyme activity in transiently transfected CHO and *ldl*D cells. CHO and *ldl*D cells were transfected with ACE cDNA, as described in Figure 7. ACE enzyme activity was assayed in the cell extracts as described under Experimental Procedures with varying levels of Hip-His-Leu. The K_m values determined for ACE expressed in CHO and *ldl*D cells were 1.16 and 1.02 mM, respectively. (▲) CHO cells; (●) *ldl*D cells.

lanes 8–11; Figure 8A, lanes 9–12), indicating that the O-linked sugars as well as most of the N-linked sugars are not necessary for proper cleavage–secretion of ACE.

Enzyme Activity of Underglycosylated ACE. As oligosaccharide chains play different roles in the physiological activity of different proteins, we were interested to learn if underglycosylated ACE has enzyme activity. The 88-kDa ACE produced in *ldl*D cells is devoid of any O-linked sugars and has some but not all of the N-linked sugars. As shown in Figure 9, this 88-kDa speceis is an active enzyme with a K_m similar to that of the 110-kDa species produced by the CHO cells. As expected, the ACE proteins produced in ACE 89 and HeLa cells are also enzymatically active (data not shown). These results suggest that the limited glycosylation of ACE, which occurs in the *ldl*D cells, is enough for its proper folding to attain an active conformation. Enzymatic activity of unglycosylated ACE produced in tunicamycin-treated cells could not be tested because of its rapid degradation. Similarly, we could not measure the enzyme activity of secreted ACE after complete enzymatic deglycosylation because the required incubation conditions inactivated the enzyme.

DISCUSSION

In this report, we have investigated the role of sugar modification in the biosynthesis and functions of rabbit testicular ACE. For this purpose, we used several expression systems to produce unglycosylated or partially glycosylated ACE and studied their cleavage–secretion, stability, and enzyme activity. We have extensively utilized the vaccinia virus–T7 RNA polymerase transient expression system to express ACE in three different cell lines. It has been shown previously that vaccinia infection does not affect the maturation of glycoproteins in mammalian cells (Jabbar & Nayak, 1990; Crise et al., 1990; Vincent et al., 1993). Hence, results obtained with this system should reflect the modifications that take place physiologically. HeLa, CHO, and CHO-*ldl*D cells, when transfected with ACE cDNA using the vaccinia virus-T7 RNA polymerase system, synthesized enzymatically active ACE and secreted the enzyme into the culture medium in a similar fashion as did our permanently transfected ACE 89 cells (Sen et al., 1991). In all cell lines, ACE acquired both N- and O-glycosylation, as evidenced by the ability of the deglycosylating enzymes Endo H and *O*-glycosidase to alter its electrophoretic mobility (Figures 1, 2, 5, and 8; data for O-glycosylation in HeLa cells are now shown). The extent of glycosylation varied from cell to cell; the mature ACE was a 116-kDa species in ACE 89 cells (Figure 1B) and a 110-kDa species in HeLa (Figure 4A) and CHO cells (Figure 7A). The rate of glycosylation as judged by pulse–chase experiments also varied considerably between cell lines. In all three cell lines, ACE is secreted into the culture medium only after it is glycosylated. Unglycosylated ACE was never detected in the medium. The mature glycosylated 116-kDa and 110-kDa forms, as well as the partially glycosylated 88-kDa form (*ldl*D cells), underwent the cleavage–secretion process in which the carboxyl-terminal region of the protein is proteolytically cleaved.

ACE_T synthesized in tunicamycin-treated HeLa cells has an apparent molecular mass of 76 kDa. The conclusion that this 76-kDa species is the unglycosylated form of ACE is supported by the following observations. First, the apparent molecular mass of 76 kDa agrees well with the calculated mass of the polypeptide chain of rabbit ACE_T without the signal peptide. Second, treatment of the 76-kDa species synthesized in the tunicamycin-treated cells with Endo H did not change its electrophoretic mobility, indicating the absence of N-linked high-mannose sugar. Finally, the 90-kDa form synthesized in the absence of tunicamycin was converted to a 76-kDa form when treated with Endo H (Figure 5). This unglycosylated ACE, unlike its glycosylated counterpart, was rapidly degraded in the cell with a half-life of about 1.5 h.

None of the newly synthesized ACE could be detected after 24 h in the tunicamycin-treated cells (Figure 4B), whereas a large amount of the enzyme was present in the untreated cells. These observations suggest that similar to a number of other proteins which become unusually susceptible to proteolytic digestion *in vivo* when synthesized in the carbohydrate-free form (Olden et al., 1978; Loh & Gainer, 1979; Schwarz et al., 1976), unglycosylated ACE is also degraded rapidly. Thus, oligosaccharide chains of ACE may protect it from proteolytic degradation in the ER. ACE synthesized in the *ldl*D cells, which are defective in glycosylation, had some N-linked sugar but no O-linked sugar. This partially glycosylated ACE, unlike the unglycosylated species, was stable and not susceptible to proteolytic degradation. Hence, the presence of some, but not all, N-linked sugars is necessary to protect ACE from degradation. O-Linked sugars do not seem to play a role in this regard.

It has been shown previously that tunicamycin treatment does not disrupt the machinery used for protein trafficking. Thus, in HeLa cells, in the presence of tunicamycin, the α-subunit of human chorionic gonadotropin is synthesized and secreted normally although its glycosylation is almost completely inhibited (Cox, 1981). Similarly in several other cells, transferrin, very low density lipoprotein, and thyroid-stimulating hormone are secreted normally in the presence of tunicamycin, although their glycosylation is drastically reduced (Elbein, 1987; Struck, 1978; Weinfraub et al., 1980). When ACE-producing HeLa cells (Figure 4A) were treated with tunicamycin, no ACE was secreted in the medium (Figure 4B), but secretion was normal in the *ldl*D cells, indicating again that only some N-linked sugar is sufficient for proper cleavage–secretion of ACE. It can be argued that in tunicamycin-treated cells the rate of degradation could be faster than the rate of secretion, resulting in ACE being degraded before it could be secreted. To address this question, EACE, lacking the membrane-anchoring domain near its carboxyl terminus, was expressed in HeLa cells. This mutant ACE is secreted out of the cell soon after it is synthesized (Figure 6). However, no EACE protein was secreted from cells that had been treated with tunicamycin, and the cell-bound protein was rapidly degraded, indicating that glycosylation indeed plays a role in proper transport of ACE through the secretory pathway but may not directly affect cleavage–secretion of the enzyme. Finally, our data indicate that O-glycosylation and complete N-glycosylation were not needed for proper folding of the ACE_T protein into an enzymatically active form.

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