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Monoclonal Antibodies 1B3 and 5C8 as Probes for Monitoring the Integrity of the C-Terminal End of Soluble Angiotensin-Converting Enzyme

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ABSTRACT

Angiotensin-converting enzyme (ACE) is a membrane-anchored ectoprotein that is proteolytically cleaved, yielding an enzymatically active soluble ACE. Two mouse monoclonal antibodies, MAbs 1B3 and 5C8, were generated to the C-terminal part of human soluble ACE. MAb 1B3 recognized the catalytically active ACE, as revealed by ELISA and precipitation assays, whereas Western blotting and immunohistochemisty on paraffin-embedded sections using MAb 5C8 detected denatured ACE. MAb 1B3 showed extensive cross-reactivity, recognizing 15 species out of the 16 tested. The binding of this MAb to ACE was greatly affected by conformational changes induced by adsorption on plastic, formalin fixation, and underglycosylation. Furthermore, MAb 1B3 binding to the mutated ACE (Pro1199Leu substitution in the juxtamembrane region, leading to a fivefold increase in serum ACE level) was markedly decreased. MAb 5C8 detected all the known expression sites of full-size ACE using formalin-fixed and paraffin-embedded human tissues. The sequential epitope for MAb 5C8 is formed by the last 11 amino acid residues of soluble ACE (Pro1193–Arg1203), whereas the conformational epitope for 1B3 is formed by a motif within these 11 amino acid residues and, in addition, by at least one stretch that includes Ala837–His839 located distal to the sequential epitope. Our findings demonstrated that MAbs 1B3 and 5C8 are very useful for the study of ACE shedding, for identification of mutations in stalk regions, and for studying alternatively spliced variants of ACE. In addition, binding of MAb 1B3 is a sensitive determinant of the integrity of soluble ACE.

INTRODUCTION

Angiotensin I–converting enzyme (ACE, CD143) is zinc metallopeptidase, which converts angiotensin I to angiotensin II, a potent vasopressor, and degrades bradykinin, a potent vasodepressor. Thus, this enzyme plays a key role in the regulation of blood pressure and the development of vascular pathology and remodeling. ACE is constitutively expressed on the surface of endothelial cells, both different absorptive epithelial and neuroepithelial cells,[1–2] and cells of the immune system—that is, activated macrophages and dendritic cells.[3] Somatic ACE contains catalytic centers in the N- and C-terminal domains,[4] whereas a short testis-specific isoform, testicular ACE (tACE), expressed in germ cells, contains an identical C-terminal domain with only one catalytic center.[5,6] ACE was assigned a new CD marker—CD143.[7,8]

Blood and other biological fluids contain a variable amount of soluble ACE. Serum ACE originates from a membrane-bound form on endothelial cells,[9] by proteolytic cleavage of the Arg1203-Ser1204 peptide bond near the transmembrane domain,[10] whereas seminal fluid ACE (exceeding blood ACE level 50-fold) likely originates from epithelial cells of the epididymis, where somatic ACE is particularly abundant.[11]

The level of ACE in the blood of healthy individuals is very

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stable,[12] whereas granulomatous diseases, particularly sarcoidosis, lead to a two- to fourfold increase of ACE activity in the blood.[13–14] Recently, a mutation in the stalk region of ACE[15–16] explained the dramatic (five- to sixfold) increase of ACE activity in the blood of affected individuals found in Japanese,[17] Italian,[18] Dutch,[15] and German[19] families.

ACE is a member of the growing family of membrane proteins that are proteolytically cleaved in the juxtamembrane stalk by metalloproteases referred to as "secretases" or "sheddases." The ACE secretase is co-localized with ACE in a number of tissues. ACE secretase has an absolute requirement for its substrate (ACE) to be anchored in a membrane for cleavage to occur.[20] However, the mechanism of production of soluble ACE *in vivo*, the components involved in this process, and the factors that regulate generation of soluble ACE are not fully understood.

Previously, two types of MAbs to human ACE were obtained: (i) MAbs recognizing conformational epitopes localized on the N-domain of human ACE,[21–22] and (ii) MAbs recognizing sequential epitopes on the denatured C-domain.[22,23] These MAbs were successfully used for ACE quantification in solution by ELISA[24–25] and on the cell surface by flow cytometry[3]; to study the structure and function of ACE[22,23,26–28]; to deliver enzymes[29,30] and genes into the pulmonary endothelium[31–32] (Brosnan et al., unpublished data); as a diagnostic tool for lung vessel visualization[33]; and for immunohistochemistry of ACE.[11,34–39] However, the absence of MAbs to the native conformation of the C-domain of ACE has prevented the study of many aspects of ACE biology, in particular those involving the testicular form of ACE.

Here, we report the production and characterization of two novel MAbs—1B3 and 5C8—that are specific for the C domain of human ACE. They recognized a motif Ser1193–Arg1203, which is located at the C-terminus of soluble testicular and somatic ACE. Thus, these MAbs are very useful for studying ACE ectodomain shedding and detecting the integrity of soluble ACE's C-terminal end.

MATERIALS AND METHODS

Purification of ACE antigen

Human testicular ACE was purified from human spermatozoa and from CHO cells expressing the testicular form of human ACE[40] using lisinopril affinity chromatography. Washed spermatozoa or CHO-tACE cells were re-suspended in 50 mM

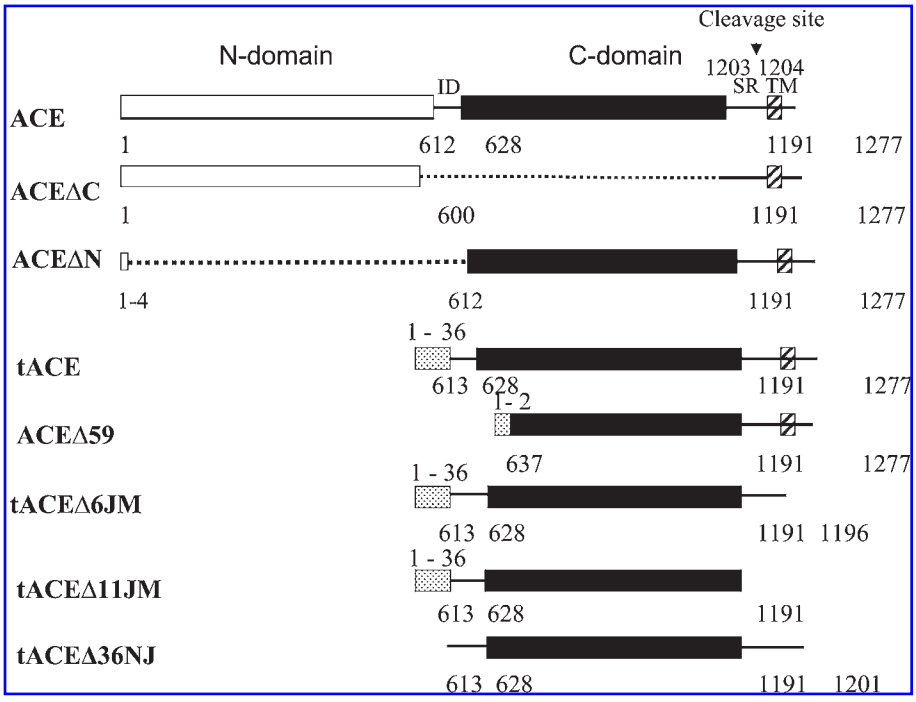

FIG. 1. Schematic diagram of different ACE constructs. Wild-type human somatic ACE is comprised of two homologous domains, N and C domains, joined by an interdomain region of approximately 16 amino acids.[58] A stalk region (SR), from residue 1192 to 1227,[46] links the ectodomain and transmembrane (TM) domains (residues 1228–1249 are indicated by a hatched box). In the construct ACEΔC, the N-terminal domain of somatic ACE (Leu1 to Pro601) is fused to the SR, TM, and cytosolic domains.[44] In the construct ACEΔN, the C-domain (coding amino acid residues 613–1277) was fused with leader sequence and first four amino acid residues from N-domain (Balyasnikova et al., unpublished data). Recombinant human testicular ACE has a unique 36–amino acid sequence, and the rest of the molecule is identical to the C-domain of somatic ACE.[40] A deletion mutant, tACEΔ36NJ, which lacks the first 36 N-terminal residues and is truncated after Ser625 (Ser 1201—somatic ACE numbering), was described previously.[45] The tACEΔ59 mutant was constructed such that 59 of the N-terminal residues of mature human testis ACE (Gln3-Gln61-tACE numbering) were deleted.[46] Deletion mutants of human tACE lacking six or 11 amino acid residues in the juxtamembrane stalk region (tACEΔ6JM and tACEΔ11JM) were described previously.[46,47]

potassium phosphate buffer (pH 8.3) containing 8 mM CHAPS and incubated for 1 h at 4°C. After centrifugation at 10,000*g* for 10 min, the supernatant was passed through a 0.45-μM filter and applied to a lisinopril-Sepharose column. After washing the column, bound tACE was eluted with 50 mM $Na_2B_4O_7$ (pH 9.5) and dialyzed against 5 mM $KHPO_4$ containing 10 μM $ZnSO_4$ (pH 8.3). For some experiments, human, rat, and bovine ACE (Chemicon Int., Temecula, CA) was also used.

ACE activity assay

Human and animal serum ACE as well as purified ACE activity was measured using a fluorimetric assay.[41–42] Briefly, aliquots were added to 200 μL of substrate for ACE (5 mM Hip-His-Leu or 2 mM Z-Phe-His-Leu) and incubated for the appropriate time at 37°C. In some experiments, we added substrates directly to the wells of microtiter plates, which were "coated" with ACE for the ELISA, or plate precipitation assay. The incubation was terminated, and the reaction product (His-Leu) was quantified fluorimetrically with *o*-phthaldialdehyde.

Immunization, fusion, and screening

Two Balb/c female mice were immunized six times at 1-day intervals with subcutaneous injections of 7 μg of human tACE in complete Freund's adjuvant (CFA). This was followed by an equal dose of the tACE in CFA by intraperitoneal (IP) injection the next day. Nine days after last IP injection, the titer of antibodies in the serum was tested, and the animal with the highest titer was chosen for a subsequent boost. The boost was done by intravenous (IV) injection of antigen without CFA for 3 days. Immediately afterwards, fusion of the spleen cells with myeloma cell line X63.Ag8.653, sub-clone P3O1, was carried out as described elsewhere.[21] Hybridoma supernatants were assayed for anti-ACE antibodies by ELISA and a plate-precipitation assay. Selected populations were cloned, and supernatants were repeatedly assayed in order to pick up the best clones. MAb Ig class and subclass determination was performed using a Mouse Typer® Sub-Isotyping Kit (Bio-Rad Laboratories, Hercules, CA).

Plate immunoprecipitation assay

96-well plates (Corning, Corning, NY) were coated with 50 μl of affinity-purified rabbit anti-mouse IgG (10 μg/ml) and stored overnight at 4°C. After washing with PBS/0.05% Tween-20, the wells were incubated with anti-ACE MAbs (2 μg/ml) in PBS/0.1% BSA for 2 h at room temperature (RT) and washed. Wells were then incubated with 50 μL of PBS/0.1% BSA containing 5–20 mU/mL of any source of ACE for 2 h at RT, washed, and assayed for plate-bound ACE activity using Hip-His-Leu as a substrate, as described previously.[22]

ELISA

96-well microtiter plates (Corning) were coated with 50 μL of purified human ACE (1–5 μg/mL) and incubated overnight at 4°C. After washing, 50 μL of hybridoma supernatants con-

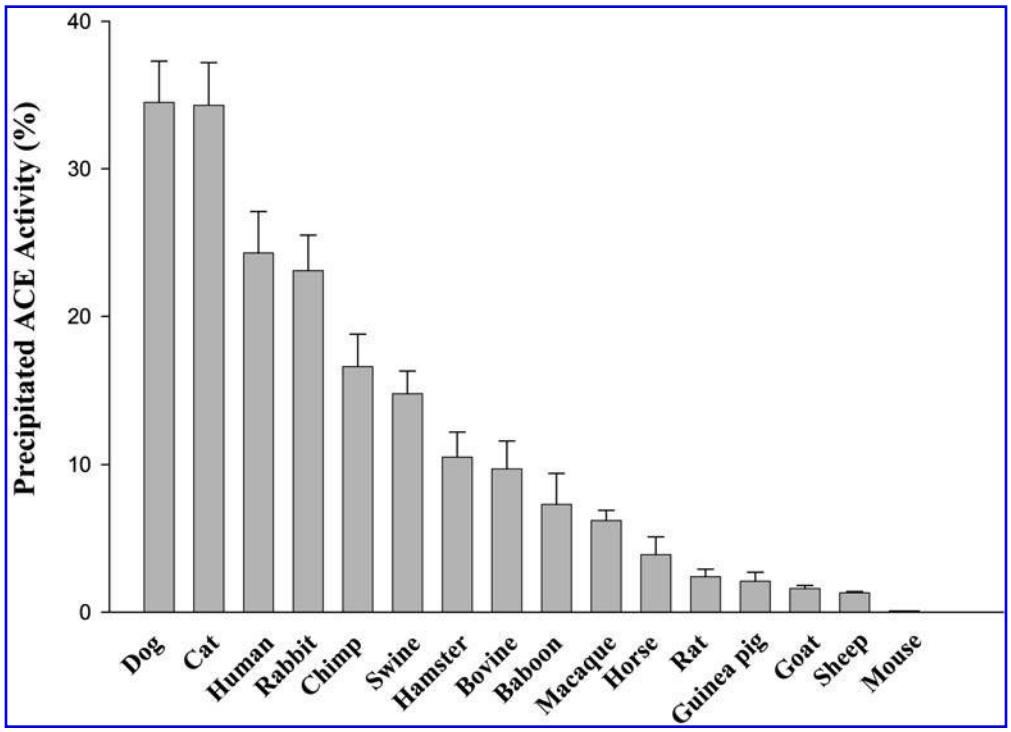

FIG. 2. Precipitation of ACE activity from sera of different species by MAb 1B3. ACE concentration in serum samples was adjusted to a final enzymatic activity of 20 mU/mL of the ACE activity using Z-Phe-His-Leu as substrate and incubated in a microtiter plate coated with MAb 1B3 via goat-anti-mouse IgG (plate precipitation assay).[22] Precipitated ACE activity was quantified by spectrofluorometric assay. Data presented are mean ± SD of triplicates. Binding of 1B3 to ACE from sera of all animal species was statistically different ($p < 0.05$) from that for ACE from human serum.

Sequence of ACEs	Stalk Region 1187 … 1203	1B3 Binding %	5C8 Binding
Human	**G E** K **L G W P** Q Y N **W** T **P** N S A R	100 [a]	++ [f]
Chimp	**G** - - - - - - - - - - - - - - - - - R	70 [a]	ND
Bovine	**G** - - - - - - - - - - - - - - - - - R	40 [a]	++ [f]
Rabbit	**G** - - - - - - - - - T - - - - - - - R	95 [a]	+/- [f]
Rat	**G** - T - - - - E - T - - - - T - R	10 [a]	- [f]
Mouse	**G** - T - - - - E - - - A - - T - R	0.3 [a]	-/+ [f]
Pro1199Leu	**G** - - - - - - - - - - - - L - - - R	44 [b]	+/- [g]
Asn1196Gln	**G** - - - - - - - - - Q - - - - - - R	102 [c,d]	- [c]
ACEΔC	**G** - V - - - - E - Q - H - - - - R	0.8 [c, e]	- [c]
tACEΔ36NJ	**G** - - - - - - - - - - - - - - - S	105 [c, e]	++ [c]
tACEΔ 6JM	**G** - - - - - - - - - **W**	20 [c, e]	- [c]
tACEΔ11JM	**G** - - - - **W**	0 [c, e]	- [c]

FIG. 3. Binding of different ACEs by MAbs 1B3 and 5C8. The amino acid sequence alignment of the C-terminal part of the somatic ACE from different species and ACE constructs (Fig. 1). Universally conserved residues, defining the C-domain boundary,[46] are shown in bold. Binding of MAb 1B3 to the studied ACEs was quantified by a plate precipitation assay as in Figure 2. Data presented as a mean of three independent determinations (which did not differ by more than 10%). [a]Serum of these species was used as a source of somatic ACE. [b]Pooled sera of four affected individuals with a Pro1199Leu substitution[15] was used as a source of mutated ACE. Data expressed as a percentage of MAb 1B3 binding to ACE from pooled sera from four of their non-affected relatives. [c]Serum-free culture medium from CHO cells expressing the indicated constructs were used as a source of mutated ACEs. Data expressed as a percentage of wild-type ACE. [d]ACE_{NQ} construct[49] was used. [e]For the description of these mutants, see Figure 1. Binding of 5C8 to the studied ACEs was estimated by Western blotting. [f]Supernatants of homogenates (1:10) of kidneys or lung (rat only) were equilibrated according to 200 mU/mL of the ACE activity (with Z-Phe-His-Leu as a substrate). SDS-PAGE with subsequent Western blotting was performed with 10 μg/mL of studied MAbs as described in Materials and Methods. [g]ACE purified from sera of these patients and their unaffected relatives was used. ND, not detected.

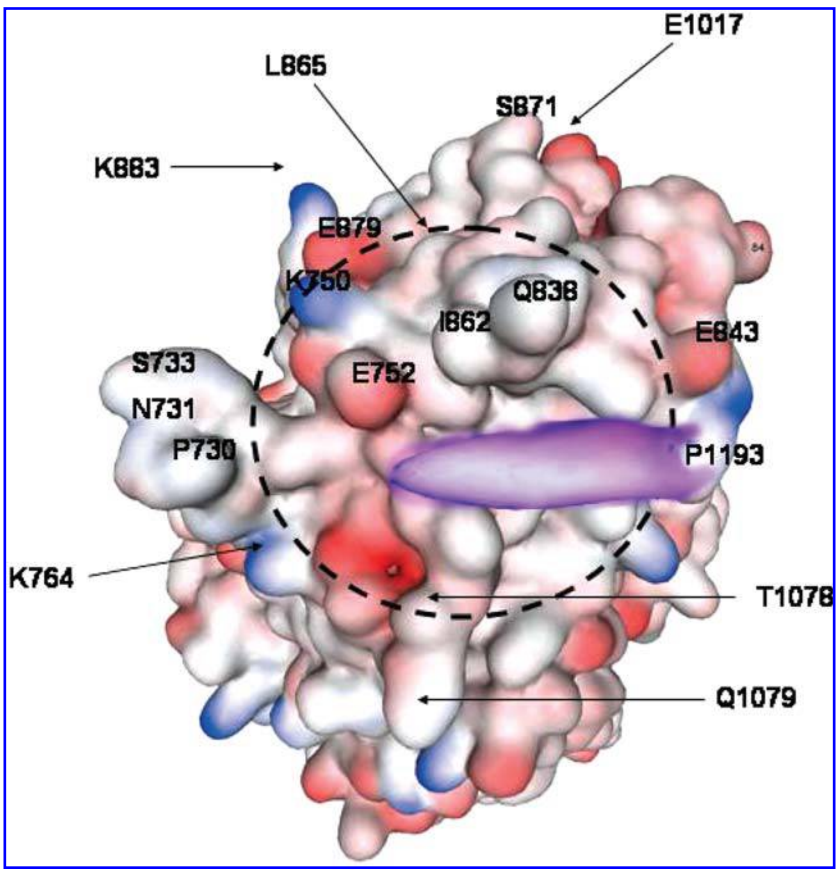

FIG. 4. Molecular surface representation of the C domain of human ACE. The negative and positive potentials are shown in red and blue, respectively. The dashed circle with a 25Å diameter shows the putative surface area covered by MAb 1B3. The proposed orientation of the C-terminus (Pro1193-Arg1203) is indicated by the purple shaded area. Key amino acids referred to in the text are denoted using somatic ACE numbering.

taining MAbs to ACE or pure MAbs (10 μg/mL) were added and incubated for 2 h at RT. The wells were then incubated with goat anti-mouse polyclonal antibody conjugated with alkaline phosphatase (Sigma, St. Louis, MO) diluted 1:1000 with PBS/BSA for 1 h at RT and washed afterwards. Alkaline phosphatase was developed using *p*-nitrophenyl phosphate as a substrate and read at 405 nm.[25]

ACE constructs and stable cell lines

Stable cell lines of CHO cells expressing wild-type human somatic ACE, testis ACE, or only the N domain of ACE (ACEΔC) were obtained and cultured as described previously.[40,43,44] CHO cells stably expressing only the C domain (Leu1-Gly4, Leu613-Ser1277) are described elsewhere (Balyasnikova et al., unpublished data). A deletion mutant tACEΔ36NJ, lacking the first 36 N-terminal residues and truncated after Ser625 (tACE numbering), was described previously.[45] The tACEAΔ59 mutant was constructed such that 59 of the N-terminal residues of mature human testis ACE (Gln3-Gln61, tACE numbering) were deleted.[46] Deletion mutants of human tACE lacking either six or 11 amino acid residues in the juxtamembrane stalk region were described previously.[46,47] ACE constructs used in this study are shown in Figure 1.

Western blotting

All samples for SDS electrophoresis were adjusted to a final concentration that had an ACE activity of 180 mU/mL. Supernatants of human, rat, bovine, rabbit, and mouse kidney homogenates, serum-free culture medium from CHO-ACE expressing cells, or lysates of these cells (40 μL per lane) were run using 7.5% Tris-HCl SDS PAGE (Bio-Rad Laboratories). The electrophoretic transfer of ACE from gels to microporous PVDF-Plus membrane was performed as described elsewhere.[48] After transfer, the membrane was incubated in 10 mM Tris-HCl (pH 8.0) buffer containing 150 mM NaCl, 0.05% Tween 20, and 5% dry milk prior to incubation with hybridoma culture fluids. The subsequent steps were carried out with ProteoQuest Kit (Sigma).

Tissues and immunohistochemistry

Routinely processed formalin-fixed and paraffin-embedded tissues of eight individuals were obtained from the archives of the Institute of Pathology (Giessen, Germany), comprising normal parts of several organs with already known and characteristic expression sites of ACE: lung, kidney, epididymis, brain, and testis. The paraffin material was sectioned at 2–4 μm, and tissue sections were mounted on super-plan slides (Superfrost, Menzel-Gläser, Braunschweig, Germany) and dried overnight at 37°C. Microwave treatment was performed in 0.01 M citrate buffer solution (pH 6.0) as previously described.[37] Immunohistochemistry was performed using the alkaline-phosphatase-anti-alkaline-phosphatase (APAAP) technique and an automated staining system (TechMate 500 Plus; Dako, Hamburg, Germany). The MAb 5C8 and 1D8 (hybridoma culture fluid supernatants) were used at a 1:10 dilution in Tris-HCl buffer (pH 7.4) and incubated for 30 min at RT each. Negative controls were performed by omitting the primary MAb from the buffer during the first incubation.

RESULTS AND DISCUSSION

Screening of hybridoma clones

To generate MAbs to the C-terminal domain of ACE, the screening of C-domain–specific hybridoma clones was carried out using an ACE ELISA on purified human testicular ACE (tACE). Out of 325 primary reactive cell populations obtained, 15 clones to tACE were further expanded. We describe the development of two hybridoma clones (1B3 and 5C8) that recognize different epitopes localized at the C terminus of soluble ACE. Both proved to be of the IgG1 isotype having a κ chain.

Species specificity of MAb 1B3

MAb 1B3 raised against human tACE recognized catalytically active somatic ACE from sera of 15 out of the 16 species tested (Fig. 2). The precipitation of ACE activity from the tested sera ranged from 2% to 35%, and interestingly, the binding of MAb 1B3 to dog and cat ACE was approximately 10% more than that to human ACE. Mouse ACE showed no cross-reactivity with MAb 1B3.

Localization of the 1B3 epitope

Different C-domain deletion constructs[44–47] were used to localize the epitope for MAb 1B3 (Fig. 1). The wide cross-reactivity of this MAb suggests that 1B3 might be directed to the N- or C-terminal part of ACE, which should be highly conserved among species. Deletion of the last 11 amino acids from the soluble form of the C-domain (tACEAΔ11JM) completely abolished binding of MAb 1B3 (Fig. 3; also see Fig. 8B below), whereas the binding of this MAb to a construct lacking the first 59 amino acid residues from the N-terminal part of tACE (tACEAΔ59) showed no change in binding (data not shown).

Alignment of protein sequences of the C-termini of soluble ACE, containing the putative epitope for MAb 1B3, and the data of the MAb 1B3 binding to the deletion constructs allowed us to identify amino acid residues involved in the binding of MAb 1B3 to soluble ACE (Fig. 3). The last two amino acid residues of soluble ACE (Ala1202 and Arg1203) do not appear to participate in the binding of 1B3, as binding to tACEAΔ36NJ did not differ from that with the soluble form of wild-type tACE. Ser1201 does not participate in the formation of the 1B3 epitope, because the precipitation of the soluble ACE_{NQ} construct, which lacks Ser1201,[49] was unaltered when compared to the wild-type tACE. Asn1196 is also not involved in the epitope for MAb 1B3, because this MAb demonstrated similar binding to both rabbit and human ACE, despite a substitution of Thr for Asn1196. This was confirmed by the fact that binding of 1B3 with the soluble form of the C-domain construct ACE_{NQ}, where Asn1196 was replaced by Gln,[49] did not differ from that of wild-type ACE.

The 10-fold decrease in binding of MAb 1B3 to rat ACE (where Gln1194 and Ser1201 are replaced by a Glu and Thr, respectively) strongly suggests that Gln1194 participates in the 1B3 epitope. Thr1198 also forms a part of the epitope, because binding to mouse ACE, which only differs from rat ACE in this region by a Thr1198Ala substitution, was almost completely

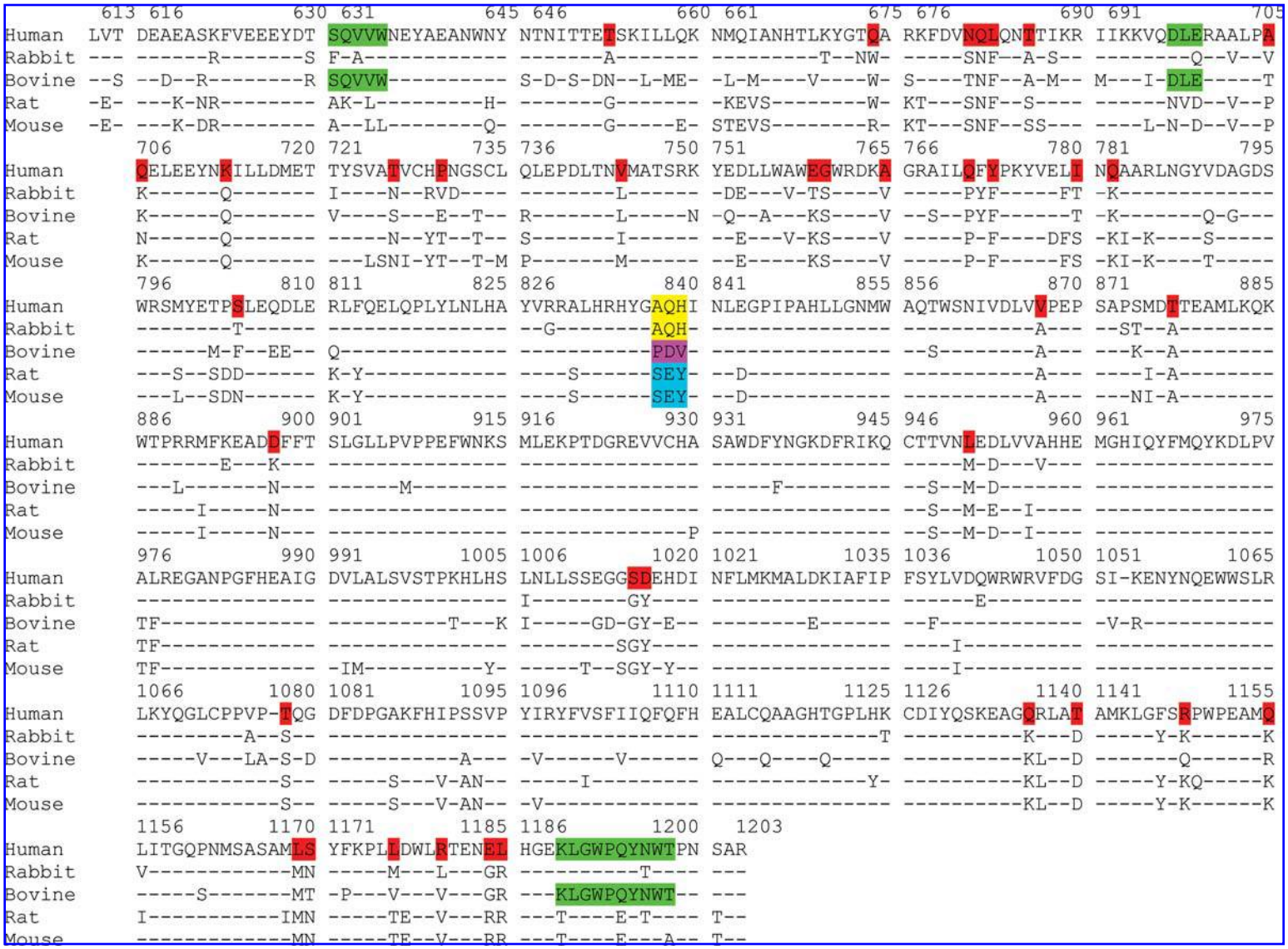

FIG. 5. Alignment of amino acid sequences of ACE from different species. Amino acid sequences of the C-domain of ACE, all deduced from cDNAs, were taken from the GenBank database. The numbering relates to the human somatic ACE.[4] Amino acid residues identical to residues in human ACE are represented by dashes. Amino acid residues which are human ACE specific are highlighted in red. Amino acid residues common to human and rabbit ACE within the motif AQH (1B3 pattern of immunoreactivity) are highlighted in yellow and their counterpart in bovine and rat/mouse mouse in purple and blue, respectively. Amino acid residues that are common to human and bovine ACE, but differ in other species (5C8 pattern of immunoreactivity) are highlighted by green.

abolished. Further, binding to an ACEAΔC mutant that has a Thr1198His substitution was also abolished. Pro1199 is likely also included in the 1B3 epitope, because binding of MAb 1B3 with serum ACE from patients having a Pro1199Leu substitution[15] decreased by 2.5-fold. These data suggest that Pro1193 to Asn1200 (PQYNWTPN) participates in the epitope formation of MAb 1B3. The fact that residual binding with tACEAΔ6JM consists of only about 20% indicates that the WTPN motif is more important than PQYN.

However, the fact that the binding of 1B3 with chimp ACE is 30% less and with bovine ACE is 60% less than that of human ACE, despite having the same sequence in this region, suggests that another stretch of the C-domain is involved in 1B3 epitope formation. Figure 4 shows a surface representation of the tACE structure centered with the putative epitope for MAb 1B3. Since the last amino acid residue of the C-domain seen in the tACE 3D-structure is Pro1193 (Pro617, tACE numbering[50]), the main motif of the 1B3 epitope could not be visualized. Nevertheless, we can speculate about the relative position of these 11 amino acid residues, representing a C-terminal end of human soluble ACE (Fig. 4). Prolines are known to form twists in peptide sequences. Thus, it is likely that Pro1193 results in a turn that permits the C terminus to be orientated in the position shown in Figure 4. Furthermore, secondary structure predictions suggest that there may be a beta strand upstream of Pro1199, followed by a loop in the hydrophilic region that contains the Arg1203-Ser1204 cleavage site, rendering it more accessible for ACE secretase cleavage.[16]

Alignment of the protein sequences of ACE from different species shows that bovine ACE has a motif PDV instead of A^{837}QH, as in human and rabbit ACE (Fig. 5). Moreover, the rat and mouse ACE also have changes in this region which might, in part, explain the decreased 1B3 binding with ACE from these two species. This suggests that a motif Ala^{837}GlnHis (AQH) is localized in close proximity to the 11 C-terminal residues of the soluble protein. The 30% decrease in 1B3 binding with chimp ACE (Figs. 2 and 3), which has a Thr substituted for Pro730[51] and the effect of glycosylation on the binding of this antibody, implicates the surface motif P^{730}NGS in the 1B3 epitope and supports the notion that the C-terminus is orientated in this direction.

Characteristics of MAb 1B3 binding

The binding of MAb 1B3 to ACE was significantly influenced by changes in the conformation of ACE owing to altered epitope accessibility. Figure 6A demonstrates that binding of MAb 1B3 to testicular ACE (tACE) immobilized on the plastic is practically negligible in comparison to binding of MAb 3F11, which recognizes another epitope in the middle of the C-domain. However, precipitation of the soluble somatic ACE (sACE) as well as tACE with MAb 1B3 was significantly higher than with MAb 3F11 (Fig. 6B). We used MAb 3F11 as a positive control for binding to a central part of the C-domain. Interestingly, the binding of MAb 1B3 to membrane-bound sACE and tACE was dramatically lower than to their soluble counterparts, while precipitation of a somatic ACE mutant, where N-domain was truncated (ACEΔN), was similar, if not identical, for both soluble and membrane-bound forms (Fig. 6B). This suggests that the attachment of the N domain (as in somatic ACE) or a highly *O*-glycosylated

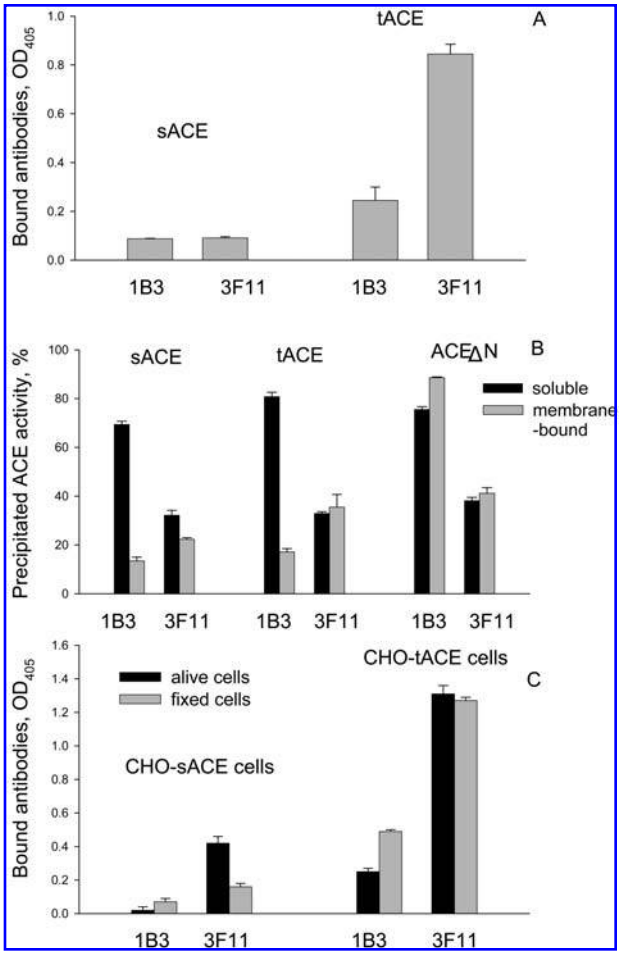

FIG. 6. Binding of somatic and testicular ACEs by mAb 1B3. (**A**) ELISA. 96-well plate coated with human sACE and tACE were incubated with mAb 1B3 and 3F11 at 10 μg/mL. Bound antibodies were detected using goat anti-mouse IgG conjugated with alkaline phosphatase and *p*-nitrophenyl phosphate as a substrate. (**B**) Plate precipitation assay. Binding of MAbs 1B3 and 3F11 to the studied ACEs was quantified by plate precipitated assay exactly as in Figure 2. The data are presented as mean ± SD of triplicates. (**C**) Cell ELISA. CHO-cells expressing somatic (clone 2C2) or testicular ACE (clone 3D10) were grown on 96-well plates until confluence. Anti-ACE MAbs or control mouse IgG (10 μg/mL), were added to the cells in serum-free medium (containing 2% BSA) and incubated for 1 h at 4°C. Bound anti-ACE MAbs were detected with goat-anti-mouse IgG, conjugated with alkaline phosphatase at 405 nm. The data represent results of several independent experiments and are expressed as the mean ± SD.

region (as in the N-terminal 36 residues of tACE) to the C-domain leads to an obstruction of the 1B3 epitope. The binding of 1B3 to both tACE and somatic ACE expressed on the cell surface was markedly reduced (Fig. 6C), suggesting that the membrane sterically hindered the binding. Denaturation of CHO-ACE cells by fixation with paraformaldehyde led to a two- to threefold increase of MAb 1B3 binding to the surface of these cells (Fig. 6C; $p < 0.05$). Moreover, pretreatment of these cells with neuraminidase increased the binding of MAb 1B3 fivefold ($p <$

0.05). Thus, we might conclude that MAb 1B3 recognizes a C-terminal part of soluble ACE that is exposed when ACE is in the solution, but is buried when ACE is immobilized (on plastic or on the cell surface).

The cultivation of CHO-ACE cells in the presence of NB-DNJ (an inhibitor of glucosidase I), which prevents the maturation of glycosylation, also increases binding of MAb 1B3 to the surface of CHO-ACE cells (fivefold, $p < 0.05$). However, there was no difference in binding of MAb 1B3 with soluble sACE or tACE from cells grown in the presence or absence of NB-DNJ. This suggests that the accessibility of the 1B3 epitope of ACE expressed on the cell surface is reduced in the presence of complex oligosaccharides, while the glycan does not participate in the 1B3 epitope itself.

MAb 1B3 as a probe for the integrity of the C-terminal end of soluble ACE

Serum proteins are subject to limited proteolysis, particularly at the C terminus, where carboxypeptidase-like trimming may occur. To evaluate the use of MAb 1B3 for detecting the integrity of the soluble ACE, we analyzed the binding of somatic ACE from sera and/or plasma samples stored under different conditions. The ratio of ACE precipitation with MAb 1B3 to that with MAb 9B9, directed to the N-domain of ACE,[22] was used to detect the extent of ACE denaturation. Using the 1B3/9B9 ratio instead of calculating the percentage of 1B3 binding has the advantage that it does not necessitate the determination of ACE activity. The latter can be problematic in the case of plasma samples with EDTA or plasma/serum samples from patients taking ACE inhibitors.

The 1B3/9B9 ratio was similar for freshly prepared serum and for plasma samples drawn with EDTA and stored for several years at −80°C, whereas it decreased significantly in plasma samples obtained with heparin and stored for 3 years at −20°C (Fig. 7A). Even plasma samples that were kept at −80°C for 3–5 years showed a 25% decrease in 1B3/9B9 ratio ($p < 0.05$, data not shown). It is interesting that the integrity of the C-terminus of soluble ACE, as reflected by this ratio, was better preserved in heparinized plasma samples from patients who were taking ACE inhibitors (Fig. 7A). This finding was further investigated by preparing sera in the presence or absence of enalapril (1 μM) and plasma samples with heparin, EDTA, or citrate and storing them for 2 months at −80°C, −20°C, and 4°C prior to analysis. MAb 1B3 binding decreased gradually with a concomitant increase in storage temperature, whereas MAb 9B9 binding and ACE activity remained unaltered (Fig. 7B). The 1B3/9B9 ratio was lower in serum, plasma heparin, and plasma citrate samples, but unchanged in serum stored with ACE inhibitor as well as plasma samples prepared with EDTA (Fig. 7C), indicating possible autolysis. Thus, proteolytic cleavage of the C-terminal end of soluble ACE may occur during prolonged storage of sera/plasma samples. Further, such cleavage of soluble ACE could be monitored by the measurement of ACE binding to MAb 1B3.

Species specificity and epitope localization of MAb 5C8

Because MAb 5C8 did not bind catalytically active human ACE in solution, we tested the cross-reactivity of this MAb to ACE from other species using Western blotting (Fig. 8A). Human and bovine ACE showed a similar binding of MAb 5C8, while the binding to rabbit ACE was low and almost negligible to rat and mouse ACE. The alignment of the primary ACE sequences of those species demonstrates that there are at least three putative motifs in the C-domain of ACE which correlate with this pattern of immunoreactivity (Fig. 5, highlighted in green).

To define the 5C8 epitope more precisely, Western blotting of several C-domain constructs with N- or C-terminal deletions (Fig. 1) was carried out. Binding of 5C8 was completely abolished with tACEΔ6JM and tACEΔ11JM mutants (Fig. 8B,C) but unaltered after deletion of 36 (Fig. 8C) or 59 N-terminal

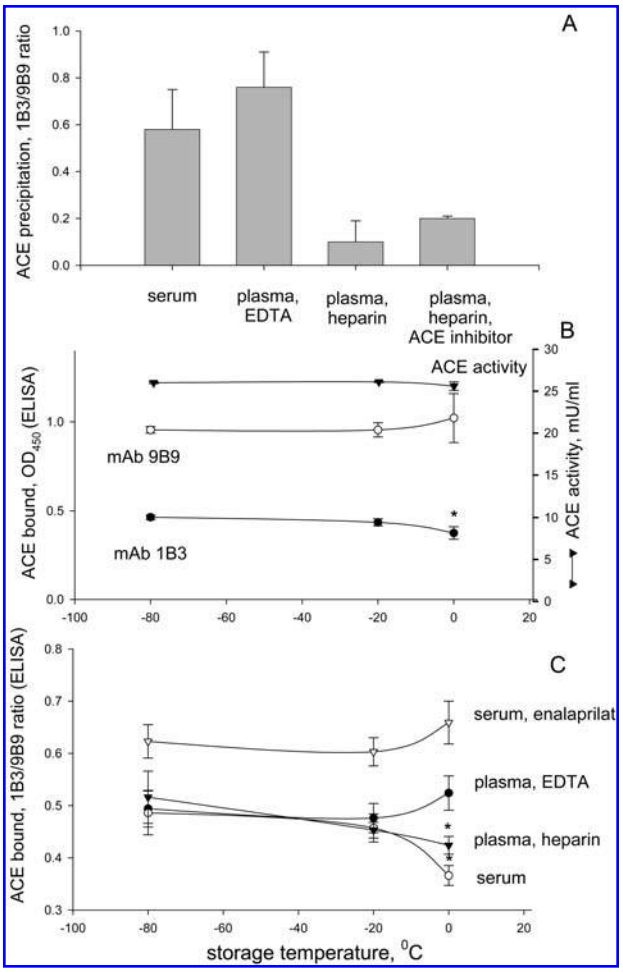

FIG. 7. Precipitation of human soluble somatic ACE by MAb 1B3 and 9B9. (**A**) Plate precipitation assay. Sera or plasma samples, diluted 1/5 with PBS, were incubated in microtiter plates coated with MAb 1B3 or 9B9 via goat-anti-mouse IgG.[22] Precipitated ACE activity was quantified by a spectrofluorometric assay. (**B,C**) ELISA. 96-well plates coated with MAbs 1B3, 9B9 or non-immune mouse IgG (as a negative control) were incubated with 1/5 dilution of sera/plasma samples. Bound ACE was visualized using polyclonal sheep-anti-human ACE IgG conjugated with horseradish peroxidase (Chemicon Int., Temecula, CA). Data presented are mean ± SD of triplicates from three to five samples.

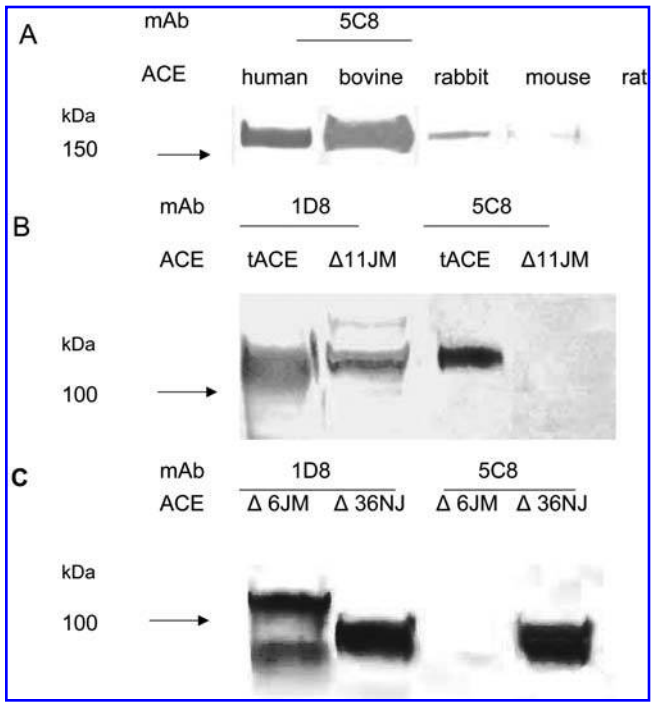

FIG. 8. Detection of ACEs by MAb 5C8 in Western blotting. (**A**) Supernatants of human, bovine, rabbit, mouse kidney, and rat lung homogenates were equilibrated to 180 mU/mL of ACE activity (Z-Phe-His-Leu as a substrate), boiled, and 20 μL of each sample run on a 7.5% gel under reducing conditions. Proteins were transferred to PVDF Plus membranes, which were developed with 2 μg of the appropriate MAbs. (**B,C**) Serum-free culture medium of CHO cells expressing tACE, tACEΔ36NJ, tACEΔ11JM, or tACEΔ6JM was equilibrated to 180 mU/mL of ACE activity (Hip-His-Leu as a substrate) and resolved as above. Molecular weight is shown on the left. MAb 1D8, a new MAb directed to the central part of the C-domain of ACE (Balyasnikova et al., unpublished data), was used as a positive control.

residues of the C-domain (data not shown). Thus, we conclude that MAb 5C8 recognizes a motif located at the very C-terminal part of soluble ACE.

Asn1196 is involved in the epitope formation for MAb 5C8, because the MAb reactivity was diminished with rabbit ACE, where Asn1196 is replaced by Thr (Figs. 3 and 8A), and completely abolished in an Asn1196Gln C-domain mutant (ACE_{NQ} construct, Fig. 9A). Pro1199 is likely also part of the 5C8 epitope, since the binding of MAb 5C8 with serum ACE of Dutch patients having a Pro1199Leu mutation[15] was abolished (Fig. 9B).

These data suggest that the motif Pro1193 to Ser1201 (PQYNWTPNS) participates in the epitope for MAb 5C8. Moreover, the fact that binding of MAb 5C8 was abolished by changes of both Asn1196 and Pro1199 indicates that both motifs—PQYNW and TPNS—are equally important for the formation of the 5C8 epitope.

Immunohistochemistry of MAb 5C8

MAb 5C8 was found suitable for the immunohistochemical detection of denatured ACE in tissues. In routinely prepared formalin-fixed and paraffin-embedded human samples, all of the known tissue sites expressing ACE[8,11,34–39] were labeled by this MAb. In comparison with MAb 1D8, MAb 5C8 demonstrates the somatic isoform of ACE in proximal but not distal kidney tubules (Fig. 10A,B), in endothelial cells of lung capillaries (Fig. 10C,D), and in Leydig cells of the testis (Fig. 10E,F). In addition, MAb 5C8 also recognizes the testicular isoform of ACE, as demonstrated by its specific occurrence in differentiating spermatides (Fig. 10E). These data were consistent with the binding of MAb 5C8 to denatured ACE from different species as revealed by Western blotting (Fig. 8).

CONCLUSION

Generation of MAbs recognizing the C-terminus provides a tool for detecting the native form of soluble ACE, and more particularly, the intact C-terminal end of the ACE molecule released from the membrane. The most important clinical application of MAb 1B3 relates to the fact that the binding of this antibody to ACE with a Pro1199Leu mutation in the stalk region was significantly decreased (Fig. 3). We demonstrated re-

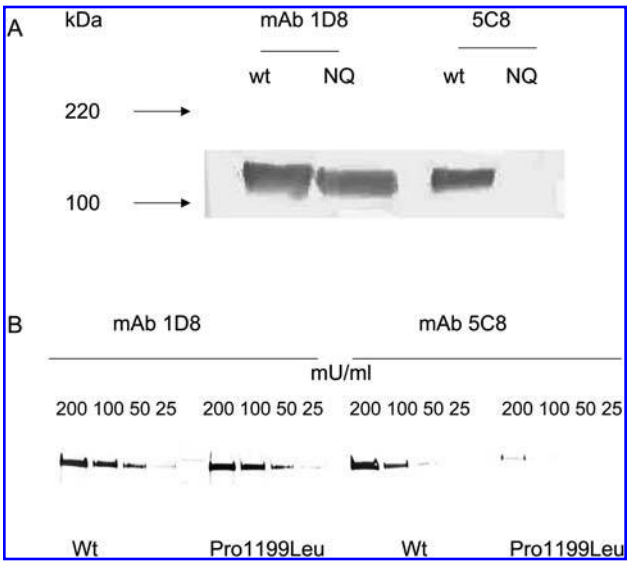

FIG. 9. Detection of the mutated forms of ACE by MAb 5C8 in Western blotting. (**A**) Serum-free culture medium of CHO cells expressing C-domain of ACE (wt) or mutant form of this fragment, where Asn1196 (somatic ACE numbering[4] was substituted by Gln (ACE_{NQ} construct[49], were equilibrated to 180 mU/mL of ACE activity (Hip-His-Leu as a substrate) and run exactly as on Figure 8. (**B**) Somatic ACE from pooled sera of healthy family members, or their affected relatives with Pro1199Leu mutation,[15] was purified by affinity chromatography on lisinopril column and equilibrated according to ACE activity (with Hip-His-Leu as a substrate), at indicated dilutions. Western blotting procedure was run as above.

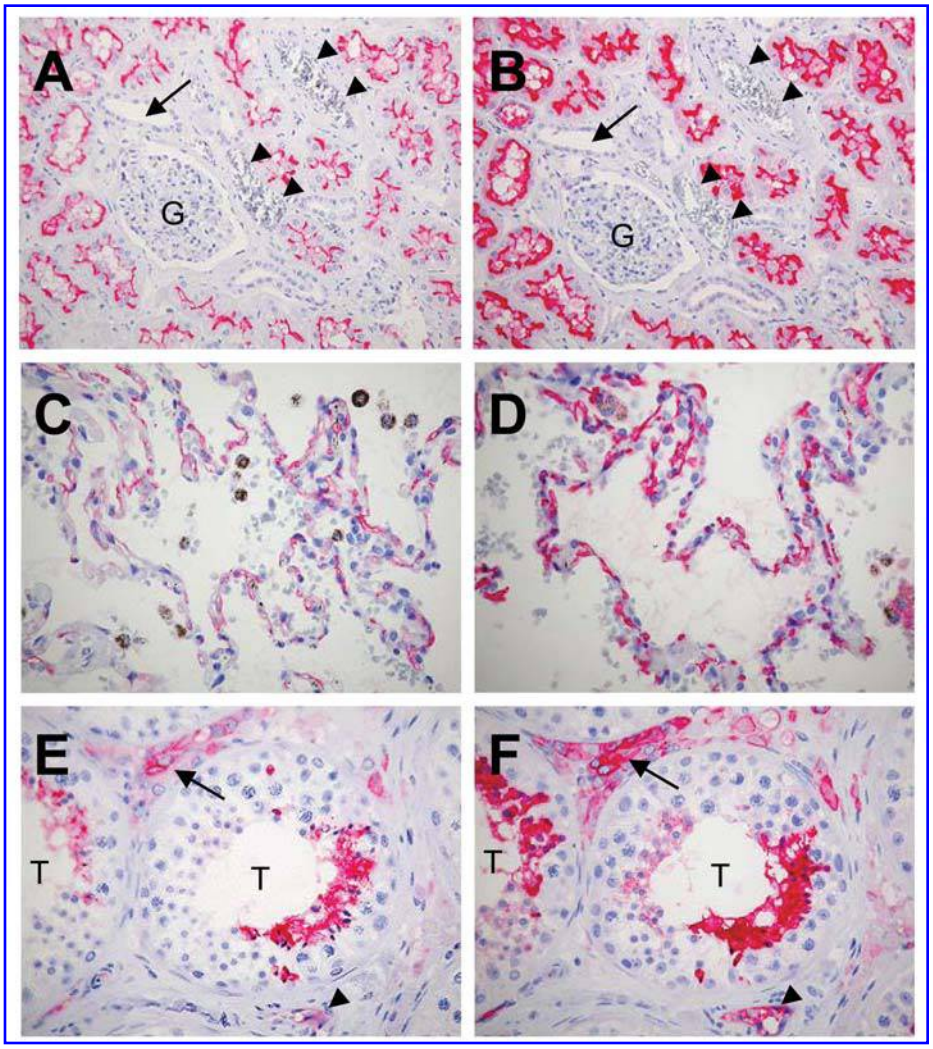

FIG. 10. Immunohistochemistry of the full-length isoforms of ACE by MAb 5C8. Paraffin-embedded tissues of the human kidney (A,B), lung (C,D) and testis (E,F) labeled by MAbs 5C8 (A,C,E) and 1D8 (B,D,F). (**A**) MAb 5C8 shows somatic ACE only at the brush borders of the proximal renal tubules, whereas distal renal tubules (arrow) and all endothelial cells (arrowheads), including glomeruli (G), are immunonegative. (**B**) MAb 1D8 on a consecutive tissue section of A. Same structures are labeled or indicated (for several other MAbs and frozen tissue sections, see also Metzger et al.[37]). (**C**) MAb 5C8 shows somatic ACE in endothelial cells of pulmonary capillaries. (**D**) MAb 1D8 on a consecutive tissue section of C, same structures are labeled (for several other MAbs and frozen tissue sections, see also Danilov et al.[34]). (**E**) MAb 5C8 shows somatic ACE on Leydig cells (arrow) and some endothelial cells (arrowhead) in the testicular interstitium, whereas the testicular isoform of ACE is also detected in differentiating germ cells (spermatids) within the seminiferous tubules (T). (**F**) MAb 1D8 on a consecutive tissue section of E. Same structures are labeled or indicated (for several other MAbs and frozen tissue sections, see also Pauls et al.[38]). Alkaline phosphatase anti-alkaline phosphatase (APAAP), Fast Red; original magnifications, ×40 (A,B), ×160 (C,D), ×240 (E,F).

cently that the reason for the marked elevation of serum ACE activity (more than four times the upper limit of normal) found in some families in different countries is a mutation in the stalk region,[15] which leads to increased shedding of ACE (presumably of the vascular endothelial cells) into the circulation. A notable increase in serum ACE activity is found in granulomatous disorders such as sarcoidosis as well as other diseases.[13,14,52,53] Therefore, the determination of ACE activity to confirm the diagnosis of sarcoidosis and for follow-up treatment is a routine clinical procedure. Recently, it was demonstrated that the hereditary elevation of ACE may result in an incorrect diagnosis of neurosarcoidosis and unwarranted treatment with immunosuppressants.[19] Therefore, a different diagnostic test for elevated ACE levels in sarcoidosis due to increased shedding or as a result of this stalk mutation would be beneficial. Based on the decreased ability of MAb 1B3 to recognize a mutated (Pro1199Leu) ACE, we developed a simple and highly sensitive method (Danilov et al., unpublished data) to distinguish mutant from wild-type soluble somatic ACE in the serum/plasma of patients.

A similar approach could be used to monitor the integrity of the ACE molecule. Proteolytic cleavage of the C terminus of soluble somatic ACE occurred after storage of plasma or serum even at low temperatures (Fig. 7). It is reasonable that plasma samples prepared with EDTA may be more resistant to proteases present in the blood, because they are inhibited by EDTA. However, the fact that ACE inhibitors can also protect plasma ACE from proteolytic cleavage is intriguing. Two possible explanations follow: (1) the conformation of ACE, when complexed with an ACE inhibitor, is more stable; and (2) if the degradation is, in part, a result of autolysis, then ACE inhibition would prevent this occurrence. In any case, regardless of the reason for proteolysis or autolysis, the binding test of any ACE or ACE construct, with MAb 1B3, has already become a routine procedure in our laboratory to monitor the integrity of the C-termini of the ACE constructs used.

Much of the work investigating the mechanism of ACE ectodomain shedding has focused on the juxtamembrane region of the ectoprotein.[47,49,54–55] Mutagenesis, metabolic labeling, and kinetic approaches have been used to elucidate this important post-translational process. Furthermore, in some membrane proteins, there is evidence that suggests regions distal to the cleavage site play a role in optimizing the interaction of the secretase and its cognate substrate, thus facilitating the proteolytic cleavage.[44,56] Hence, the use of MAbs such as 1B3 should be valuable weapons in our arsenal for elucidating these interactions and unraveling the ACE shedding machinery.

The expression of alternatively spliced ACE mRNA coding for a soluble form of ACE was demonstrated using cultured human endothelial cells.[57] This mRNA may theoretically code for a shorter variant of soluble ACE spanning 1–1107 amino acid residues of human ACE, plus an additional nine amino acids coded by the part of 3′ untranslated sequence before the stop codon. However, the presence of this shorter form of ACE has not been shown previously. Using combinations of MAbs 1B3 and 5C8, which are directed to the part of wild-type ACE that lacks the putative alternatively spliced variant of soluble ACE, and mAbs directed to the epitopes localized proximally to the N-terminus and recognized both forms of ACE, we demonstrated the presence of shorter variants of soluble ACE in human plasma (Balyasnikova, Sun, and Danilov, unpublished data). Moreover, this approach can be extended using immunohistochemistry with MAb 5C8 in combination with an antibody such as 1D8 recognizing the more central part of the molecule to detect alternatively spliced forms of ACE in human tissue. Although this is only a semiquantitative analysis, it could provide an important diagnostic tool for pathologies that may be linked to the expression of alternatively spliced variants of ACE.

In summary, we report on two novel MAbs—1B3 and 5C8—directed to the C-terminal end of soluble ACE. These MAbs are sensitive to even subtle conformational changes and thus can be used as probes for monitoring the integrity of the C-terminal domain of ACE as well as a tool to study the proteolytic processing of ACE from the cell surface.

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