J. Biochem. 2009;145(1)115–121
doi:10.1093/jb/mvn148

Testicular Angiotensin-Converting Enzyme with Different Glycan Modification: Characterization on Glycosylphosphatidylinositol-Anchored Protein Releasing and Dipeptidase Activities

Gen Kondoh[1,2,*], Hitomi Watanabe[1], Yuko Tashima[3], Yusuke Maeda[3] and Taroh Kinoshita[2,3,4]

[1]Laboratory of Animal Experiments for Regeneration, Institute for Frontier Medical Sciences, Kyoto University, Kyoto 606-8507; [2]CREST Program, Japan Science and Technology Society, Kyoto, Japan; [3]Department of Immunoregulation, Research Institute for Microbial Diseases, Suita 565-0871; and [4]Laboratory of Immunoglycobiology, Immunology Frontier Research Center, Osaka University, Suita 565-0871, Osaka, Japan

Received August 10, 2008; accepted October 23, 2008; published online November 04, 2008

We have previously found that the angiotensin-converting enzyme (ACE) carries GPI-anchored protein releasing activity (GPIase) as well as dipeptidase activity. Testicular ACE (tACE), the male germinal specific isozyme, plays a crucial role in male fertilization. The amino-terminal region of this isozyme is different from that of somatic isozyme (sACE) and contains potential O-linked glycosylation sites. By multiple mutagenesis after an *in silico* prediction, amino acid residues acquiring O-glycans were assigned. Both GPIase and dipeptidase activities were compared between O-glycan null mutant and wild-type molecules, but no differences were found. Furthermore, the wild-type tACE was produced in two different cells (COS7 and CHO) and its activities compared. The GPIase activity, but not dipeptidase, was apparently higher for CHO-derived molecule than COS7. Sensitivity to neuraminidase and O-glycosidase digestions and the profile of glycosylation were quite different between these two molecules. Moreover, serial digestions with neuraminidase and O-glycosidase have no influence on GPIase activity of both molecules, suggesting that the sialylation and the presence of O-glycan has no influence on tACE enzyme activities, while the set of glycans modulate GPIase activity.

Key words: angiotensin-converting enzyme, enzyme activity, glycan, gpi-anchored protein, post-translational modification.

Abbreviations: GPI, glycosylphosphatidylinositol; tACE, testicular angiotensin-converting enzyme; sACE, somatic angiotensin-converting enzyme; ZP, zona pellucida.

INTRODUCTION

The angiotensin-converting enzyme (ACE) (EC 3.4.15.1) is a key regulator of blood pressure and also plays a crucial role in male reproduction. Peptides such as angiotensin I and bradykinin that regulate blood pressure and induce inflammation are catalysed via the dipeptidase activity of somatic isozyme, somatic ACE (sACE). sACE is broadly distributed in body fluids, suggesting involvement of this isozyme in various biologic processes (*1–3*).

In contrast, the male germinal specific isozyme, testicular ACE (tACE) is less characterized. It is encoded by the second half of *Ace*, and its expression is driven by a testis-specific promoter located in intron 12 of the gene (*4*, *5*). The dipeptidase domain of tACE is identical to one of the catalytic domains of sACE and shows similar activity *in vitro*. *Ace* knockout mice show impaired male fertilization, such as defects in sperm uterotubular migration and ability to bind the zona pellucida (ZP) of the egg (*6–8*). These defects are rescued by introducing tACE, but not sACE, to the germ cells, suggesting that tACE possesses crucial functions in male fertility (*9*, *10*).

The N-terminal amino acid sequence of tACE is completely different from that of sACE. It consists of 38 amino-acid residues and is known to contain potential O-glycosylation sites. However, the dipeptidase activity of tACE did not change even after deletion of these sequences, suggesting that O-glycan is neither required for dipeptidase activity nor modulates it (*11*).

We have previously identified another enzymatic activity of ACE, the GPI-anchored protein-releasing activity (GPIase) (*12*). The sperm-ZP binding insufficiency of *Ace* knockout sperm can be rescued by a dipeptidase-inactivated tACE, indicating that the GPIase activity is crucial for the function of the enzyme in the sperm.

In the present study, we assessed the effects of glycosylation on the GPIase and dipeptidase activities of tACE. The sialylation and the O-glycosylation in the tACE-specific region had no influence on the basal GPIase activity, whereas sets of glycans modulated this activity.

*To whom correspondence should be addressed. Tel/Fax: +81-75-751-4860, E-mail: kondohg@frontier.kyoto-u.ac.jp

MATERIALS AND METHODS

Mutagenesis of tACE—To introduce multiple mutations in tACE, we developed a simple method illustrated in Fig. 1A. A mutated product was developed by amplifying two fragments, the left fragment and right fragments, using fixed primers located out of the coding region and back-to-back primers that carry mutations. The fixed primers carried a unique restriction enzyme site at the 5′ end. The back-to-back primers were phosphorylated at the 5′ end and ready for ligation. The PCR products amplified by Pfx polymerase, which does not add a nucleotide on the 5′ end, were digested with each restriction enzyme (here *Xho*I for the left and *Eco*RI for the right fragments). Finally, the CAAG expression vector (*13*), which is cleaved with both restriction enzymes, together with the left and right fragments were ligated at once. All ligated constructs were sequenced to certify the introduced mutations or excluding errors by PCR. Table 1 provides a list of the primers used in this study.

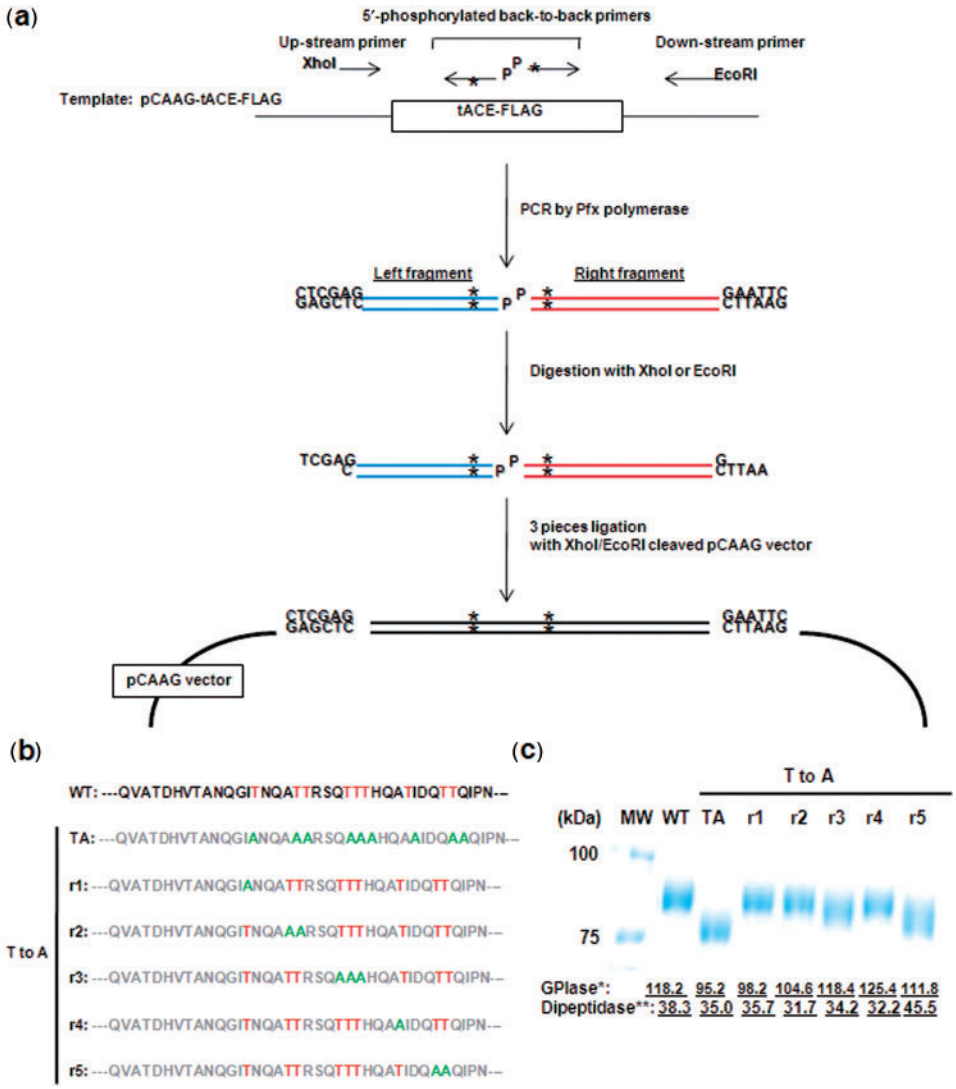

Fig. 1. **Generation of O-glycan-binding mutants of tACE.** (a) Schematic diagram of mutagenesis. FLAG-tagged murine tACE ligated to pCAAG vector was used as a template. A mutated product was developed by amplifying two fragments, the left fragment and right fragment using primers that are located out of the coding region and back-to-back primers that carry mutations. The back-to-back primers are phosphorylated at the 5′ end. PCR products amplified by Pfx polymerase, which does not add a nucleotide on 5′ end, were digested with *Xho*I for the left and *Eco*RI for the right fragments. Finally, these fragments are ligated with the CAAG expression vector, which had been cleaved with both restriction enzymes previously. Primers used in this study are listed in Table 1. (b) Amino-acid alignments of tACE-specific region and its mutants. T residues indicated in red are predicted O-glycan binding sites. Replaced A residues are indicated in green. WT, wild-type. (c) Production of recombinant mutant proteins. The expression vector of each mutant was transfected into COS7 cells and recombinant proteins were purified from the culture supernatants. Five micrograms of each protein were applied to SDS–PAGE, followed by staining with GelCode Blue Stain Reagent. The GPIase and dipeptidase activities of each protein are indicated at the bottom. *arbitrary unit per microgram protein per hour; **U per mg protein.

Production of Recombinant Proteins—Expression vectors carrying a FLAG-tagged version of the soluble tACE or its mutants were transiently transfected into COS7 cells. The same wild-type tACE vector was also transfected into CHO cells and cells permanently producing tACE were selected. The culture supernatants were collected and recombinant protein was purified by anti-FLAG M2-agarose affinity column (Sigma Chemical Co., St Louis, MO). Purified proteins were applied for SDS–PAGE and stained with GelCode Blue Stain Reagent (Pierce, Rockford, IL).

Neuraminidase and O-glycosidase Digestions—The wild-type tACE proteins were digested with α-(2→3,6,8,9)-Neuraminidase (Sigma N8271) and/or O-glycosidase (Sigma G1163) using the instructions provided by the supplier. The digested samples were applied to SDS–PAGE followed by staining with GelCode Blue Stain Reagent (Pierce) or to GPIase assay.

Lectin Chip Analysis of Recombinant tACE—Recombinant TA/COS7, WT/COS7 and WT/CHO proteins were Cy3-labelled and applied to LecChip™ ver.1.0 (Moritex Corporation, Yokohama, Japan). The fluorescent intensity of each chip was acquired by an evanescent-field fluorescent scanner, GlycoStation™ Reader1200 (Moritex Corporation). All data were analysed by Array-Pro™ Analyzer (Media Cybernetics, Inc., Bethesda, MD) (*14*).

GPIase Assay—GPIase activity was examined by a placental alkaline phosphatase (PLAP) conversion assay on Triton X-114 partition, as described previously (*15*). Briefly, recombinant proteins were mixed with 0.3 IU/ml of detergent-soluble PLAP in a buffer containing 100 mM 2-(*N*-morpholino)ethanesulfonic acid, pH 6.0 and 5 mM $CaCl_2$, and reaction was performed for 60 min at 37°C. The reaction was stopped by adding Triton X-114 at a final concentration of 2%, followed by microcentrifugation at 35°C. The water-soluble phase was collected, and PLAP activity was measured.

Dipeptidase Assay—The ACE dipeptidase activity was measured by colorimetry, as described previously, using p-hydroxyhippuryl-L-histidyl-L-leucine (pHHHL) as a substrate (*16*).

Statistical Analyses—All data were expressed as mean ± SD. Differences between GPIase and dipeptidase assays were examined for statistical significance using the Student's *t*- test. A *P*-value less than 0.05 denoted the presence of a statistically significant difference.

RESULTS AND DISCUSSIONS

Generation and Characterization of O-glycosylation Site Mutants—To investigate the effects of O-glycosylation on enzyme activities of tACE, amino acid residues for O-glycan binding were predicted by NetOGlyc 3.1 server of Technical University of Denmark. The amino acid T42, T46, T47, T51, T52, T53, T57, T61 and T62 were predicted as potential O-glycan-binding sites.

We classified these sites into five regions and introduced T to A mutations using our simple and defined method (Fig. 1a). We also developed a mutant, called TA, in which all nine predicted T residues were exchanged with A. These constructs driven under a strong promoter, CAAG, were introduced into COS7 cells and recombinant proteins were purified from culture supernatants. We found size differences among these mutants and the wild-type by SDS–PAGE (Fig. 1b). High mobility was observed for TA, r3 and r5 mutants, implying that the O-glycan binding sites are T51/T52/T53 and T61/T62.

We also assessed both GPIase and dipeptidase activities of these molecules but found no differences, even in the TA mutant, compared with the wild-type molecule (Figs 1c, 3b and c). Furthermore, the profile of glycosylation was compared between TA and wild-type molecules by the lectin chip analysis. As shown in Fig. 2a, TA did not bind with BPL, ABA, Jacalin and WFA lectins, indicating that O-glycan is absent in TA. The above findings also implied that O-glycan binding is not required for both GPIase and dipeptidase activities of tACE.

Characterization of tACE Produced in Different Cells—We produced tACE molecule in COS7 and CHO cells, and termed them WT/COS7 and WT/CHO, respectively. We then compared the profile of glycosylation and enzyme activities of these two molecules. The size of proteins appeared similar by SDS–PAGE, while the mobility after neuraminidase alone or serial digestions with O-glycosidase were faster for WT/CHO than WT/COS7, indicating that sensitivity to neuraminidase and O-glycosidase is

Table 1. **PCR primers used in this study.**

Type of primer	Primer name	Sequences
Upstream primer	ACE-delU	GGCGGCTCTAGAGCCTCTGCTAACC
Downstream primer	ACE-delD	AGGAGGAATTCGTCACTTATCATC
Back-to-back primer	T to A-U	GGCTGCTGCCTGGTTTGCTATCCCCTGGTTGGCTGCCAC
	T to A-D	AGAGCCCAGGCAGCAGCTCACCAAGCAGCCATTGACCAGGCAGCC
	R1-U	TGTTGCCTGGTTTGCTATCC
	R1-D	ACCAGAAGCCAGACAACAACT
	R2-U	TGCCTGGTTTGTTATCCCCTGG
	R2-D	GCAGCCAGAAGCCAGACAACAACT
	R3-U	CTGGCTTCTGGTTGTTGCC
	R3-D	GCAGCAGCTCACCAAGCAACC
	R4-U	CTGGTCAATGGCTGCTTGGT
	R4-D	ACAACCCAGATCCCAAACCTA
	R5-U	CTGGTCAATGGTTGCTTGGT
	R5-D	GCAGCCCAGATCCCAAACCT

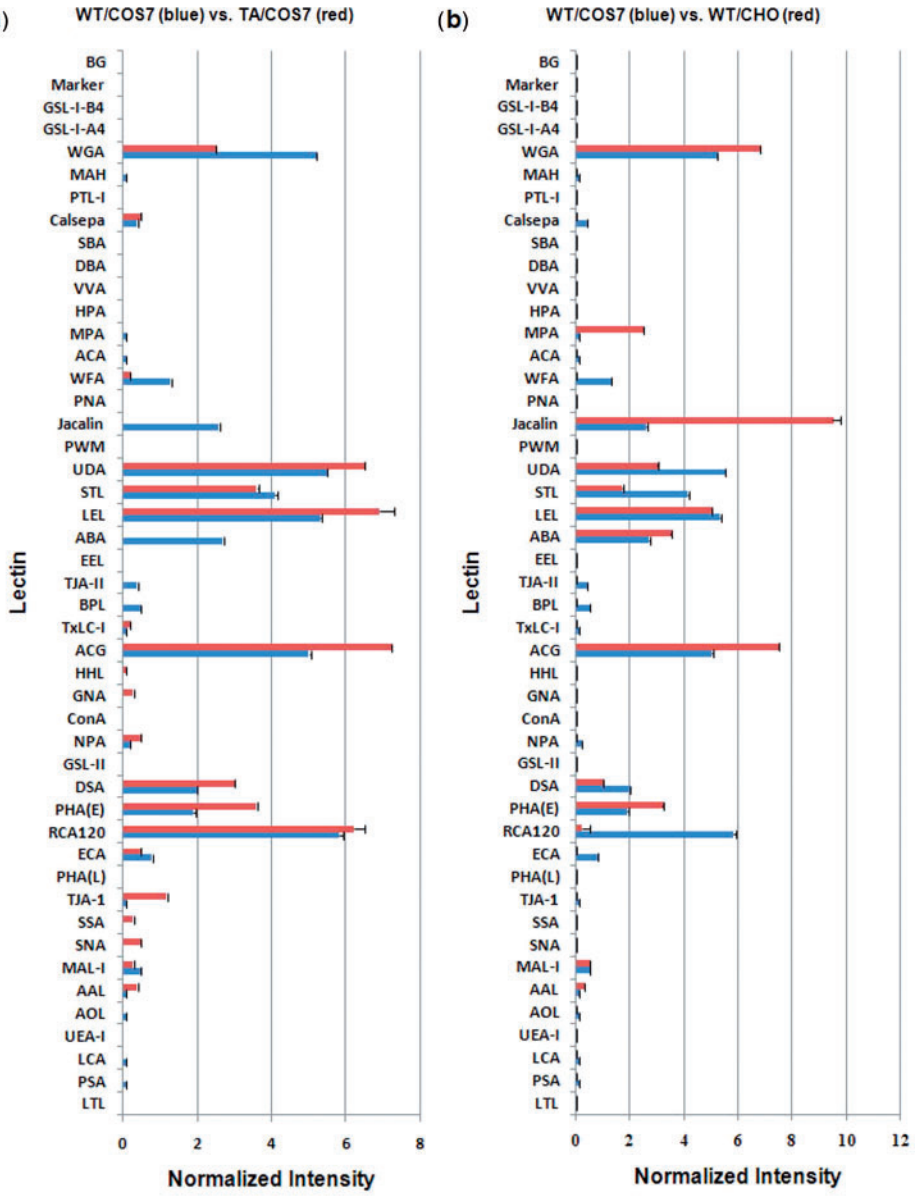

Fig. 2. **Lectin chip analysis of recombinant tACE proteins.** Comparison of glycosylation profile between WT/COS7 and TA/COS7 (a) and between WT/COS7 and WT/CHO (b). Data represent the normalized intensity of each lectin binding. Lectins used in this study was listed in Table 2.

different between these two proteins (Fig. 3a). We also digested these molecules with N-glycosidase (PNGase F, Sigma G5166) but found no difference in mobility (data not shown).

Based on the lectin chip analysis, the profile of glycosylation was different between WT/COS7 and WT/CHO. As shown in Fig. 2b, BPL binding was absent in WT/CHO, ABA even, Jacaline reduced in WT/COS7, WFA absent in WT/CHO and MPA absent in COS7, suggesting that the profile of O-glycan is apparently different. Using *in silico* analysis, WT/COS7 was found to contain T-antigen, Tn-antigen and Sialyl-T, while WT/CHO carried almost Sialyl-T only. For N-glycans, TJA-II binding was detected in WT/COS7, indicating the presence of Fucα1-2. In WT/COS7, RCA120 and LEL bindings indicated higher expression of N-acetyl lactosamine.

In the next step, we examined GPIase and dipeptidase activities of these molecules. We found two-fold higher activity of GPIase in WT/CHO than in WT/COS7, while dipeptidase activity was equal in the two molecules (Fig. 3b and c). Finally, the GPIase activity of both molecules have not changed after neuraminidase and/or O-glycosidase treatments, implying that the sialylation and the O-glycosylation have no influence on GPIase action (Fig. 3d).

These results suggest that the glycans sets modulate GPIase activity but not dipeptidase of tACE.

The profile of glycosylation was different between these two proteins, suggesting that glycan species

Table 2. **Lectins used in the lectin chip assay.**

Lectin	Hapten sugar
GSL-I-B4	α-Linked GalNAc
GSL-I-A4	α-Linked Gal
WGA	Chitin oligomers, Sia
MAH	Siaα2-3Galβ1-3(Siaα2-6)GalNAc
PTL-I	α-Linked terminal GalNAc
Calsepa	Mannose, Maltose
SBA	α- or β-linked terminal GalNAc, GalNAcα1–3Gal
DBA	Blood A antigen, GalNAcα1–3GalNAc
VVA	α-Linked terminal GalNAc, GalNACα1–3Gal
HPA	α-Linked terminal GalNAc
MPA	Galβ1–3GalNAc, GalNAc
ACA	Galβ1–3GalNAc
WFA	GalNAcβ1–4GlcNAc, Galβ1–3(-6)GalNAc
PNA	Galβ1–3GalNAc
Jacalin	Galβ1–3GalNAc, GalNAc
PWM	$(GlcNAc\beta1–4)_n$
UDA	GlcNAcβ1–4GlcNAc, Mixture of Man5 to Man9
STL	GlcNAc oligomers, oligosaccharide containing GlcNAc and MurNAc
LEL	GlcNAc trimers/tetramers
ABA	Galβ1–3GalNAc
EEL	Blood B antigen, Galα1–3Gal
TJA-II	Fucα1–2Galβ1→ or GalNAcβ1→groups at their non-reducing terminals
BPL	Galβ1–3GalNAc, GalNAc
TxLC1	Manα1–3(Manα1–6)Man, bi- and tri-antennery complex-type N-glycan, GalNAc
ACG	Siaα2–3Galβ1–4GlcNAc
HHL	High-mannose, Manα1–3Man, Manα1–6Man
GNA	High-mannose, Manα1–3Man
ConA	High-mannose, Manα1–6Man(Manα1–3)Man
NPA	High-mannose, Manα1–6Man
GSL-II	Agalctosylated tri/tetra antennary glycans, GlcNAc
DSA	(GlcNAcβ1–4)*n*, Galβ1–4GlcNAc
PHA(E)	Bi-antennary complex-type N-glycan with outer Gal and bisecting GlcNAc
RCA120	Galβ1–4GlcNAc
ECA	Galβ1–4GlcNAc
PHA(L)	Tri/tetra-antennary complex-type N-glycan
TJA-I	Siaα2–6Gal/GalNAc
SSA	Siaα2–6Gal/GalNAc
SNA	Siaα2–6Gal/GalNAc
MAL	Siaα2–3Galβ1–4GlcNAc
AAL	Fucα1–6GlcNAc, Fucα1–3(Galβ1–4)GlcNAc
AOL	Fucα1–6GlcNAc(core Fuc)
UEA-1	Fucα1–2Galβ1–4GlcNAc
LCA	Fucα1–6GlcNAc, α-D-Glc, α-D-Man
PSA	Fucα1–6GlcNAc, α-D-Glc, α-D-Man
LTL	Fucα1–3(Galβ1–4)GlcNAc, Fucα1–2Galβ1–4GlcNAc

bound on the same protein modulated the GPIase activity. Several reports described the correlation between glycosylation and protein function. The change in glycosylation profile was mainly due to deficiencies of some sugar binding enzymes, resulting in dysfunction of the protein. The targets of these enzymes are cell surface receptors, adhesion molecules and/or matrix proteases. Such proteins with extra-ordered glycosylation may induce abnormal cell-to-cell interaction and lead to the development of diseases (*17–20*). In contrast, our finding in this study may be showing that glycosylation profile modulates enzymatic activity.

In the present study, our results suggested that glycosylation of an enzyme molecule modulates its enzyme activity. It is possible that the same enzyme produced by different tissues exhibits different enzymatic activity. Thus, the glycosylation of enzyme may represent a post-translational regulatory process involved in the modulation of enzymatic activity *in vivo*.

ACKNOWLEDGEMENTS

We thank S. Takeishi, M. Yamada and T. Suzuki for helpful discussions.

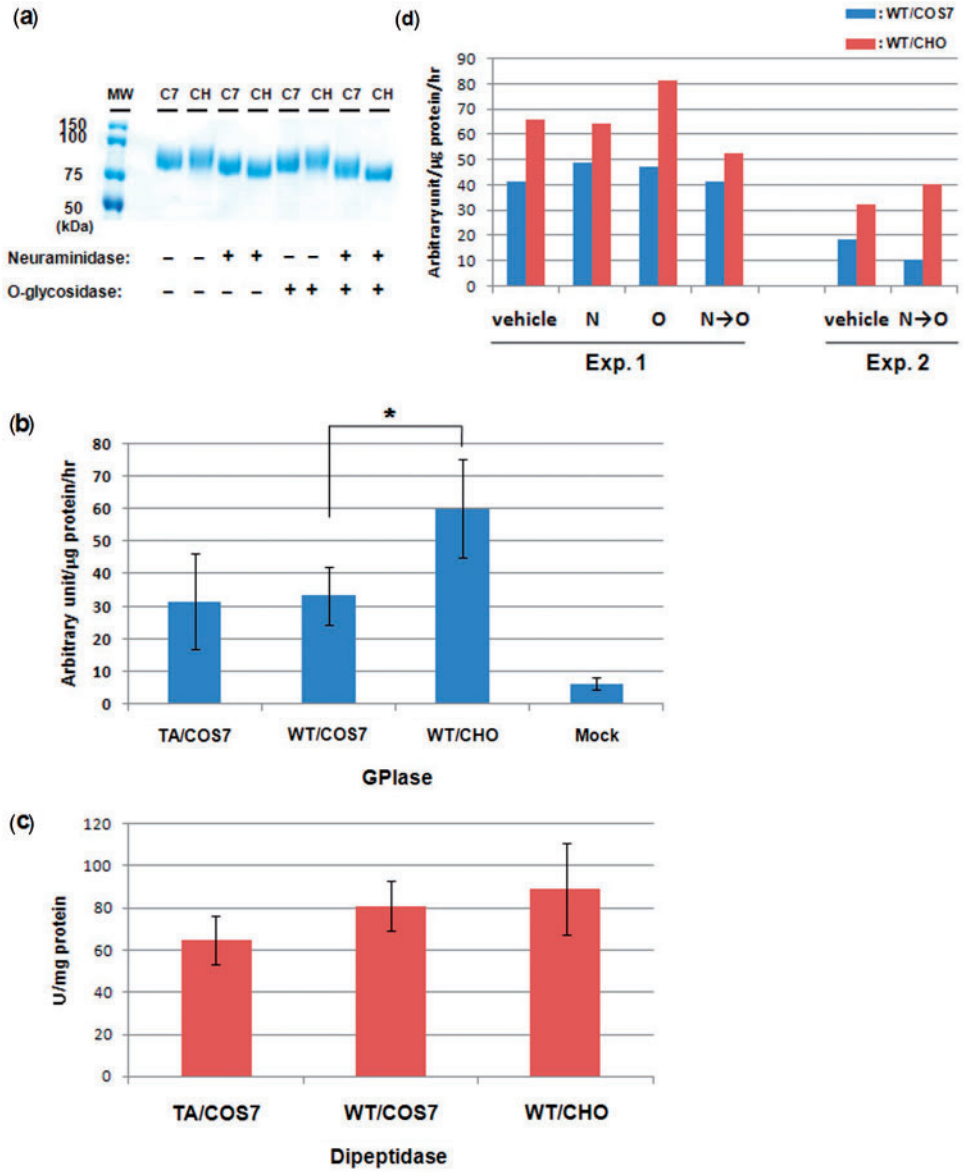

Fig. 3. **Comparison of WT/COS7 and WT/CHO**. (a) Neuraminidase and/or O-glycosidase digestions of WT/COS7 (C7) and WT/CHO (CH). After enzyme treatments, samples are applied to SDS–PAGE, followed by staining with GelCode Blue Stain Reagent. (b) GPIase activity. Values are mean ± SD. The numbers of samples examined were four in TA/COS7, five in WT/COS7, five in WT/CHO and four in Mock. $^{*}P < 0.01$. (c) Dipeptidase activity. Values are mean ± SD. The numbers of samples examined were four in TA/COS7, six in WT/COS7 and five in WT/CHO. (d) GPIase activity of WT/COS7 and TA/COS7 after neuraminidase and/or O-glycosidase treatments. Results from two experiments were indicated. N, neuraminidase; O, O-glycosidase; N→O, serial neuraminidase and O-glycosidase treatments.

CONFLICT OF INTEREST

None declared.

REFERENCES

1. Bernstein, K.E. (2006) Views of the renin-angiotensin system: brilling, mimsy, and slithy tove. *Hypertension* **47**, 509–514
2. Hooper, N.M. (1991) Angiotensin converting enzyme: implications from molecular biology for its physiological functions. *Int. J. Biochem.* **23**, 641–647
3. Turner, A.J. and Hooper, N.M. (2002) The angiotensin-converting enzyme gene family: genomics and pharmacology. *Trends Pharmacol. Sci.* **23**, 177–183
4. Howard, T.E., Shai, S.I., Langford, K.G., Martin, B.M., and Bernstein, K.E. (1990) Transcription of testicular angiotensin-converting enzyme (ACE) is initiated within the 12th intron of the somatic ACE gene. *Mol. Cell Biol.* **10**, 4294–4302
5. Langford, K.G., Shai, S.Y, Howard, T.E., Kovac, M.J., Overbeek, P.A., and Bernstein, K.E. (1991) Transgenic mice demonstrate a testis specific promoter for angiotensin-converting enzyme (ACE). *J. Biol. Chem.* **266**, 15559–15562
6. Krege, J.H., John, S.W.M., Langenbach, L.L., Hodgin, J.B., Hagaman, J.R., Bachman, E.S., Jennettte, J.C., O'Brien, D.A., and Smithies, O. (1995) Male-female differences in fertility and blood pressure in ACE-deficient mice. *Nature* **375**, 146–148

7. Esther, C.R. Jr, Howard, T.E., Marino, E.M., Goddard, J.M., Capecchi, M.R., and Bernstein, K.E. (1996) Mice lacking angiotensin-converting enzyme have low blood pressure, renal pathology, and reduced male fertility. *Lab. Invest.* **74**, 953–965
8. Hagaman, J.R., Moyer, J.S., Bachman, E.S., Sibony, M., Magyar, P.L., Welch, J.E., Smithies, O., Krege, J.H., and O'Brien, D.A. (1998) Angiotensin-converting enzyme and male fertility. *Proc. Natl Acad. Sci. USA* **95**, 2552–2557
9. Ramaraj, P., Kessler, S.P., Colmenares, C., and Sen, G.C. (1998) Selective restoration of male fertility in mice lacking angiotensin-converting enzymes by sperm-specific expression of the testicular isozyme. *J. Clin. Inv.* **102**, 371–378
10. Kessler, S.P., Rowe, T.M., Gomos, J.B., Kessler, P.M., and Sen, G.C. (2000) Physiological non-equivalence of the two isoforms of angiotensin-converting enzyme. *J. Biol. Chem.* **275**, 26259–26264
11. Ehlers, M.R.W., Chen, Y.-N.P., and Riordan, J.F. (1992) The unique N-terminal sequence of testis angiotensin-converting enzyme is heavily O-glycosylated and unessential for activity and stability. *Biochem. Biophys. Res. Commun.* **183**, 199–205
12. Kondoh, G., Tojo, H., Nakatani, Y., Komazawa, N., Murata, C., Yamagata, K., Maeda, Y., Kinoshita, T., Okabe, M., Taguchi, R., and Takeda, J. (2005) Angiotensin-converting enzyme is a GPI-anchored protein releasing factor crucial for fertilization. *Nat. Med.* **11**, 160–166
13. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene* **108**, 193–199
14. Kuno, A., Uchiyama, N., Koseki-Kuno, S., Ebe, Y., Takashima, S., Yamada, M., and Hirabayashi, J. (2005) Evanescent-field-fluorescence-assisted lectin microarray: a new strategy for glycan profiling. *Nat. Methods* **2**, 851–856
15. Deguchi, E., Tani, T., Watanabe, H., Yamada, S., and Kondoh, G. (2007) Dipeptidase-inactivated tACE action in vivo: selective inhibition of sperm-zona pellucida binding in the mouse. *Biol. Reprod.* **77**, 794–802
16. Kasahara, Y. and Ashihara Y. (1981) Colorimetry of angiotensin-I converting enzyme activity in serum. *Clin. Chem.* **27**, 1922–1925
17. Seitz, O. (2000) Glycopeptide synthesis and the effects of glycosylation on protein structure and activity. *ChemBioChem* **1**, 214–246
18. Lowe, J.B. and Marth, J.D. (2003) A genetic approach to mammalian glycan functions. *Annu. Rev. Biochem.* **72**, 643–691
19. Brockhausen, I. (2006) Mucin-type O-glycans in human colon and breast cancer: glycodynamics and functions. *EMBO Rep.* **7**, 599–604
20. Taniguchi, N., Miyoshi, E., Jianguo, G., Honke, K., and Matsumoto, A. (2006) Decoding sugar functions by identifying target glycoproteins. *Curr. Opin. Struct. Biol.* **16**, 561–566