Epitope-Dependent Blocking of the Angiotensin-Converting Enzyme Dimerization by Monoclonal Antibodies to the N-Terminal Domain of ACE: Possible Link of ACE Dimerization and Shedding from the Cell Surface†

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ABSTRACT: In a biomembrane modeling system, reverse micelles, somatic ACE forms dimers via carbohydrate-mediated interaction, providing evidence for the existence of a carbohydrate-recognizing domain on the ACE molecule. We localized this putative region on the N-domain of ACE using monoclonal antibodies (mAbs) to seven different epitopes of ACE. Two mAbs, 9B9 and 3G8, directed to distinct, but overlapping, epitopes of the N-domain of ACE shielded the CRD. Only "simple" ACE−antibody complexes were found in the system. Five mAbs allowed the formation of "double" antibody−ACE−ACE−antibody complexes via carbohydrate-mediated interactions. The results were confirmed using the ACE N- and C-domains. Testicular ACE was unable to form carbohydrate-mediated ACE dimers in the reverse micelles, while the N-domain of ACE, obtained by limited proteolysis of the parent full-length ACE, retained the ability to form dimers. Furthermore, mAb 3G8, which blocked ACE dimerization in micelles, significantly inhibited ACE shedding from the surface of ACE-expressing cells. Galactose prevented ACE dimerization in reverse micelles and also affected antibody-induced ACE shedding in an epitope-dependent manner. Restricted glycosylation of somatic ACE, obtained by the treatment of CHO-ACE cells with the glucosidase inhibitor *N*-butyldeoxynojirimycin, significantly increased the rate of basal ACE shedding and altered antibody-induced ACE shedding. A chemical cross-linking approach was used to show that ACE is present (at least in part) as noncovalently linked dimers on the surface of CHO-ACE cells. These results suggest a possible link between putative ACE dimerization on the cell surface and the proteolytic cleavage (shedding) of ACE.

Angiotensin I-converting enzyme (ACE)[1] (kininase II, CD 143, EC 3.4.15.1) is a Zn^{2+} peptidyl dipeptidase that cleaves two vasoactive peptides, angiotensin I and bradykinin. This enzyme plays an important role in blood pressure regulation, the development of vascular pathology, and endothelium remodeling in some disease states. The enzyme is also involved in neuropeptide metabolism as well as reproductive and immune functions (for reviews see refs *1*−*4*). Inhibitors of ACE are widely employed for the treatment of hypertension and various cardiovascular diseases (*5*). Recently, ACE was assigned as a new CD marker, CD 143 (*6*).

The somatic isoform of ACE has two highly homologous domains (N- and C-domains), each bearing a functional zinc-dependent catalytic site (*7*). ACE is a type 1 integral membrane protein that is anchored in the plasma membrane near its C-terminus. Somatic ACE also exists as a soluble form, e.g., in plasma, amniotic and cerebrospinal fluid, and seminal plasma (*1*−*3*), that lacks its transmembrane domain (*8*). The testicular isoform of ACE is smaller and contains a unique N-terminal sequence of 36 amino acid residues, whereas the other 665 amino acid residues are identical to the C-domain of somatic ACE (*9*). The form of ACE that corresponds to the N-terminal domain can be obtained by the limited proteolysis of the full-length somatic ACE (*10*−*12*).

ACE is a member of the growing family of membrane proteins that are proteolytically cleaved in the juxtamembrane stalk region by enzymes referred to as a secretases or sheddases. ACE secretases are metalloproteases that are colocalized with ACE in a number of tissues. ACE secretase has an absolute requirement for its substrate (ACE) to be anchored in the membrane for cleavage to occur (for review see ref *13*). However, the mechanism of ACE solubilization in vivo, the components involved in this process, and the factors that regulate generation of soluble ACE have not been

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[1] Abbreviations: ACE, angiotensin-converting enzyme; CRD, carbohydrate-recognizing domain; AOT, aerosol OT, bis(2-ethylhexyl)-sulfosuccinate sodium salt; FA-Phe-Gly-Gly, *N*-[3-(2-furyl)acryloyl]-L-phenylalanylglycylglycine; Hip-His-Leu, hippuryl-L-histidyl-L-leucine; lisinopril, *N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline; CHO, Chinese hamster ovary; mAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay; CD markers, cluster designation markers; NB-DNJ, *N*-butyldeoxynojirimycin; BS^3, bis(sulfosuccinimidyl) suberate; PBS, phosphate-buffered saline; PFA, paraformaldehyde.

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specifically delineated. Recently, we demonstrated that binding of monoclonal antibodies (mAbs) to ACE on the cell surface affects the rate of ACE cleavage (shedding) in an epitope-dependent manner (*14*).

ACE is extensively glycosylated. The degree of glycosylation (14−33%), the number and position of sites of glycosylation, and the structure of the oligosaccharide chains depend on the ACE source. In somatic ACE the majority of carbohydrate chains are complex-type *N*-glycans (*15*, *16*), whereas in testis ACE the unique N-terminal sequence is heavily O-glycosylated (*15*). It has been shown that deglycosylation of mature somatic ACE does not abolish its enzymatic activity (*17*−*19*). Similarly, a mutant testicular ACE devoid of most of its O-linked sugars retains enzymatic activity (*15*). However, numerous studies demonstrated that glycosylation plays an important role in the folding of ACE and that the glycosylation effects on transport and enzyme release might be site dependent (*20*−*22*).

Recently, we have shown, in the system of reverse micelles modeling the biomembrane environment of enzymes (*23*, *24*), that both human and bovine somatic ACE can form carbohydrate-mediated dimers and both enzymes contain a specific carbohydrate-recognizing domain (*25*, *26*). ACE, therefore, can be considered as an enzyme with lectin-like properties. This region is not directly involved in the catalytic activity of ACE but can affect the structural state of the enzyme. In the cell membrane, this carbohydrate-recognizing domain may be responsible for ACE dimerization and/or interaction with other glycoproteins. The structural requirements of the carbohydrate-recognizing domain on the ACE molecule were estimated on the basis of the ability of different saccharides to inhibit ACE dimerization in reverse micelles. We demonstrated that the motif neuraminic acid−galactose is crucial for the interaction of the oligosaccharide with the carbohydrate-recognizing domain of ACE (*26*).

In this report, using various forms of ACE in reverse micelles and a panel of monoclonal antibodies to different ACE epitopes, we localized the CRD to the N-terminal half of the ACE molecule. We also demonstrated that one of the mAbs to human somatic ACE, which blocked effectively ACE dimerization in the reverse micelles, inhibited significantly ACE shedding from the surface of ACE-expressing cells. Moreover, galactose, which prevents ACE dimerization in reverse micelles, also affected antibody-induced ACE shedding in an epitope-dependent manner. A chemical cross-linking approach with a membrane-impermeable cross-linker, bis(sulfosuccinimidyl) suberate (BS^3), demonstrated that a population of ACE is present in a dimeric form on the surface of CHO-ACE cells. These results suggest a possible link between the dimerization and proteolytic cleavage (shedding) of ACE.

MATERIALS AND METHODS

Chemicals. *N*-[3-(2-Furyl)acryloyl]-L-phenylalanylglycylglycine (FA-Phe-Gly-Gly), hippuryl-L-histidyl-L-leucine (Hip-His-Leu), *N*-[(*S*)-1-carboxy-3-phenylpropyl]-L-lysyl-L-proline (lisinopril), phenylmethanesulfonyl fluoride, trypsin, papain, pepsin, and protein molecular mass markers were from Sigma (St. Louis, MO); sodium bis(2-ethylhexyl)sulfosuccinate (aerosol OT, AOT) was from Fluka (Buchs, Switzerland); Triton X-100 and Triton X-114 were from Ferak (Berlin, Germany).

Enzyme Purification. Bovine somatic ACE was isolated from bovine lungs by extraction with 50 mM phosphate buffer, pH 7.5, containing 150 mM NaCl, 1 mM EDTA, and 0.1% Triton X-100. Soluble and membrane fractions of the enzyme were separated by hydrophobic chromatography as described previously (*27*). Human somatic ACE was isolated from the membrane fraction of human kidney by extraction with 50 mM phosphate buffer, pH 7.5, containing 150 mM NaCl and 0.1% Triton X-100. Bovine and human ACEs were purified to electrophoretic homogeneity by lisinopril affinity chromatography (*28*). The elution was performed by the change of eluent from 50 mM phosphate, pH 7.5, and 150 mM NaCl to 50 mM borate, pH 9.5. Elution buffer used for the purification of human ACE contained 0.1% Triton X-100. Bovine testicular ACE (soluble form) was purified to homogeneity by hydrophobic and affinity chromatography as described (*27*). The N-domain of bovine ACE was obtained by limited proteolysis of the parent somatic enzyme with trypsin (*12*). Bovine ACE was partially denatured by the addition of NH_4OH to pH 10.8 and then treated with trypsin for 16 h. Tryptic digestion of ACE was terminated by adding a 10-fold excess of phenylmethanesulfonyl fluoride (in dioxane). The N-terminal domain was isolated by gel-filtration chromatography on Sephadex G-200 superfine. The purity of the isolated ACE forms was checked by SDS−PAGE in the presence of β-mercaptoethanol.

Protein concentration was determined by the modification (*29*) of Lowry's method. Phase separation of different ACE forms in Triton X-114 was performed as described in ref *30*.

Antibodies. Properties of a set of monoclonal antibodies directed to different epitopes located on the N-terminal domain of ACE were described previously (*31*). $F(ab')_2$ and Fab fragments of mAb 9B9 were obtained from the parent antibody by treatment with pepsin and papain according to refs *32* and *33*.

ACE Activity Measurements. Kinetic experiments in reverse micelles of the ternary system AOT−water−octane were carried out as described (*25*, *26*). The required hydration degree ($W_o = 16-50$) in micelles was obtained by varying the volume of the buffer. In a typical experiment, 47−225 μL of 25 mM Hepes buffer, pH 7.5, containing 75 mM NaCl, 1 μM $ZnCl_2$, 20 μL of 0.5 μM ACE stock solution, and 20 μL of acetonitrile was solubilized in 1 mL of 0.3 M AOT solution in octane. After 20 min of preincubation, the hydrolysis was initiated by adding, with vigorous shaking, 30 μL of 3 mM FA-Phe-Gly-Gly in Hepes buffer. The reaction was followed by a decrease in absorbance at 328 nm (*34*).

ACE−mAb Complexes: Activity Measurements. ACE with mAb (ratio from 1:1 to 1:30, mg/mg) in 50 mM Hepes buffer, pH 7.5, containing 150 mM NaCl and 1 μM $ZnCl_2$ was incubated overnight. Then the activity of the complex in reverse micelles was determined as described above.

Sedimentation Measurements. The sedimentation coefficients (*s*) of ACE-containing reverse micelles were measured at 20000−30000 rpm using a Beckman Optima LE 80 analytical ultracentrifuge (Beckman Instruments, Inc., Fullerton, CA). The scanning was carried out at 280 nm for ACE-containing micelles (the enzyme concentration was approximately 0.3 mg/mL) and at 400 nm for buffer-containing micelles (in the latter case picric acid was added

to the final concentration of 20 μM). The values of the molecular mass of the protein incorporated into the reverse micelles were calculated from *s* values (*35*).

ACE Shedding Assay. A stable line of CHO cells (2C2 clone) expressing wild-type human ACE was obtained and cultured as described previously (*36*). In the experiments with restricted glycosylation, the cells were fed with "complete culture medium" (Mediatech, Inc., Herndon, VA) containing 2 mM *N*-butyldeoxynojirimycin (NB-DNJ). This medium was changed twice over a period of 5 days before harvesting. When CHO-ACE cells growing in 96-well microtiter plates reached confluence, they were washed three times with serum-free medium and incubated with galactose diluted in the same medium at least 30 min prior to addition of mAbs (10 μg/mL), also diluted in a complete culture medium. After 4 h at 37 °C, the culture medium was collected, whereas cells were washed several times and fixed with 4% paraformaldehyde (PFA) for subsequent estimation of bound mAbs in cell ELISA assays. To determine cell-associated ACE activity as well as to quantify correctly the rate of ACE release (by referring ACE level in the culture medium to that on the cell surface), cells were lysed with 100 μL of 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanosulfonate (CHAPS). Both supernatants and lysates were centrifuged, and ACE activity was determined using a fluorometric assay. Aliquots (10−50 μL) were added to 200 μL of 5 mM Hip-His-Leu (substrate for ACE) and incubated for the appropriate time at 37 °C. The reaction was terminated with 0.28 N NaOH, and the His-Leu product was estimated fluorometrically (*37*).

Cell ELISA. CHO-ACE cells (2C2 clone) were grown in 96-well microtiter plates. Cells were chilled on ice for at least 30 min and were washed several times with cold PBS. Control mouse IgG or 10 μg/mL anti-ACE mAbs in PBS/casein (0.2%) were added and incubated for 2 h on ice. After washing, cells were fixed with 4% PFA for 15 min at room temperature and washed several times with PBS; then bound mAbs (that reflect membrane-bound ACE) were quantified by incubation with alkaline phosphatase-conjugated anti-mouse Ab followed by spectrophotometric assay at 405 nm.

ACE Plate Precipitation Assay. Microtiter plates coated with different anti-ACE mAbs were incubated with serum-free culture medium from control CHO-ACE cells or from the cells which were underglycosylated in the presence of NB-DNJ (see above). The precipitated ACE activity was estimated directly in the wells using the substrate Hip-His-Leu (*31*). Negligible background hydrolysis of the substrate in the wells coated by nonimmune mouse IgG (negative control) was subtracted from each value with specific anti-ACE mAbs.

Chemical Cross-Linking and Western Blotting of ACE. Cross-linking was performed using BS^3 (Pierce, Rockford, IL) according to the manufacturer's instructions. Cross-linking of ACE on the surface of confluent CHO-ACE cells was performed at 4 °C using 1−2 mM BS^3. Cells were washed in phosphate-buffered saline (PBS) and then lysed on ice for 1 h in lysis buffer containing 8 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanosulfonate (CHAPS) in 10 mM Tris, pH 8.0, with a protease inhibitor cocktail, Complete, Mini, EDTA-free (Roche Diagnostics GmbH, Mannheim, Germany). Cell lysates were centrifuged to remove detergent-insoluble components, concentrated 5-fold, and subjected to electrophoresis on 7.5% SDS−PAGE. After SDS−PAGE, proteins were transferred electrophoretically to PVDF membrane, and Western blotting of ACE was performed with a new, highly sensitive mAb to denatured ACE, 1D8 (Balyasnikova et al., manuscript in preparation), at a concentration of 5 μg/mL using a ProteoQwest kit (Sigma, St. Louis, MO). Bound antibody was visualized with a WestPico Super Signal chemiluminescence substrate (Pierce, Rockford, IL).

RESULTS

Dimerization of Somatic ACE in Reverse Micelles. The interactions between different ACE forms were investigated on the basis of their ability to form carbohydrate-mediated oligomeric forms in reverse micelle modeling biomembranes. In these systems, proteins are incorporated into the polar intramicellar spaces and are surrounded by a monomolecular layer of hydrated surfactant. The formation of enzymatic forms that differ in size can be monitored using this system. The catalytic activity of the enzymes in reverse micelles depends on the hydration of the surfactant, $W_o = [H_2O]/[AOT]$, i.e., the amount of water phase in the system (*23*, *24*). The change in the extent of hydration results in a corresponding change in the intramicellar space (*38*). The enzymatic activity vs W_o dependence curves are usually bell-shaped, and the activity maximum corresponds to the degree of hydration at which the intramicellar space radius equals that of the protein globule. For enzymes with quaternary structure or enzymes capable of forming protein complexes (homo or hetero), the shapes of the dependence curves are more complex, with several maxima related to the functioning of various enzymatically active subunits or various protein complexes, respectively (*39*). Thus, the presence or absence of a definite maximum on the dependence curve can be considered as a reliable indicator of the presence or absence of a protein of certain size.

We have purified ACE from two mammalian tissues, bovine lung and human kidney, to electrophoretic homogeneity. Phase separation using Triton X-114 demonstrated that both enzyme preparations were comprised of predominantly amphipathic proteins. For both bovine and human ACE, the dependence of catalytic activity on the degree of hydration of reversed micelles is represented by a curve with several maxima (Figure 1) corresponding to the existence of different enzymatic forms of both enzymes. For human ACE, the maximum at $W_o = 27$, micelle radius 44.4 Å, and the maximum at $W_o = 31$, micelle radius 50.5 Å, were previously attributed to active monomer and dimer, respectively (*26*), whereas the third maximum could correspond to an oligomeric form of ACE (Figure 1A). Whereas soluble bovine ACE (without its hydrophobic anchor) was also found in reverse micelles in both monomeric and dimeric forms with activity maxima at $W_o = 27$ and $W_o = 31$ (*26*, *40*), the amphipathic form of bovine ACE did not show the first maximum at $W_o = 27$. This amphipathic form exhibits four distinctive activity maxima at $W_o = 31$, 35, 40, and 47 (micelle radii 50.5, 56.5, 64, and 74.5 Å, respectively) corresponding to at least four types of bovine ACE oligomeric forms (Figure 1B). Sedimentation analysis of the ACE-containing micelles allowed us to correlate these maxima to those of the ACE compact dimer, two larger dimers, and a

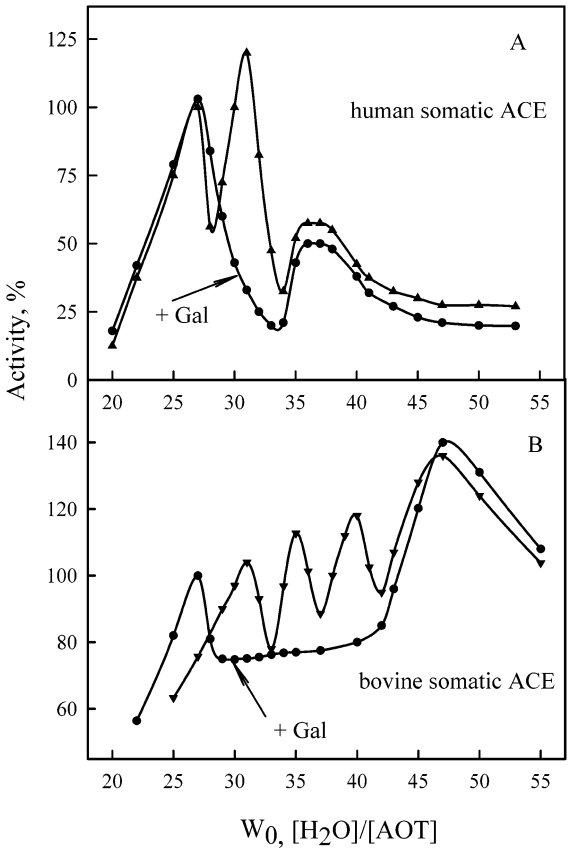

FIGURE 1: Dependence of catalytic activity of somatic ACE on hydration degree of reverse micelles of the ternary system AOT−water−octane. The required hydration degree (W_o) in micelles was obtained by varying the volume of buffer. After preincubation of ACE stock solution in reverse micelles (0.3 M AOT, 10 μM ACE), ACE activity was measured with FA-Phe-Gly-Gly (75 μM) and expressed in percent; ACE activity in the first maximum was taken as 100%. In parallel experiments 10 μM galactose (Gal) in the water phase was added. Panels: (A) human somatic ACE; (B) bovine somatic ACE.

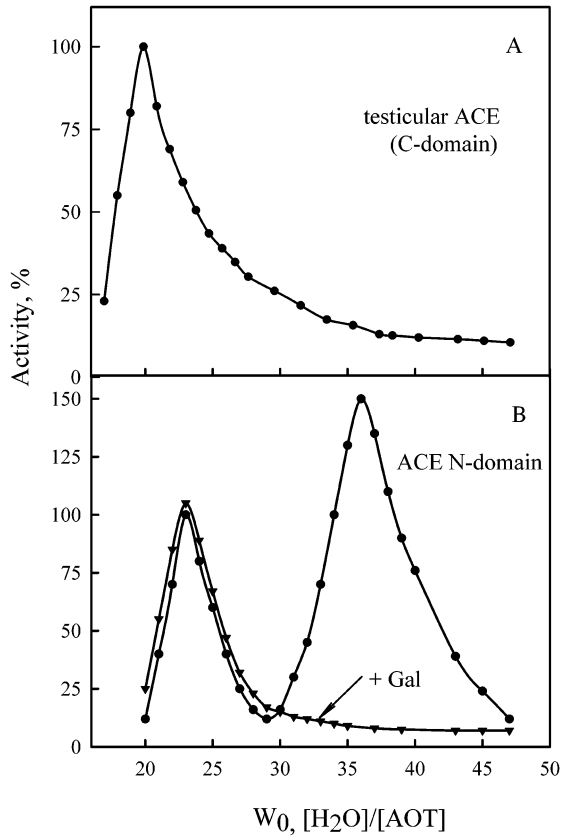

FIGURE 2: Dependence of ACE catalytic activity on hydration degree of reverse micelles of the ternary system AOT−water−octane. Panels: (A) bovine testicular ACE (C-domain); (B) human N-terminal domain of ACE. Other details as in Figure 1.

tetramer, respectively (*40*). Thus, the presence of the ACE hydrophobic anchor can affect its ability to function as a monomer in reverse micelles. However, different two-domain ACE forms are found as dimers.

The addition of 10 μM galactose to human ACE, shown previously to suppress the formation of bovine ACE compact dimers (*25*−*26*, *40*), did not change the position and value of the first and third maxima on the "activity−hydration degree" dependence curve but led to disappearance of the second maximum (Figure 1A). Thus, the human ACE compact dimer was not formed in the presence of galactose in accordance with previous observations (*26*), while the third maximum should be referred to some oligomeric form, which is not formed via carbohydrate chains of the enzyme. The addition of 10 μM galactose to bovine ACE led to disappearance of the maxima at $W_o = 31$, 35, and 40, while the maximum at $W_o = 47$ did not change. Simultaneously, the maximum at $W_o = 27$ corresponding to the functioning of ACE monomer (*40*) appeared on the activity profile (Figure 1B). Sedimentation analysis proved that only the monomer and tetramer of bovine ACE were present in the system in the presence of galactose (data not shown). So, all three dimers of bovine ACE, but not the tetramer, are carbohydrate-mediated forms or aggregates.

Ability of Single-Domain Forms of ACE To Generate Carbohydrate-Controlled Dimers. We have purified the soluble form of bovine testicular ACE, corresponding to the C-domain of somatic ACE (*9*), and the ACE N-domain obtained by limited proteolysis of bovine somatic ACE (*12*) to electrophoretic homogeneity. For soluble testicular bovine ACE, the profile of enzymatic activity versus hydration degree has only one optimum at $W_o = 19$, micelle radius 32.5 Å (Figure 2A). The micelle of this size can be occupied by a spherical protein with a molecular mass of about 110 kDa (calculated according to ref *40*), which is very close to the 100 kDa molecular mass of testicular ACE (*9*). We can conclude, therefore, that soluble testicular ACE is present in micelles only in the monomeric form and is unable to form carbohydrate-controlled dimers, suggesting that it does not have an exposed carbohydrate-recognizing domain. For the N-domain of bovine ACE, the profile of enzymatic activity versus hydration degree had two maxima (Figure 2B) at $W_o = 23$, micelle radius 38.5 Å, and at $W_o = 36$, micelle radius 58 Å. The first maximum was identified previously (*12*) as a nonspherical N-domain monomer, whereas the second maximum could be a "loose" dimer. The addition of 10 μM galactose to the reaction medium did not change the position and the value of the first maximum but led to the disappearance of the second maximum (Figure 2B) as we observed for somatic ACEs (Figure 1). So, the carbohydrate appeared to have similar mechanisms of interaction in the case of both parent somatic ACE and single ACE N-domain. Thus, we can conclude that the N-domain bears a carbohydrate-recognizing domain.

Table 1: Inhibition of Dimerization of Bovine Somatic ACE and Its Single N-Domain by Carbohydrates[a]

carbohydrate	IC_{50}, μM somatic ACE	IC_{50}, μM N-domain
Gal	4 ± 1	4 ± 1
Neu5Ac	5 ± 1	6 ± 1
Lac	400 ± 100	500 ± 100
3′SiaLac	0.5 ± 0.1	0.5 ± 0.1
6′SiaLac	6 ± 1	5 ± 1

[a] Tested carbohydrates were incubated with bovine somatic ACE or the N-terminal domain isolated from the parent bovine somatic ACE, and then the dependence of ACE activity in the reversed micelles on hydration degree was determined (see Materials and Methods). The addition of free carbohydrates to the reaction media did not change the position and the value of the first maximum on the curve of the dependence of enzyme activity on hydration degree but led to disappearance of the second maximum (Figures 1 and 2). The ability of the tested carbohydrates to prevent ACE−ACE interaction was expressed as 50% of the concentration needed for complete inhibition of ACE dimerization.

Comparative Characteristics of Carbohydrate Specificity of CRDs on Somatic ACE and the Single N-Domain. We estimated the effectiveness of the binding of several saccharides to the carbohydrate-recognizing domain of the N-domain on the basis of their ability to suppress ACE dimerization according to ref *26*. The enzyme activity detected in reverse micelles at the hydration degree $W_o = 36$ in the absence of carbohydrates can be related to an oligomeric (dimeric) N-domain state. The corresponding activity determined in the presence of high concentrations of carbohydrates should be attributed to the N-domain monomer or, more correctly, to the N-domain−carbohydrate complex. The changes in enzymatic activity at $W_o = 36$ were measured with increase of carbohydrate concentration in the reaction medium, and the relative affinity of carbohydrates to the N-domain−carbohydrate complex was expressed as 50% of the concentration needed for complete inhibition of ACE dimerization. The results, together with corresponding data for the parent somatic enzyme, are presented in Table 1. The IC_{50} values for carbohydrates of different structures were found to be the same for somatic ACE and the N-domain, indicating similar structural requirements of the carbohydrate-recognizing domain on both ACE forms. Therefore, the carbohydrate-recognizing domain is located on the N-domain of somatic ACE.

Effect of Anti-ACE mAbs on ACE Dimerization in the Reverse Micelles. In an attempt to identify the location of the carbohydrate-recognizing domain within the N-domain of the ACE molecule, we used a panel of seven monoclonal antibodies recognizing different epitopes of human ACE. These antibodies, 9B9, 3G8, i1A8, 5F1, 3A5, i2H5, and 1G12, bind to different epitopes on the N-domain of human ACE within several antigenic regions (*31*). All of these are immunoglobulin G1 antibodies and are characterized by their high-affinity binding to the ACE molecule. The hypothesis was that certain antibodies might shield the carbohydrate-recognizing domain on the protein surface and in this case ACE would lack the ability to bind to another enzyme molecule. Other antibodies recognizing epitopes more distal to the carbohydrate-recognizing domain would not prevent ACE−ACE interaction, and in these cases the formation of "double" mAb−ACE−ACE−mAb complexes could be monitored.

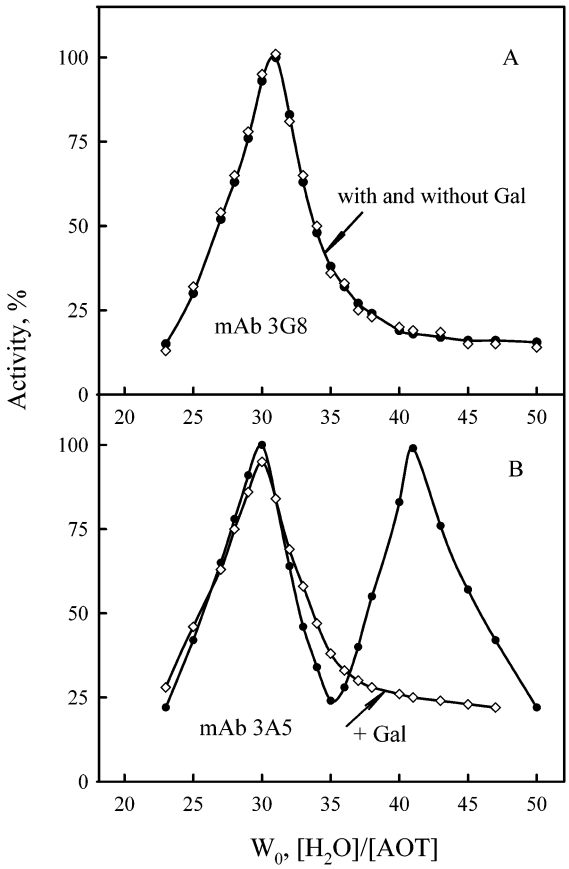

FIGURE 3: Dependence of catalytic activity of the complexes of ACE−mAbs on hydration degree of reverse micelles of the ternary system AOT−water−octane. Human somatic ACE was incubated overnight with anti-ACE mAbs 3G8 (A) or 3A5 (B) at a ratio (mg/mg) of 1:30. Then the ACE activity of the complex in the reverse micelles was determined as described in Figure 1.

We followed the formation of the ACE−mAb complexes by the profile of ACE catalytic activity versus the reverse micelle degree of hydration. The profile of activity of bovine ACE did not change in the presence of antibodies (data not shown), because none of mAbs recognized bovine ACE (*31*). Human ACE, however, exhibited an epitope-dependent profile of ACE catalytic activity (Figures 3 and 4, Table 2) after incubation with anti-ACE mAbs. In the presence of all mAbs, the first maximum at $W_o = 30-34$ could be attributed to the enzyme−antibody complex. Binding of five of the mAbs, 3A5 (Figure 3B), i1A8, i2H5, 1G12, and 5F1 (Table 2), with the ACE molecule resulted in the appearance of the second maximum at $W_o = 36-40$ on the "activity versus hydration degree" dependence curve. This maximum could be attributed to the double mAb−ACE−ACE−mAb complex. However, in the presence of mAbs 3G8 and 9B9, the ACE activity profile did not display the second maximum (Figures 3 and 4).

To confirm the nature of the two maxima, we carried out three series of experiments. In the first series, we varied the ratio ACE/mAb 9B9 (mg/mg) from 1:1 to 1:30 and followed the activity−hydration degree dependence curve. In the presence of an excess of antibody, there was a single activity maximum at $W_o = 33$, which indicated that only one enzymatically active ACE form was present. Lowering the ratio of ACE/mAb led to the appearance of another maxi-

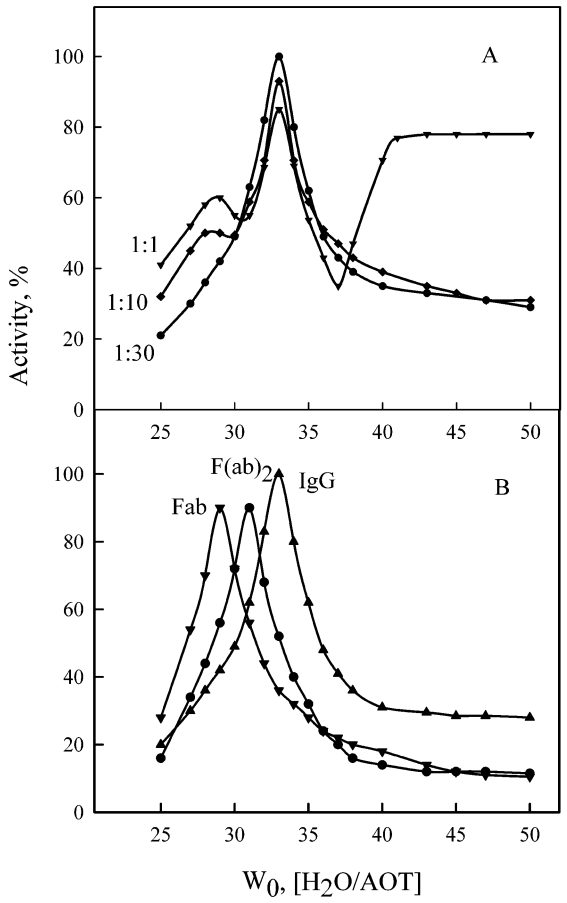

FIGURE 4: Dependence of catalytic activity of the complexes of ACE−mAb 9B9 on hydration degree of reversed micelles AOT−water−octane. Human somatic ACE was incubated overnight with (A) anti-ACE mAb 9B9 (whole molecule) at ratios (mg/mg) from 1:1 to 1:30 and (B) anti-ACE mAb 9B9 fragments at a ratio (mg/mg) of 1:30. Then ACE activity of the complex in the reverse micelles was determined as described in Figure 1. The activity of the ACE−mAb complex obtained at a ratio of 1:30 was taken as 100%.

Table 2: Positions of Maxima on the Curve of the Dependence of Human ACE−mAb Complexes and Catalytic Activity on Hydration Degree (W_o) of Reverse Micelles AOT−Water−Octane[a]

	first maximum		second maximum	
mAb	W_o	r_{opt}	W_o	r_{opt}
9B9	33	53.5		
3G8	31	50.5		
i1A8	31	50.5	36	58.0
3A5	30	49.0	40	64.0
5F1	34	55.0	41	65.5
i2H5	30	49.0	37	59.5
1G12	31	50.5	37	59.5

[a] Human somatic ACE was incubated overnight with anti-ACE mAbs at a ratio (mg/mg) of 1:30. Then the ACE activity of the complex in the reverse micelles was determined as described in Materials and Methods. The intramicellar radii r_{opt} were determined as described in Grinstein et al. (*40*).

mum at $W_o = 27-28$, which corresponds to unbound enzyme (Figure 4A).

In the second series of experiments, we used Fab and $F(ab)_2$ fragments of the same monoclonal antibody, 9B9, as smaller compounds (40 and 90 kDa, respectively) with the ability to bind to ACE molecule. As expected, the positions of the maxima of ACE catalytic activity on hydration degree of reversed micelles in the presence of Fab fragments were shifted to the lower sized micelles compared to the position of the maximum exhibited in the presence of the whole antibody (Figure 4B).

In the third series of experiments, we checked the influence of galactose on the activity profiles of ACE in the reverse micelles in the presence of different mAbs. Addition of 10 μM galactose to the reaction system did not influence the position and value of the first maximum on ACE catalytic profile in the reverse micelles (Figure 3). However, galactose led to the disappearance of the second maximum on the dependence curve (Figure 3B).

Summarizing these results, we can conclude that the first maximum corresponds to the functioning of the "simple" ACE−mAb complex, whereas the second maximum represents the carbohydrate-controlled double complex mAb−ACE−ACE−mAb. Moreover, we can also state that the carbohydrate-recognizing domain on the ACE molecule is shielded by mAbs 9B9 and 3G8 directed to overlapping epitopes located on the N-domain of the enzyme.

Epitope-Dependent Antibody-Induced Shedding of ACE from the Cell Surface. To investigate a possible link between the ability of some mAbs to block ACE dimerization and the effect of these mAbs on ACE shedding, we determined the rate of epitope-dependent ACE cleavage from the surface of CHO-ACE cells in the presence of different carbohydrates or in the presence of the inhibitor of N-glycosylation. Previously, we demonstrated that the antibody-induced ACE shedding was strongly epitope specific: most of the antibodies increased the shedding by 20−40% and mAbs 9B9 and 3A5 increased the shedding by 2−4-fold, whereas binding of mAb 3G8 decreased ACE shedding by 36% (*14*).

Because ACE dimerization in the reverse micelles is specifically controlled by carbohydrates of different structure (*26*), we studied antibody-induced ACE shedding from CHO-ACE cells in the presence of galactose ("effective" monosaccharide) and glucose ("noneffective" monosaccharide). The latter neither altered the binding of mAbs to the ACE molecule nor affected ACE shedding from the surface of CHO-ACE cells (data not shown). Binding of mAbs to the cell surface of ACE-expressing cells (at 4 °C in the absence of shedding) was also not altered in the presence of up to 5 mM galactose (Figure 5A). However, galactose influenced antibody-induced ACE shedding (at 37 °C) in an epitope-specific manner: the changes in ACE shedding induced by mAbs 9B9 and 3G8 (although opposite, increasing and decreasing, respectively) were completely abolished by galactose, whereas 3A5-induced increase of ACE shedding was not influenced by the presence of galactose (Figure 5B). Note that the differences between 3G8-dependent ACE shedding (and obviously 9B9-dependent as well) in the presence or absence of galactose are significant. Basal ACE shedding from the surface of CHO-ACE cells was increased by the indicated concentration of galactose by 40% ($p < 0.05$).

These experiments show that at least one antibody to the N-terminal domain of human ACE, 3G8, which effectively blocked ACE dimerization in reverse micelles, also significantly inhibited proteolytic ACE shedding from the surface of ACE-expressing cells, and another antibody, 9B9, blocked ACE dimerization as well but significantly induced ACE shedding from the membrane.

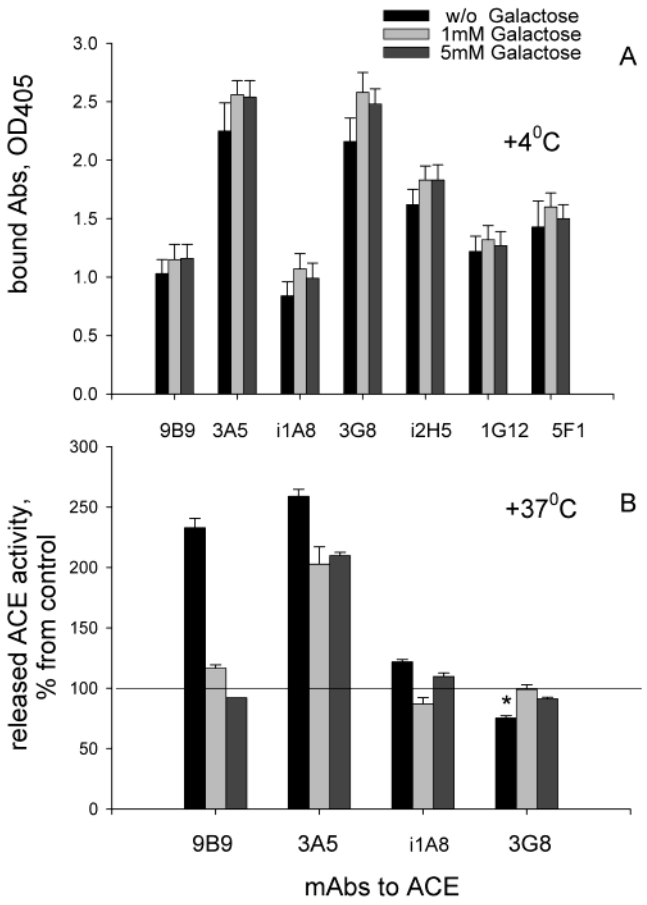

FIGURE 5: Effect of galactose on the binding of anti-ACE mAbs to the ACE on the cell surface (A) and antibody-induced ACE shedding (B). The CHO-ACE cell line (clone 2C2) was grown on a 96-well plate until confluence. Anti-ACE mAbs or control mouse IgG at a concentration of 10 μg/mL was added to the cells in serum-free medium (containing 2% BSA) and incubated for 1 h at 4 °C for the mAb binding assay (A) and for 4 h at 37 °C for the ACE shedding assay (B) in the absence or presence of the indicated concentrations of galactose. (A) mAb binding assay (cell ELISA). Bound anti-ACE mAbs were revealed with goat−anti-mouse IgG conjugated with alkaline phosphatase at 405 nm. (B) ACE shedding assay. The culture fluids were collected and centrifuged (for the precipitation of the detached cells), and the ACE activity, released from the plasma membrane of cells, was measured fluorometrically with Hip-His-Leu as a substrate. Antibody-induced shedding is expressed as percent from the basal shedding of ACE (in the presence of control mouse IgG only). The data represent results of several independent experiments and are expressed as mean ± SD. (*) $p < 0.05$; differences are statistically significant in comparison with galactose-treated cells.

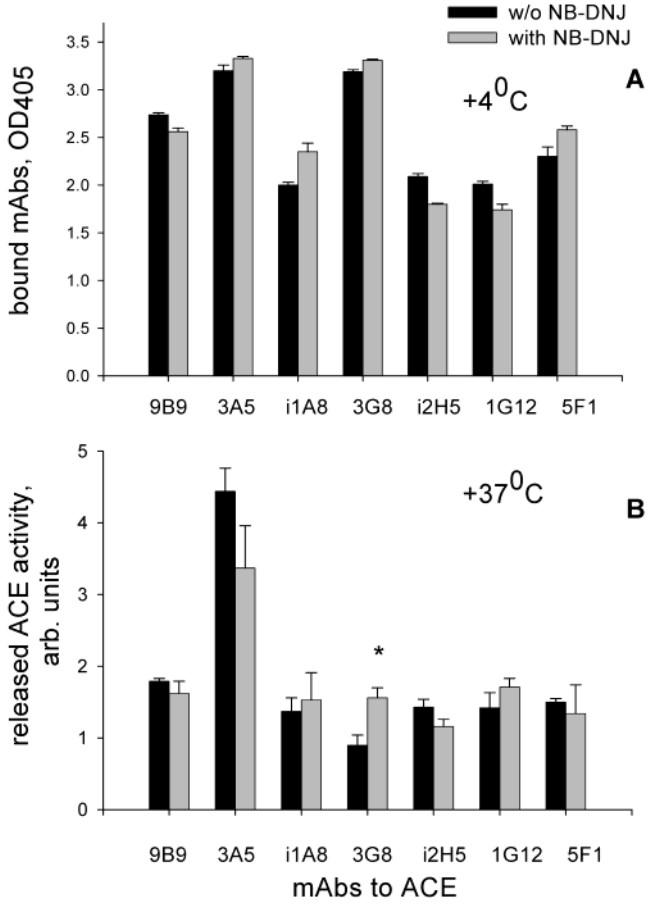

FIGURE 6: Effect of NB-DNJ on the binding of anti-ACE mAbs to the ACE on the cell surface (A) and antibody-induced ACE shedding (B). The CHO-ACE cell line (clone 2C2) was grown on a 96-well plate until confluence in the presence (2 mM) or absence of NB-DNJ in serum-free (complete) medium. (A) mAb binding assay (cell ELISA), as in Figure 5. (B) ACE shedding assay, as in Figure 5. (*) $p < 0.05$; differences are statistically significant in comparison with NB-DNJ-treated cells.

To examine the relationship between ACE N-glycosylation and ACE shedding, we studied antibody binding and antibody-induced (as well as basal) ACE shedding in the presence of *N*-butyldeoxynojirimycin (NB-DNJ), an inhibitor of the oligosaccharide-processing enzymes α-glucosidases I and II. This inhibitor prevents maturation of N-linked oligosaccharides on ACE molecules expressed in CHO cells so that these sugars remain as an oligomannose core (*22*).

Inhibition of ACE N-glycosylation in the presence of NB-DNJ resulted in the following findings: (i) The rate of the basal shedding of somatic ACE increased by 80% (from 18.6 ± 3.7% during 24 h to 33.6 ± 9.2%, $p < 0.05$). (ii) The pattern of mAb binding to ACE on the cell surface was altered in an epitope-specific manner (Figure 6A). MAb 3A5 binding, which induced ACE shedding, remained unchanged. mAbs 3G8, 1A8, and 5F1 bind better with partially glycosylated ACE, whereas mAbs belonging to the i2H5 group (*31*) and 9B9 bind better with fully glycosylated ACE. (iii) The pattern of antibody-induced ACE shedding also changed dramatically (Figure 6B). Shedding of underglycosylated ACE in the presence of all tested mAbs (with an exception of mAb 3G8) decreased in comparison to fully glycosylated ACE, whereas the effect of mAb 3G8 binding was the opposite (Figure 6B). In other words, restricted glycosylation of ACE completely abolished the inhibitory effect of mAb 3G8 on the ACE shedding from the cell surface.

Chemical Cross-Linking of Cell Surface Proteins. The chemical cross-linker BS^3 (which is membrane impermeable and has a spacer arm of 11.4 Å) reacts with lysine residues. Cross-linking experiments followed by Western blotting showed that the somatic ACE from the lysate of the untreated CHO-ACE cells ran as a monomer (lane 1) with an apparent molecular mass of 170 kDa (Figure 7). Addition of the cross-linker significantly decreased the density of the ACE band corresponding to the monomer and produced additional bands, corresponding to a dimeric form (lane 2). In addition, higher molecular weight aggregates were detected. These aggregates may represent dimer−monomer or dimer−dimer interactions.

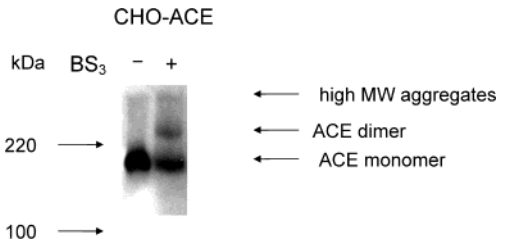

FIGURE 7: Western blot analysis of cross-linking of ACE on the surface of CHO-ACE cells. The CHO-ACE cell line (clone 2C2) was grown until confluent. Cross-linking was carried out on ice using 1 mM BS^3 and incubating the cells for 1 h. Cells were lysed with CHAPS and separated by 7.5% SDS−PAGE, followed by immunoblotting using anti-ACE mAb 1D8 at 5 μg/mL. The position of the molecular mass markers is shown on the left-hand side. The monomeric and dimeric forms of ACE are marked. (This blot is representative of three experiments.)

DISCUSSION

Dimerization plays an important role in the functioning of many membrane proteins (*41*−*42*). In contrast to what is known about the dimerization of several membrane proteins such as ICAM (*43*), PECAM (*44*), VE-cadherin (*45*), and P-selectin (*46*), there is little information on ACE dimerization. Numerous studies on the isolation of somatic ACE from different tissues and species show the presence of several peaks of ACE activity, including high molecular mass aggregates, during gel-filtration or ultracentrifugation steps. However, electrophoresis under denaturing conditions reveals only canonical forms of somatic ACE (around 180 kDa), indicating that high molecular mass aggregates might represent noncovalent complexes of ACE (*47*, *48*). A study performed on the brush-border membrane from the organ culture of human intestinal mucosa did not reveal ACE dimers, while dimers of another brush-border enzyme (aminopeptidase N) were demonstrated (*49*, *50*). Despite the fact that each domain of ACE has one unpaired cysteine (*51*), the molecular mass of ACE isolated from the cell membrane did not differ in reducing versus nonreducing conditions, thus excluding the existence of disulfide-linked dimers of ACE. The possibility of intramolecular disulfide linkages via this unpaired cysteine was also excluded (*51*).

Given the clinical significance of tissue ACE expression (*4*, *52*), it is important to understand the topology of ACE on the cell membrane. To study a organization of ACE within the membrane architecture, we have examined ACE in a reverse micellar system (*25*, *40*, *53*, *54*). The reverse micelle is one of many models thought to have properties resembling the biological cellular environment (*23*, *24*). In these systems, proteins are incorporated into the polar intramicellar spaces and are protected by monomolecular cover of hydrated surfactant against action of organic solvent. Previously, we demonstrated that bovine somatic ACE can be detected in reverse micelles not only as a monomer but also as a homodimer as well (*25*, *40*, *53*). Moreover, ACE dimerization in the reverse micelles is suppressed by carbohydrates, the extent of this effect being highly dependent on the structure of the definite carbohydrate; e.g., the effect of glucose (noneffective monosaccharide) and galactose (effective monosaccharide) differed 10000 times (*25*, *26*). These data allowed us to propose the existence of a carbohydrate-recognizing domain on the ACE molecule (*26*).

The present study shows that both bovine and human somatic ACE bear a carbohydrate-recognizing domain, but the variety of protein complexes formed is larger for bovine ACE (Figure 1). The reason for this observation can be that bovine ACE is more glycosylated (*26*) and therefore is able to interact more extensively with the carbohydrate-recognizing domain on another ACE molecule. It is worth noting, however, that the ACE molecule providing the oligosaccharide chain for this interaction appears to be unable to use its own carbohydrate-recognizing domain for subsequent interactions, as trimers or chains of ACE molecules were not observed. This suggests that a putative structural asymmetry with respect to the disposition of the oligosaccharide chains on the ACE globule may exist.

The fact that the N-domain, but not the C-domain, bears a carbohydrate-recognizing domain (Figures 2−4) adds to the functional differences between these two domains of ACE, despite the high degree of sequence homology. Thus, the rate of hydrolysis of various synthetic and natural substrates by the two domains differs, with the C-domain of human ACE having higher catalytic constants for Hip-His-Leu, angiotensin I, bradykinin, and substance P, whereas the N-domain hydrolyzes LH-RH and the negative hematopoietic regulator *N*-Ac-Ser-Asp-Lys-Pro at a much higher rates (*55*−*57*). The C-terminal domain is more sensitive to chloride activation (*55*, *58*). Most of the competitive ACE inhibitors bind tightly with the C-domain active center (*59*). The two domains of ACE differ also in conformation (*31*, *60*), and the N-domain seems to be more immunogenic (*31*). The N-domain expresses higher thermostability (*61*, *62*) and contains more putative glycosylation sites (10 sites for human enzyme and 11 sites for bovine ACE) than the C-terminal domain (7 and 5 sites for human and bovine ACEs, respectively) (*7*, *22*, *62*). And, finally, the C-terminal domain of ACE (or, perhaps more correctly, testicular ACE containing the C-domain and 36 additional amino acids, heavily O-glycosylated) determines male fertility (*63*).

The inhibition of carbohydrate-controlled ACE−ACE interactions in reverse micelles by certain N-domain-specific monoclonal antibodies confirmed the location of the carbohydrate-recognizing domain on the ACE N-domain.

In the presence of all mAbs (the panel of seven), the first maximum of enzymatic activity on the enzymatic activity−hydration degree dependence curve was observed at W_o = 30−34. This maximum was attributed to the enzyme−antibody complex. Some differences in the position of the first maximum in the presence of different antibodies (Table 2) are probably due to the location of the particular epitope on the ACE globule. The molecule of ACE was shown to be nonspherical but could be considered as an ellipsoid (*40*, *53*). So, the binding of antibody to the "side" or to the "top" of the enzyme could produce complexes of different size.

The second activity maximum is obtained in the presence of five different mAbs (Table 2) and may correspond to a mAb−ACE−ACE−mAb complex. Such complexes could be formed through the binding of an oligosaccharide chain of one simple ACE−mAb complex with the carbohydrate-recognizing center on the ACE molecule within another ACE−mAb complex and would be sensitive to the presence of free carbohydrates. Indeed, addition of galactose, specific for the carbohydrate-recognizing domain of ACE (*25*, *26*),

resulted in the disappearance of the second maximum on the profile of ACE−mAb complex activity (Figure 3B).

Thus, out of seven monoclonal antibodies to different ACE epitopes, five antibodies, 3A5, i1A8, i2H5, 1G12, and 5F1, were able to form ACE−mAb complexes with sequential mAb−ACE−ACE−mAb complex formation, suggesting that these antibodies do not shield the carbohydrate-recognizing domain on the enzyme globule. However, two partially competitive antibodies to the N-domain, 9B9 and 3G8, abrogated formation of these double complexes in reverse micelles. Thus, mAbs 9B9 and 3G8 shield the CRD upon binding, so that this center becomes unable to interact with oligosaccharide chains of another glycoprotein molecule. Moreover, we can state that the CRD region is restricted by the area overlapped by smaller Fab fragments of mAb 9B9 (Figure 4B).

The binding of mAbs 9B9 and 3G8 to ACE appears to affect the functional activity of the enzyme. Recently, we demonstrated epitope-dependent antibody-induced ACE shedding, as only two mAbs (9B9 and 3A5) out of eight assayed (all recognizing different epitopes on ACE molecule) dramatically induced ACE shedding (*14*; see also Figure 6). We proposed that the binding of mAbs 9B9 and 3A5 (directed to distinct, but overlapping epitopes; *31*) alters the conformation of the large, extracellular domain of ACE, thus exposing the stalk region and facilitating access of the secretase(s) involved in this process (*14*). The epitopes for mAbs 9B9 and 3A5 are located between Lys535 and Lys572 on the N-domain of ACE (I. V. Balyasnikova, and S. M. Danilov, unpublished work). It was surprising that mAb 3G8, whose epitope overlaps significantly with both mAbs 9B9 and 3A5 (*31*), inhibited ACE shedding from the cell surface (Figure 5). Therefore, this scenario is more complex than it seems: binding of mAb 3G8 to the surface of ACE somewhere between Tyr521 and Lys572 (I. V. Balyasnikova and S. M. Danilov, unpublished work) effectively inhibits ACE−ACE interaction via the carbohydrate-recognizing domain in artificial membranes and inhibits shedding of ACE from natural cell membrane. Binding of mAb 3A5 (to approximately the same region) dramatically induces ACE shedding without affecting ACE−ACE interaction, whereas binding of mAb 9B9 to ACE shields the CRD on the ACE globule (as in case of mAb 3G8) in artificial membranes but induces ACE shedding on real cell membranes. The effect of galactose ("specific" monosaccharide) on antibody-induced ACE shedding also elicits different effects in the presence of these three mAbs (Figure 5).

The mechanism of ACE dimerization seems to be similar to the noncovalent dimerization of membrane proteins such as P-selectin (*46*) and ICAM-1 (*43*) but different from dimerization of other membrane proteins, where disulfide links are involved, for example, P-selectin ligand PSGL-1 (*64*) or PDGF (*65*). In some cases such as VEGF a combination of noncovalent, hydrophobic interaction and disulfide-linked bridges is invoked in dimer formation (*66*, *67*).

It is worth noting that carbohydrate-mediated ACE−ACE interaction should be considered as a weak one which is difficult to determine in water solutions. ACE−ACE dimerization was not found in solution unequivocally at the enzyme concentrations up to 10^{-6} M (O. A. Kost, unpublished results). However, these interactions are likely more pronounced where the interacting partners are in closer proximity, e.g., on cell membrane. The term "weak" is not equivalent to the definition "insignificant", as these interactions can finely regulate other protein functions. We have shown recently (*40*, *54*) that ACE can function in different oligomeric forms in an artificial membrane with dimers and tetramers being more catalytically active than ACE monomers. Moreover, the microenvironment on the membrane can dramatically influence both the oligomeric state and the catalytic activity of the enzyme (*54*). Evaluating the effect of the self-association of proteins in membranes due to the presence of other proteins in high local concentrations and due to the preoriented state of the reactive species allowed to calculate (*68*) that these factors alone can increase the likelihood of forming dimers 10^6-fold.

The lectin-like properties of the ACE molecule (the existence of a specific carbohydrate-recognizing center), although not necessarily important for enzymatic function in vitro, could facilitate the orientation of the ACE globule on the cell surface and invoke the enzyme in cell recognition events. Our work suggests that the carbohydrate-recognizing domain may be important for ACE shedding from the cell surface.

Cross-linking of ACE on the surface of CHO-ACE cells (Figure 7) indicates that ACE might form dimers on the cell surface, albeit that the population of ACE in the dimeric form is relatively small. Similarly, a small percentage (10−15%) of the total EGF receptor population was present as dimers (*69*). Previous attempts to show ACE dimerization on brush-border membranes in using sucrose gradient sedimentation and cross-linking with a bifunctional reagent (*49*) were unsuccessful. However, these experiments were performed under very different conditions: (1) membrane vesicles, isolated from biosynthetically labeled intestinal tissue explants, were used; (2) the cross-linking was carried out with the hydrophobic cross-linker DSS [dithiobis(succinimidyl propionate)] as opposed to the polar membrane-impermeable BS^3; (3) the hybridoma HBB 3/264/18 was used to detect the dimers, whereas we used the very sensitive monoclonal antibody mAb 1D8. Using this approach, however, it is impossible to quantify the effect of galactose or mAb 3G8 on ACE dimerization on the cell surface, and further experiments with cross-linking agents are required.

Moreover, we demonstrated the ability of the ACE globule to form complexes with carbohydrate-containing molecules (free carbohydrates, another ACE molecule, or ACE−mAb complexes) through the interaction of the carbohydrates with the ACE CRD. At this stage, it is uncertain whether the putative ligand for the ACE CRD on the cell membrane is the another ACE molecule or a still unknown glycoprotein of similar molecular mass. The physiological role of ACE dimerization on the cell surface still remains to be elucidated.

Numerous studies have demonstrated that three-dimensional conformation, rather than a linear sequence, is the target for different (including ACE) secretases (*13*, *70*). Our data on both the inhibition of ACE−ACE interactions in reverse micelles and the increase in ACE shedding from the cell surface by mAb 9B9 allow us to speculate that putative dimerization of ACE via its carbohydrate-recognizing domain on the surface of ACE-expressing cells prevents the enzyme from proteolytic cleavage by the ACE secretase(s). Thus, the ACE monomer appears to be shed more efficiently, and

the increase of basal ACE shedding in the presence of galactose specifically suppressing ACE−ACE interaction supports this hypothesis. This proposed mechanism is in accordance with the mechanism of shedding for growth hormone receptor (GHR): dimerization of GHR induced by growth hormone significantly inhibited proteolytic cleavage of GHR (*71*).

Results of the present study also indicate the possible link between ACE glycosylation and shedding. Antibody-induced ACE shedding in the presence of galactose was affected in an epitope-specific manner (Figure 5B). Restriction of ACE glycosylation also dramatically changed basal as well as antibody-induced ACE shedding (Figure 6). The increase in basal shedding of underglycosylated ACE, lacking carbohydrate−CRD interactions, serves as additional evidence for the more favored proteolytic cleavage of the monomeric ACE monomer by the ACE secretase(s). As numerous studies have demonstrated that glycosylation affects the association of membrane proteins (*69*, *72*), it is possible that both putative dimerization (or interaction with a still unknown glycoprotein partner on the cell membrane) and shedding may be regulated via carbohydrates.

In summary, we have demonstrated that carbohydrate-controlled ACE−ACE interactions (via a carbohydrate-recognizing region on the N-terminal domain of ACE) occur in reverse micelles, and we have provided evidence for the participation of carbohydrates in ACE shedding, suggesting a link between ACE dimerization and shedding. However, the exact mechanism of ACE dimerization and it physiological significance still require further investigation.

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