nature medicine

Angiotensin-converting enzyme is a GPI-anchored protein releasing factor crucial for fertilization

Gen Kondoh[1,7,8], Hiromasa Tojo[2], Yuka Nakatani[1], Nobuyasu Komazawa[1], Chie Murata[3], Kazuo Yamagata[4,9], Yusuke Maeda[5], Taroh Kinoshita[5,8], Masaru Okabe[4], Ryo Taguchi[3,8] & Junji Takeda[1,6]

The angiotensin-converting enzyme (ACE) is a key regulator of blood pressure. It is known to cleave small peptides, such as angiotensin I and bradykinin and changes their biological activities, leading to upregulation of blood pressure. Here we describe a new activity for ACE: a glycosylphosphatidylinositol (GPI)-anchored protein releasing activity (GPIase activity). Unlike its peptidase activity, GPIase activity is weakly inhibited by the tightly binding ACE inhibitor and not inactivated by substitutions of core amino acid residues for the peptidase activity, suggesting that the active site elements for GPIase differ from those for peptidase activity. ACE shed various GPI-anchored proteins from the cell surface, and the process was accelerated by the lipid raft disruptor filipin. The released products carried portions of the GPI anchor, indicating cleavage within the GPI moiety. Further analysis by high-performance liquid chromatography–mass spectrometry predicted the cleavage site at the mannose-mannose linkage. GPI-anchored proteins such as TESP5 and PH-20 were released from the sperm membrane of wild-type mice but not in *Ace* knockout sperm *in vivo*. Moreover, peptidase-inactivated E414D mutant ACE and also PI-PLC rescued the egg-binding deficiency of *Ace* knockout sperms, implying that ACE plays a crucial role in fertilization through this activity.

In mammals, more than 200 cell surface proteins with various functions, such as hydroxylation, cellular adhesion and receptor, are anchored to the membrane by a covalently attached GPI moiety[1,2]. GPI deficiency causes developmental abnormalities, failure of skin barrier formation and female infertility in mice, indicating that a GPI anchor is essential for cell integrity[3–5]. Patients with the acquired hematopoietic disorder paroxysmal nocturnal hemoglobinuria have defective biosynthesis of GPI in hematopoietic stem cells[1,2]. The discovery of the *PIGA* gene and its mutations in paroxysmal nocturnal hemoglobinuria has opened the avenue for investigating the mechanisms involved in GPI biosynthesis, although the information on the metabolic system of GPI-anchored proteins is still limited[6,7].

Prions are infectious pathogens that cause fatal neurodegenerative diseases in both human and cattle through the modification of prion protein[8,9]. Because prion protein is a GPI-anchored protein and its release is strictly linked to its pathogenic role[10,11], studies on the general mechanisms and biological meaning of released GPI-anchored protein are necessary to establish new strategies for defeating these incurable neurodegenerative diseases.

To investigate the fate of GPI *in vivo*, we previously developed GPI-anchored enhanced green fluorescent protein (EGFP-GPI) transgenic mice and found a considerable amount of EGFP-GPI in the extracellular fluid[12]. This phenotype prompted us to identify GPI-anchored protein releasing factors, in order to identify new mechanisms of GPI-anchored protein turnover other than the recycling between plasma membrane and endosomes[13,14].

The angiotensin-converting enzyme (ACE) is a well-characterized zinc peptidase that regulates bioactivities of circulating peptides such as angiotensin I and bradykinin as a dipeptidyl carboxypeptidase[15,16], leading to upregulation of blood pressure. Two isoforms of ACE, the somatic and testicular forms, have been characterized and both contain the zinc binding motif HEXXH in the center of the active site[17]. Its peptidase activity is completely abolished by either chelating the zinc ion or exchanging the conserved amino acid residues[18]. Furthermore, ACE inhibitors, which are widely used as antihypertensive agents, specifically bind and compete with substrate peptides at this active site, indicating that the active site of ACE is indispensable for the peptidase activity[19].

Because the molecular size of ACE is rather large (150–180 kDa and 100–110 kDa for somatic and testicular isoforms, respectively), it is conceivable that the enzyme has other undiscovered functions. In this regard, a homolog of ACE, the ACE-2 (ref. 20), which is also a zinc peptidase and acts as an antagonist for ACE peptidase function, was recently found to act as a receptor for the virus that causes severe acute respiratory syndrome (SARS)[21]. In this report, we describe a new function for ACE; it has a GPI-anchored protein releasing activity and that it has a crucial role in fertilization through this activity.

[1]Department of Social and Environmental Medicine and [2]Department of Biochemistry and Molecular Biology, Graduate School of Medicine, Osaka University, Suita, 2-2 Yamadaoka, Osaka 565-0871, Japan. [3]Department of Metabolome, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-0033, Japan. [4]Genome Information Research Center, and [5]Research Institute for Microbial Diseases, Osaka University, 3-1 Yamadaoka, Suita, Osaka 565-0871, Japan. [6]Center for Advanced Science and Innovation, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan. [7]Present address: Laboratory of Animal Experiments for Regeneration, Institute for Frontier Medical Sciences, Kyoto University, Japan. [8]Present address: CREST, Japan Science and Technology Society, 53 Syogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. [9]Present address: Institute of Applied Biochemistry, University of Tsukuba, 1-1-1 Tennoudai, Tsukuba Science City, Ibaraki 305-8572, Japan. Correspondence should be addressed to G.K. (kondohg@frontier.kyoto-u.ac.jp) or J.T. (takeda@mr-envi.med.osaka-u.ac.jp).

Published online 23 January 2005; doi:nm1179

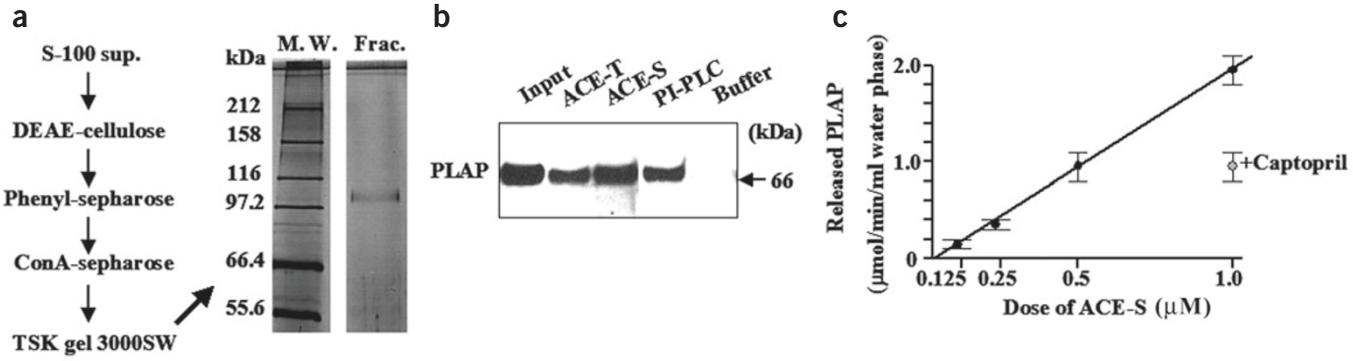

Figure 1 ACE acts as a GPI anchored protein–releasing factor. (**a**) A single 100-kDa band isolated by the TSK gel 3000SW column was subjected to SDS-PAGE and silver stained. (**b**) Immunoblotting of released PLAP from reaction with purified ACE-t, ACE-S, PI-PLC or vehicle alone (Buffer). 'Input' indicates substrate of the reaction. The size of the products released by ACE and PI-PLC was similar to that of the substrate. (**c**) Dose dependence of ACE-S GPIase activity. The results of experiments in which captopril was applied are also indicated. Values are mean ± s.d., $n = 3$.

RESULTS

Identification of ACE as a GPI-anchored protein releasing factor

First, we established an assay system to monitor the GPIase activity, taking advantage of the temperature-dependent phase separation of the aqueous TritonX-114 solution, which can separate water-soluble released proteins (products) from detergent-soluble GPI-anchored proteins (substrates) at 37 °C. When GPI-anchored proteins are deprived of their lipid moiety, they shift from the detergent-soluble phase to the water-soluble phase. This assay system was used for monitoring GPIase activity through purification. We examined this activity in various organs using both EGFP-GPI and the placental alkaline phosphatase (PLAP) as probes for GPI-anchored proteins and found a substantial activity in testicular germ cells (see **Supplementary Fig. 1** online). We also examined the expression of *Gpld1*, the gene that encodes a GPI anchor–cleaving enzyme in mammals[22,23], in various tissues. Because testicular germ cells do not express *Gpld1*, this activity may be compensated by other factors (**Supplementary Fig. 2** online). Therefore, we decided to identify the molecular entity of the enzyme responsible for GPIase activity in these cells. Using serial liquid chromatography that started from detergent-soluble fraction of mouse testicular germ cells, we purified a 100-kDa protein and subsequently identified it as ACE by proteomics analysis (**Fig. 1a**, **Table 1** and **Supplementary Fig. 3** online). To confirm that this GPIase activity involved ACE, we performed a similar assay using either a recombinant product of testicular isoform ACE (ACE-t; **Supplementary Fig. 4** online) or a commercially available rabbit ACE (ACE-S) and purified PLAP as substrate (**Supplementary Fig. 5** online). The products released by this activity had the same size as those of bacterial phosphatidylinositol-specific phospholipase (PI-PLC), a commonly used GPI anchor–cleaving enzyme, implying that this activity cleaves GPI-anchored protein located in proximity to the cleavage site of PI-PLC (**Fig. 1b**). Similar activities were shown by both molecules in a dose-dependent manner (**Fig. 1c**, data not shown), confirming that ACE was responsible for GPIase activity and did not require other factors for the action.

Characterization of ACE GPIase activity

To examine whether the ACE GPIase activity is identical to the PI-PLC activity, we used several PI-PLC inhibitors, such as myo-inositol, inositol monophosphate and antibody specific for PI-PLC, as well as PLC inhibitors such as A23187, U-73122 and C48/80 on ACE GPIase assay. None of these compounds had any inhibitory effects, even when used at a high dose (100 mM each), indicating that ACE GPIase activity is not an endogenous PI-PLC-like activity (data not shown).

We also assessed the effect of ACE-specific inhibitors, such as captopril and lisinopril, which bind to the catalytic center with ligation of its thiol to the zinc ion and completely inhibit the peptidase activity, but found only a minor inhibitory effect on the GPIase assay (**Fig. 1c**, 1×10^{-3} M captopril produced 40% inhibition and data not shown). Moreover, we assessed the effect of chloride ion, which activates the peptidase activity of ACE[15,16], but found no effects on the GPIase activity (data not shown).

We then assessed the metal requirement for GPIase activity. First, ethylenediaminetetraacetic acid (EDTA), which can remove various metal ions from proteins, was added at different doses to the PLAP conversion assay. Because EDTA has some inhibitory effects on PLAP enzyme action[24], immunoblotting was performed using antibody specific for PLAP to check for PLAP release. GPIase activity was inhibited by 1 mM of EDTA but not by an amount (10% less) at which peptidase activity was considerably inhibited (**Fig. 2a**). We also assessed the effects of other metal chelating reagents, such as *trans*-1,2-diaminocyclohexane-N, N, N′, N′-tetraacetic acid (CyDTA) and ethylene glycol bis (beta-aminoethyl ether)-N, N, N′, N′-tetraacetic acid (EGTA).

Table 1 Purification of GPI-anchored protein releasing activity from male germ cells

Column	Volume (ml)	Total protein (mg)	Total activity (AU)*	Specific activity (U/mg protein)	Fold purification
S-100 sup.	393	19,100	336,160	18	1.0
DEAE-cellulose	8	228	14,410	63	3.5
Phenyl-sepharose	8	212	17,596	83	4.6
Con-A-sepharose	1	6	6,100	1,017	56.5
TSK gel 3000SW	1	1	2,500	2,500	138.9

The membrane-rich fraction of germ cells from mouse testis was solubilized in a buffer containing 1% Triton X-100, centrifuged and the supernatant was collected and subjected to chromatographic fractionation. The PLAP conversion assay was performed on the eluted fractions and the maximum values are used here.

$$\text{*Arbitrary Unit} = \frac{[\text{OD595: sample}] - [\text{OD595: background}]}{[\text{OD595: PI-PLC}] - [\text{OD595: background}]}$$

All reactions were performed using PI-PLC (1.0 IU/ml), the value of which was defined as maximum reaction.

The peptidase activity was vigorously inhibited by 1 mM of each of these reagents, but found no inhibition of GPIase activity. To confirm the difference in GPIase and peptidase activities, we replaced Glu414 with aspartate or replaced both His413 and His417 with lysine in ACE-t (**Supplementary Fig. 4** online), which activates a water nucleophile as a general base or captures a zinc ion, thus becoming essential for peptidase activity[18]. These mutants showed only a trace peptidase activity but retained GPIase activity at a level comparable with that of wild-type ACE-t (**Fig. 2b**). The GPIase activity of H413K-H417K mutant, which cannot capture the zinc ion at the peptidase active site, was also inhibited by high-dose EDTA but not by CyDTA or EGTA (**Fig. 2c**), suggesting that EDTA-induced inhibition of GPIase activity is mediated in a non-metal-chelating manner[25,26] and that the GPIase action of ACE does not require zinc ion. Considered together, these results suggest that the active site element for GPIase activity differ from that for peptidase activity.

ACE sheds various GPI-anchored proteins from the cell surface

To examine whether ACE GPIase activity catalyzes GPI-anchored proteins in intact cells, we developed the F9 cell clone, which stably expressed EGFP-GPI on the cell surface. Although ACE exerted little effect on EGFP-GPI expression, pretreatment of cells with filipin, a commonly used reagent for lipid raft disruption[27], allowed ACE to shed EGFP-GPI from the cell surface (**Fig. 3a**,**b**). Most of the GPI-anchored proteins are localized and packed in the lipid raft[28–30]. Furthermore, increased accessibility of GPI-anchored proteins to shedding enzyme was observed upon disruption of lipid raft[31]. Because filipin has no pharmacological effects on ACE GPIase activity, as assessed by PLAP conversion assay (data not shown), exogenous ACE seems to be prevented from accessing the substrate molecules by this membrane microstructure. In contrast, both ACE and PI-PLC had no effect on the transmembrane protein E-cadherin, implying that the activity of ACE is unique for GPI-anchored protein shedding. Moreover, ACE displayed both time- and dose-dependent enzymatic characteristics (data not shown).

We examined the effect of this activity on various endogenous GPI-anchored proteins including Sca-1 and Thy-1 in F9 cells; CD59 and the decay-accelerating factor (DAF) in HeLa cells; and prion protein (PrP) and CD59 in HEK293 cells (**Fig. 3c**). All proteins, except transmembrane protein E-cadherin, were efficiently shed but at various degrees. In contrast to F9 cell molecules, GPI-anchored proteins on human cells were readily released from the cell surface without filipin treatment.

ACE cleaves GPI moiety

To determine the ACE cleavage profile on the substrate, we performed the following studies. First, we used radiolabeling to clarify whether the released molecules contained GPI components. We metabolically labeled the F9 cells expressing EGFP-GPI with radioactive phosphate or ethanolamine, both of which could be incorporated into the GPI anchor moiety but not in the EGFP protein. We treated cells with ACE, PI-PLC or mouse glandular kallikrein (mGK), which digests EGFP protein near the carboxy termini (data not shown) and trapped the released products from the supernatants using antibody specific for GFP. Following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and transfer onto nitrocellulose membrane, we performed autoradiography followed by immunoblotting to detect EGFP-GPI protein. The ACE-released EGFP-GPI was labeled with [^{32}P]-phosphate or [^{3}H]-ethanolamine, but not mGK-treated products, indicating the presence of a portion of GPI anchor structure in the ACE-released molecules (**Fig. 4a**). The radioactivity of released products was about one-third when it was labeled on the phosphate and one-half on the ethanolamine compared with those of PI-PLC-released molecules, suggesting that ACE cleaves GPI anchor between mannoses or at the linkage between phosphate and the third mannose.

We then examined the difference in the cleavage mechanism between ACE and PI-PLC. K562 human erythroleukemia cell line was treated with either enzyme to test the shedding of the surface DAF. Because an additional fatty acylation on the inositol hydroxyl is frequently found on mature GPI anchor[2] and this modification is prominent in K562

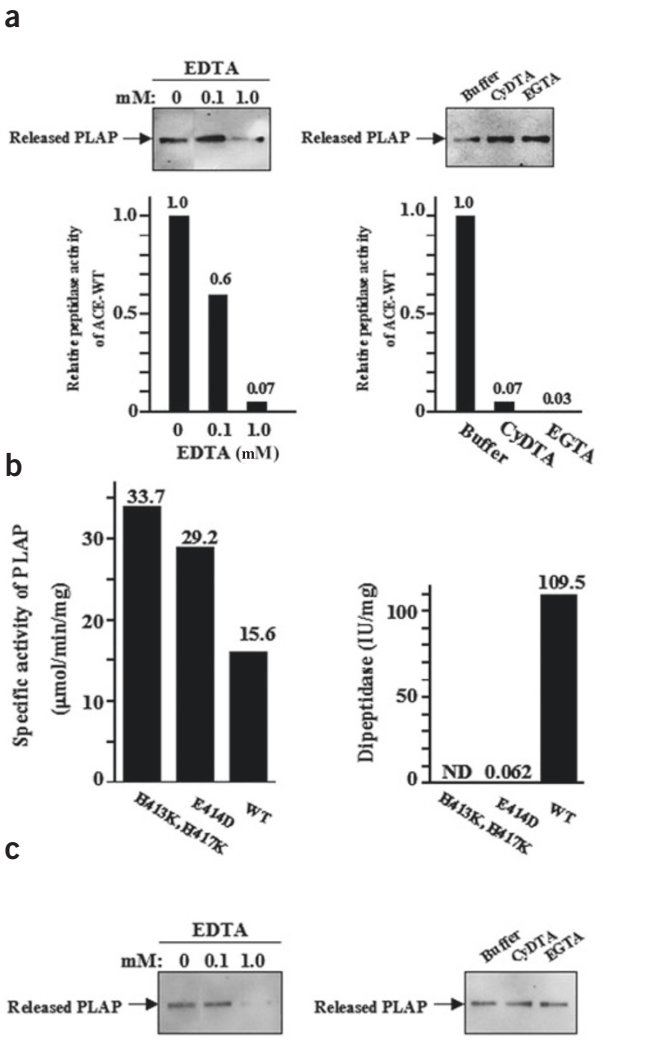

Figure 2 Differences in GPIase and peptidase activities of ACE. (**a**) Effects of metal chelating reagents on the wild-type Ace. EDTA, CyDTA or EGTA was applied to the PLAP conversion assay and the released PLAP was detected by immunoblotting (top). Bottom panels indicate effects of these reagents on the peptidase activity (activity under control condition; *i.e.*, no reagent, was considered 1.0). Dose effect of EDTA on GPIase and peptidase activities. EDTA was applied at 0.1 mM or 1 mM (left). Effects of high dose (1 mM) CyDTA and EGTA on GPIase and peptidase activities (right). Buffer indicates no reagent applied. (**b**) GPIase and peptidase activities of the wild-type (WT), E414D and H413K-H417K mutants. GPIase activity is expressed as released PLAP activity (left). The dipeptidyl carboxypeptidase (Dipeptidase) activity of the same samples (right). ND, not detected. (**c**) Effects of metal chelating reagents on the H413K-H417K mutant. EDTA, CyDTA or EGTA was applied to the PLAP conversion assay and the released PLAP was detected by immunoblotting. Dose effect of EDTA on GPIase activity (left). EDTA was applied at 0.1 mM or 1 mM. Effects of high-dose (1 mM) CyDTA and EGTA on GPIase activity (right).

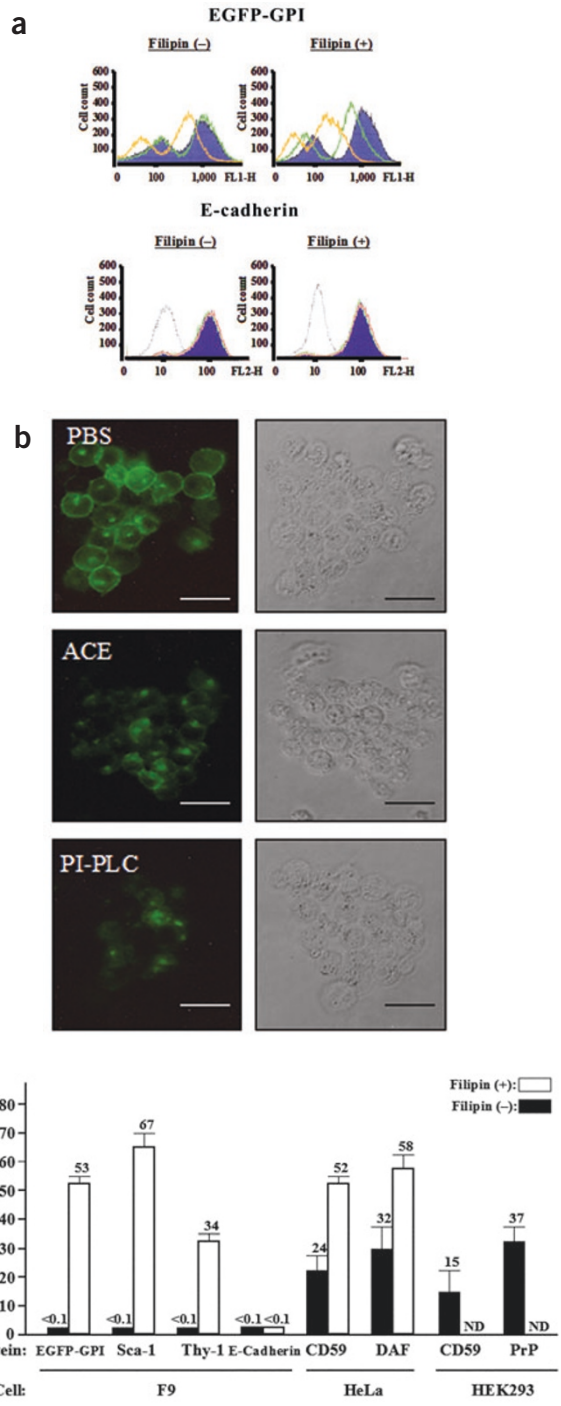

Figure 3 Shedding activity of ACE on the cell surface. (**a**) Expression of EGFP-GPI was examined by FACS analysis. Purple area, ACE (–); green line, ACE (+); yellow line, PI-PLC-treated; black dot, background. (**b**) Expression of EGFP-GPI after ACE or PI-PLC treatment examined by fluorescence microscopy. Note that the fluorescence of Golgi complex remained the same. Magnification, ×200. (**c**) ACE caused shedding of various endogenous GPI-anchored proteins. The amount of ACE used was equivalent to the endogenous ACE activity. Values are mean ± s.d., $n = 3$. ND, not determined.

(ref. 32), resistance to PI-PLC treatment was apparent, whereas ACE considerably shed DAF (**Supplementary Fig. 6** online).

We also attempted to identify the structure of the carboxy-terminal peptides by high-performance liquid chromatography–mass spectrometry with trypsin treatment of the released EGFP-GPI. Under high-peptide coverage, we found a single charged fragment at mass-to-charge ratio (m/3) 660.5, corresponding to the carboxy-terminal peptide LEN, carrying an ethanolamine-phosphate-mannose structure, suggesting that ACE cleaves GPI anchor between the first and second mannose of the GPI moiety (**Fig. 4b**). This conclusion was supported by the fact that its tandem mass spectra showed intense dehydrated ions of $(M + H)^+$ at m/z 642 and less intense ions at m/z 498 that underwent neutral loss of mannose-H_2O. These results suggest that ACE cleaves GPI anchor as an endo-mannosidase, different from PI-PLC cleavage, which is more proximate to the protein.

Rescue of *ACE* knockout sperm by mutant ACE and PI-PLC

The most prominent phenotype of *Ace* knockout mouse is male infertility. Although the physiological parameters of *Ace* knockout sperm, such as number, shape, viability, mobility and rates of capacitation and acrosomal reaction, were not different from those of wild-type sperm, they showed defective sperm-egg binding at the zona pellucida[33,34].

To show a functional ACE GPIase activity *in vivo* and assess its role in fertilization, we first checked the state of GPI-anchored proteins in the sperm. We collected epididymal sperm from both wild-type and *Ace* knockout mice[33] and compared the distribution of GPI-anchored proteins in water-soluble and detergent-soluble fractions. In the sperm, the water-soluble fraction contains soluble ingredients of the acrosome, whereas the detergent-soluble fraction consists of membrane components. Two GPI-anchored proteins, Tesp5 and Ph-20, were examined, because both proteins are released from the sperm during fertilization[35,36]. Immunoblotting showed both Tesp5 and Ph-20 in the water-soluble fraction of wild-type sperm but not in the *Ace* knockout sperm (**Fig. 5a**), implying that ACE participates in converting GPI-anchored proteins from the membrane-bound form to a soluble form.

Finally, we examined the effect of ACE on sperm-egg binding. We prepared epididymal sperm of both genotypes and treated them with either wild-type or peptidase-inactivated ACE (ACE-E414D) or PI-PLC and then incubated them with unfertilized eggs from C57BL/6 mice. These treatments had no effect on wild-type sperm–zona pellucida binding (data not shown). In contrast, treatment with wild-type or ACE-E414D vigorously restored sperm–zona pellucida binding of the *Ace* knockout mice (**Fig. 5b**). Moreover, PI-PLC treatment apparently restored the egg-binding ability of *Ace* knockout sperm to a level comparable with both ACE treatments, confirmed by inhibition with inositol monophosphate, a PI-PLC-specific inhibitor (**Fig. 5c**). Then, we transplanted the wild-type oocytes fertilized with ACE-pretreated knockout sperm into pseudopregnant females to assess their developmental potential. Normal $ACE^{+/-}$ pups were born (**Supplementary Fig. 7 online**). These findings indicate that ACE GPIase activity is crucial for the egg-binding ability of sperm.

DISCUSSION

The major finding of the present study was the discovery that ACE is a GPI-anchored protein releasing factor. Furthermore, this activity of ACE might also be different from that of GPI-PLD, the only enzyme known so far to cleave GPI anchor in mammals[22,23]. It has been reported that GPI-PLD releases GPI-anchored protein only when it is expressed intracellularly in culture cells[23]. In contrast, ACE efficiently released GPI-anchored protein from the cell surface. Because GPI-PLD cleaves GPI anchor at the phosphorus-oxygen bond of the phosphatidylinositol backbone[22], inositol acylation may also prevent completion of GPI-anchored protein release by GPI-PLD. Indeed, GPI-PLD could not release DAF from the intact erythrocyte[37]. In contrast,

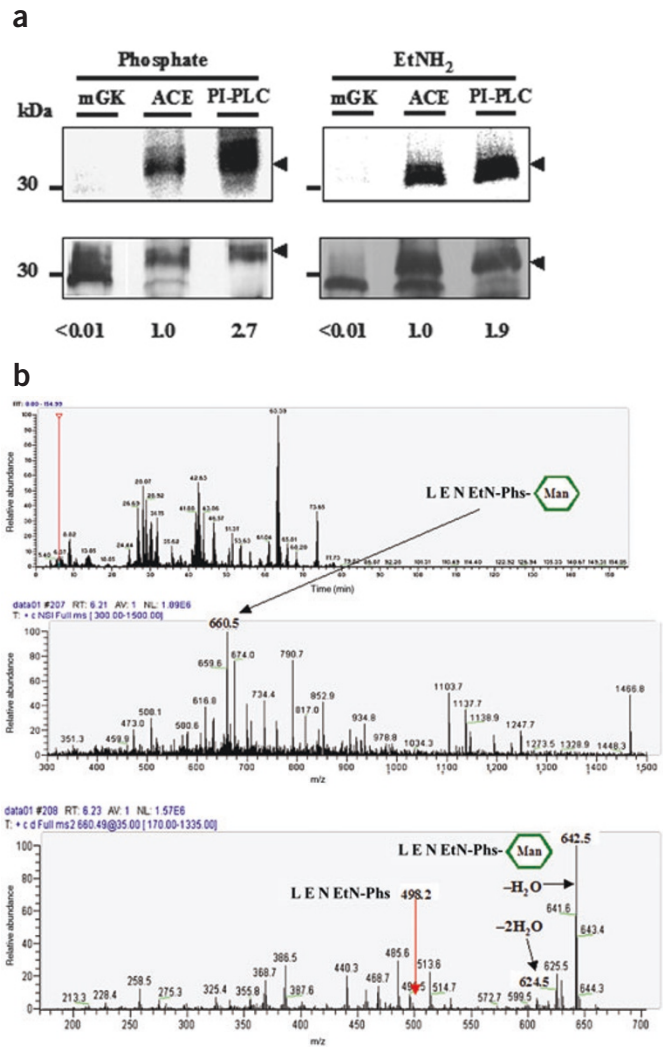

Figure 4 Characteristics of GPI cleavage by ACE. (**a**) Detection of GPI anchor moiety in the released products. Autoradiography using radioactive phosphate (top left) and ethanolamine (top right); immunoblotting of GFP (bottom panels). The radioactivity of the cognate band detected by immunoblotting (arrowhead) was determined. The radioactivity per quantity of protein is indicated (amount of ACE-treated product was considered 1.0). (**b**) Identification and characterization of carboxy-terminal peptide by high-performance liquid chromatography–mass spectrometry. Spectra of the eluted peptides are shown. The peak at the retention time (RT) of 6.2 min was considered a carboxy-terminal peptide with the indicated modification. EtN, ethanolamine; Phs, phosphate; Man, mannose. Base peak chromatogram (top). The fraction at RT = 6.2 min is indicated (red). A full-scan spectrum at RT = 6.2 min (middle). A tandem mass spectrum of m/z-660 ions (bottom).

ACE cleaved GPI anchor distal to the inositol moiety, with no influence on inositol acylation.

A recent crystallographic study has shown that positioning of Glu414 is consistent with its function and that ACE has a single fissure-like groove with zinc buried in the center[19]. Two histidines in the well-characterized core sequences of the catalytic site, HEMGH and downstream glutamate[17], are ligated with zinc. ACE-specific inhibitors also bind here, implying that this structure is indispensable for the peptidase activity. On the other hand, the results of amino acid replacements and metal chelating experiments suggest that the microstructure necessary for the GPIase activity is different from that required for peptidase activity.

High-performance liquid chromatography–mass spectrometry predicted that ACE cleaves the mannose linkage in the GPI moiety. We searched for consensus motifs of glycosidase or lectin on the ACE amino acid sequences *in silico* but found no significant similarities. Because ACE does not seem to cleave conventional sugar chain and specifically cleaves the mannose linkage of the GPI anchor, there might be some unique motifs for this activity. Serial mutagenesis of ACE could be used to determine such motifs.

Shedding of GPI-anchored proteins on the cell surface was accelerated by filipin treatment, suggesting that GPI-anchored proteins, the majority of which are packed in the lipid raft, were inherently protected from ACE attack, whereas spontaneous disruption of lipid raft was suggested on sperm capacitation, which might be caused by cholesterol depletion[38]. We believe that the sperm capacitation process includes a similar reaction caused by filipin treatment of culture cells. In this regard, GPI-anchored proteins were not released in sperms of *Ace* knockout mice (**Fig. 5a**). Considered together, our results indicate that ACE participates in the release of GPI-anchored proteins *in vivo*.

Both the peptidase-inactivated mutant of ACE and PI-PLC efficiently cured the phenotype of *Ace* knockout sperm, indicating that the release of GPI-anchored protein is crucial for the sperm binding ability. In this regard, male mice lacking angiotensinogen and kallikrein were fertile, excluding the involvement of ACE substrates such as angiotensin I and bradykinin in fertilization[39,40]. Based on these findings, our results suggest two scenarios: (i) functional activation of some GPI-anchored proteins on the sperm surface upon their release; (ii) exposure of a zona pellucida–binding factor after the shedding of some GPI-anchored proteins. By using a cell-surface biotinylation technique, we found that ACE released several proteins from the membrane fraction of germ cells (G.K., unpublished data). These proteins might also contain GPI-anchored proteins that do not directly contribute to the sperm-egg binding, although how such proteins influence sperm-egg binding remains to be investigated.

Our results also showed shedding of cell surface PrP by ACE (**Fig. 3c**). With regard to the pathogenesis of prion-related diseases, shedding of surface molecules seems to reduce the chance of pathogenic conversion[10,11]. Moreover, a soluble form of PrP also prevented the pathogenic conversion and prolonged the lifespan of scrapie-form PrP (PrP^{SC})–transferred mice[41]. Based on this scheme, we propose that ACE might be a promising compound that could prevent the production of pathogenic prion proteins. The peptidase-inactivated ACE mutant, which reduced the side effect of peptidase activity, might be potentially useful for the treatment of prion disease.

METHODS

Purification of GPI-anchored protein releasing activity. Germ cells prepared from 500 testes of adult ICR mice were crushed in a buffer containing 3 mM Tris pH 7.4, 2 mM $MgCl_2$, 1 mM EDTA, 0.25 M sucrose and Complete protease inhibitor (Boehringer Mannheim), and homogenates were centrifuged at 100,000*g*. We solubilized the pellet in a buffer containing 20 mM Tris, pH 8.0, 1% TritonX-100 and Complete protease-inhibitor. We ultracentrifuged lysates (100,000*g*), and collected the supernatant. This sample was applied to serial liquid chromatography: (i) DEAE-cellulose (Seikagaku); (ii) phenyl-Sepharose CL-4B (Amersham Bioscience); (iii) ConA-Sepharose 4B (Amersham Bioscience); and (iv) TSK gel 3000SW (Tosoh).

Mass spectrometry. To identify the 100-kDa protein, the peak fraction of TSK gel chromatogram was separated by SDS-PAGE and stained with GelCode blue stain reagent (Pierce). We excised the cognate band and digested in gel with 1.5 μg/ml trypsin (Sigma) in 50 mM ammonium bicarbonate and 5 mM $CaCl_2$ for

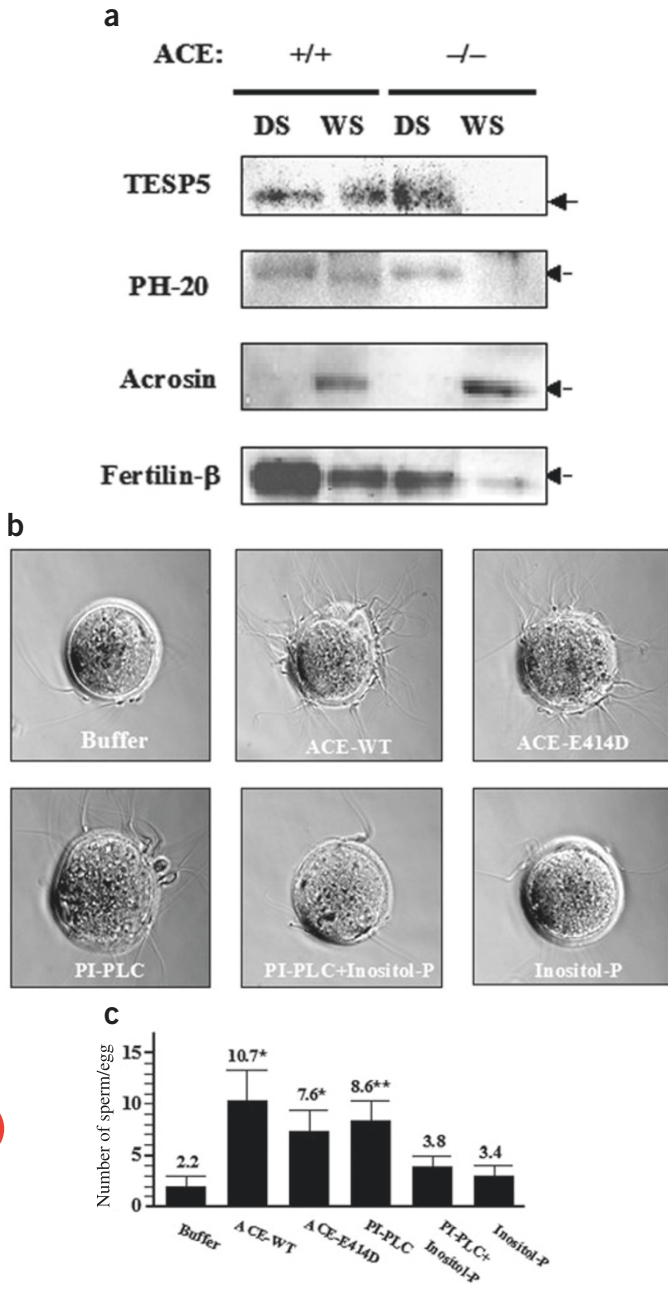

Figure 5 Involvement of ACE GPIase activity in sperm-egg binding. (**a**) Distribution of Tesp-5 (TESP5) and Ph-20 (PH-20) in the sperm of wild-type and *Ace* knockout mice detected by immunoblotting. The acrosin and fertilin-β are indicated for water-soluble and detergent-soluble fraction controls, respectively. +/+, wild-type; −/−, *Ace* knockout. DS, detergent-soluble fraction; WS, water-soluble fraction. (**b**) Binding of *Ace*-knockout sperm to the zona pellucida after various treatments. The amount of Ace used for treatment was equivalent to the endogenous ACE activity. (**c**) Number of sperm bound to the egg. Values are mean ± s.e.m. $^{*}P < 0.001$, $^{**}P < 0.005$, compared with buffer control; $P < 0.3$, comparison of wild-type ACE with ACE-E414D; $P < 0.5$, comparison of wild-type ACE with PI-PLC; $P < 0.05$, comparison of PI-PLC with PI-PLC + Inositol-P (Student's *t*-test).

16 h at 37 °C. After eluting peptides with 50% acetonitrile/5% formic acid several times, we applied them to the capillary high-performance liquid chromatography (Magic, Michrom)/LCQ ion-trap mass spectrometry (ThermoElectron). A perfluorinated polymer-coated electrospray tip (Fortis Tip) was used[42].The tandem mass spectra were subjected to database search using Sequest and Mascot engines. We performed two independent experiments with similar results.

We prepared the sample for investigating the structure of released EGFP-GPI as follows: we treated 1×10^9 F9 EGFP-GPI-expressing cells with 10 μg/ml filipin/phosphate-buffered saline (PBS; Sigma) for 1 h at 0 °C and then with 1.0 μM of ACE-S for 1 h at 37 °C. The released EGFP-GPI molecules were trapped with an anti-GFP antibody column, eluted with 0.1 M glycine, pH 2.8, and subjected to SDS-PAGE. We stained the gels with copper stain and destain kit (Bio-Rad) and excised the cognate band. In our experiments, the amount of released EGFP was about 30 μg. We treated all samples with trypsin and applied eluted peptides to the capillary high-performance liquid chromatography, eluted with acetonitrile gradient from 5% to 90% vol/vol and analyzed using the LCQ mass spectrometer. The tandem mass spectra were subjected to database search.

PLAP conversion assay on TritonX-114 partition. We prepared PLAP by expressing cDNA in COS7 cells, extracted it using buffer containing 20 mM Tris, pH 8.0, 150 mM NaCl, 1% TritonX-114, Complete protease inhibitor and collected the detergent-soluble phase after partitioning at 37 °C. PLAP was purified by DEAE-cellulose and anti-PLAP antibody columns. We measured PLAP activity with an alkaline phosphatase detection kit (Nacalai tesque). The conversion reaction was performed in 100 mM Tris, pH 7.5, 5 mM $CaCl_2$, 150 mM NaCl and 0.3 IU/ml of PLAP for 90 min at 37 °C. We stopped the reaction by adding TritonX-114 at a final concentration of 2% and 1 mM EDTA, followed by microcentrifugation at 25 °C. The water-soluble phase was collected and PLAP activity was measured by the alkaline phosphatase detection kit. The GPIase activity represented PLAP activity released in the water phase using the extinction coefficient of the product (32.4 $cm^{-1}mM^{-1}$) at 595 nm. To examine the metal requirement of GPIase activity, we added EDTA (Nacalai Tesque), CyDTA (Dojindo) or EGTA (Nacalai Tesque) to the assay. After stopping the reaction, we subjected the water phase to immunoblotting using a polyclonal anti-PLAP antibody (Biomeda) to detect released PLAP.

Immunoblotting. Tissue extracts were prepared as described previously[12]. The protein-transferred membranes were probed with a rabbit polyclonal antibody against GFP (Medical & Biological Laboratories), PLAP (Biomeda), TESP5 (ref. 35), PH-20 (ref. 36), acrosin or mouse monoclonal antibody against fertilin-β and detected using the ECL system (Amersham Bioscience).

ACE samples. *Ace* cDNA was obtained by reverse transcription-polymerase chain reaction (RT-PCR) using mouse testis cDNA as a template and primer pairs, 5′-TGAATTCCACCATGGGCCAAGGTTGGGCTACTCCAGG-3′ and 5′-GAATTCGTCACTTATCATCATCATCCTTATAATCCTGCTGTGGCTCCAG GTACAGGC-3′. This encodes a FLAG-tagged version of the soluble testicular isoform. Peptidase-inactivated mutants with amino acid Glu414 replaced by aspartate or His413 and His417 replaced by lysine were synthesized by site-directed mutagenesis using 5′-CTTGGTGATAGCGCACCACGATATGGGCC ACATCCAGTATTTCATGCA-3′ and 5′-CATGGAGGACTTGGTGATAGCGC ACAAGGAAATGGGCAAGATCCAGT ATTTCATGC-3′ as mutation primers, respectively. The culture supernatants of transfected COS7 cells were collected and recombinant ACE was purified by anti-FLAG M2-agarose affinity column (Sigma) and TSK gel 3000SW (Tosoh). In this study, we also used the somatic isoform of ACE (ACE-S) from rabbit lung (Sigma A-6778). The ACE peptidase activity was measured as described previously[43]. We measured both GPIase and peptidase activities of recombinant proteins and ACE-S three times independently and obtained similar results.

FACS analysis. Suspended cells were treated with 10 μg/ml filipin/PBS (Sigma) for 1 h at 0 °C. Cells (1×10^6) were then treated with 1.0 μM of ACE, 1.0 IU/ml of PI-PLC (GLYKO) or PBS alone for 1 h at 37 °C, stained with biotin-conjugated antibodies for human CD59 (ref. 44), human DAF (ref. 44), mouse Sca-1 (Pharmingen-Fujisawa), mouse Thy1.2 (Pharmingen-Fujisawa), mouse E-cadherin (Takarasyuzo) or human prion protein (3F4, Signet Laboratories), then treated with phycoerythrin-conjugated streptavidin (Pharmingen-Fujisawa) and applied to a FACScan cell sorter. EGFP-GPI expressed in F9 cells was directly detected. For quantification of shedding, we used the mean fluorescence values for each cell population to generate the percent shedding value:

$$\text{Percent shedding} = \frac{\text{ACE}(-) - \text{ACE}(+)}{\text{ACE}(-) - \text{PI-PLC}} \times 100$$

The mean fluorescence value of PI-PLC-treated population was defined as the maximum shedding and that of ACE(–) as no shedding. We performed three independent experiments and obtained similar results.

Radiolabeling analysis. We metabolically labeled F9 cells expressing EGFP-GPI with 0.2 mCi/ml of [^{32}P]-orthophosphoric acid (Amersham Bioscience) or 0.1 mCi/ml of [^{3}H]-ethanolamine (Amersham Bioscience) for 16 h. Filipin-pretreated cells were incubated with 1.0 μM of ACE-S, 1.0 IU/ml of PI-PLC or 10% lysate of mouse submaxillary gland (containing mGK) for 1 h at 37 °C. Released EGFP was immunoprecipitated with antibody specific for GFP, subjected to SDS-PAGE and transferred onto nitrocellulose membrane. We evaluated the quantity of EGFP-GPI protein by measuring the density of bands detected on EGFP immunoblotting by using a densitometer (Molecular Device) and determined the radioactivity of the cognate band using a liquid scintillation counter (Aloka). Three independent experiments were performed with similar results.

Sperm-egg binding assay. All gametes were handled as described previously[35]. Incubated sperm (approximately 2.0×10^6 sperm/ml) were treated for 90 min with 1.0 μM wild-type Ace, 1.0 μM ACE-E414D, 1.0 IU/ml PI-PLC, or 1.0 IU/ml PI-PLC with 4 mM inositol monophosphate (Sigma), 4 mM inositol monophosphate and buffer (PBS) alone. For the sperm-egg (zona pellucida) binding assay, gametes were coincubated in a TYH droplet covered with mineral oil (Sigma) for 1 h, washed gently in PBS and fixed with 4% paraformaldehyde/PBS. The oocytes were visualized (magnification, ×200) under a light microscope (Olympus) and the number of sperm was counted at a focus showing the widest diameter of eggs. The numbers of oocytes examined were 18 in buffer only without reagents, 20 with wild-type ACE, 17 with ACE-E414D, 18 with PI-PLC, 18 with PI-PLC + inositol-P, and 17 with inositol-P alone. Three independent experiments were performed with similar results. All animal experiments were performed under approval of the Osaka University Animal Experiment Committee.

Note: Supplementary information is available on the Nature Medicine website.

ACKNOWLEDGMENTS

We thank K. Ohishi and Y. Tashima for technical assistance, T. Baba for providing anti-TESP5 antibody and P. Primakoff for providing anti-PH-20 antibody. We are also grateful to V.W. Keng and D.G. Myles for the critical reading of the manuscript. This work was supported by grants from the Osaka Medical Research Foundation for Incurable Diseases and the Ministry of Education, Science, Sports, and Culture of Japan.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

Received 14 May; accepted 8 December 2004
Published online at http://www.nature.com/naturemedicine/

1. Kinoshita, T., Ohishi, K. & Takeda, J. GPI-anchor synthesis in mammalian cells: genes, their products, and a deficiency. *J. Biochem.* **122**, 251–257 (1997).
2. Ikezawa, H. Glycosylphosphatidylinositol (GPI)-anchored proteins. *Biol. Pharm. Bull.* **25**, 409–417 (2002).
3. Nozaki, M. *et al.* Developmental abnormalities of glycosylphosphatidylinositol-anchor-deficient embryos revealed by Cre/loxP system. *Lab. Invest.* 79, 293–299 (1999).
4. Tarutani, M. *et al.* Tissue-specific knockout of the mouse Pig-a gene reveals important roles for GPI-anchored proteins in skin development. *Proc. Natl. Acad. Sci. USA* **94**, 7400–7405 (1997).
5. Alfieri, J. A. *et al.* Infertility in female mice with an oocyte-specific knock of GPI-anchored proteins. *J. Cell Sci.* **116**, 2149–2155 (2003).
6. Miyata, T. *et al.* The cloning of PIG-A, a component in the early step of GPI-anchor biosynthesis. *Science* **259**, 1318–1320 (1993).
7. Takeda, J. *et al.* Deficiency of the GPI anchor caused by a somatic mutation of the PIG-A gene in paroxysmal nocturnal hemoglobinuria. *Cell* **73**, 703–711 (1993).
8. Prusiner, S. B. Prions. *Proc. Natl. Acad. Sci. USA* 95, 13363–13383 (1998).
9. Aguzzi, A. & Polymenidou, M. Mammalian prion biology: one century of evolving concepts. *Cell* **116**, 313–327 (2004).
10. Marella, M., Lehmann, S., Grassi, J., & Chabry, J. Filipin prevents pathological prion protein accumulation by reducing endocytosis and inducing cellular PrP release. *J. Biol. Chem.* **277**, 25457–25464 (2002).
11. Vincent, B. *et al.* Phorbol ester-regulated cleavage of normal prion protein in HEK293 human cells and murine neurons *J. Biol. Chem.* **275**, 35612–35616 (2000).
12. Kondoh, G. *et al.* Tissue-inherent fate of GPI revealed by GPI-anchored GFP transgenesis. *FEBS Lett.* **458**, 299–303 (1999).
13. Mayor, S., Sabharanjak, S. & Maxfield, F. R. Cholesterol-dependent retention of GPI-anchored proteins in endosomes. *EMBO J.* **17**, 4626–4638 (1998).
14. Sabharanjak, S., Sharma, P., Parton, R. G. & Mayor, S. GPI-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated, clathrin-independent pinocytic pathway. *Dev. Cell* **2**, 411–423 (2002).
15. Hooper, N. M. Angiotensin converting enzyme: implications from molecular biology for its physiological functions. *Int. J. Biochem.* **23**, 641–647 (1991).
16. Turner, A. J. & Hooper, N. M. The angiotensin-converting enzyme gene family: genomics and pharmacology. *Trends Pharmacol. Sci.* **23**, 177–183 (2002).
17. Hooper, N. M. Families of zinc metalloproteases. *FEBS Lett.* 354, 1–6 (1994).
18. Wei, L., Alhenc-Gelas, F., Corvol, P. & Clauser, E. The two homologous domains of human angiotensin I-converting enzyme are both catalytically active. *J. Biol. Chem.* **266**, 9002–9008 (1991).
19. Natesh, R., Schwager, S. L. U., Sturrock, E. D. & Acharya, K. R. Crystal structure of the human angiotensin-converting enzyme-lisinopril complex. *Nature* **421**, 551–554 (2003).
20. Crackower, M. A., *et al.* Angiotensin-converting enzyme 2 is an essential regulator of heart function. *Nature* **417**, 822–828 (2002).
21. Li, W. *et al.* Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. *Nature* **426**, 450–454 (2003).
22. Scallon, B. J. *et al.* Primary structure and functional activity of a phosphatidylinositol-glycan-specific phospholipase D. *Science* **252**, 446–448 (1991).
23. Tujioka, H., Misumi, Y., Takami, N. and Ikehara, Y. Posttranslational modification of glycosylphosphatidylinositol (GPI)-specific phospholipase D and its activity in cleavage of GPI anchors. *Biochem. Biophys. Res. Commun.* **251,** 737–743 (1998).
24. Watanabe, T., Wada, N., Kim, E. E., Wyckoff, H. W. & Chou, J. Y. Mutations of a single amino acid converts germ cell alkaline phosphatase to placental alkaline phosphatase. *J. Biol. Chem.* 266, 21174–21178 (1991).
25. Carvajal, N. *et al.* Non-chelating inhibition of the H10N variant of human liver arginase by EDTA. *J. Inorg. Biochem.* **98**, 1465–1469 (2004).
26. Nyborg, J. K. & Peersen, O. B. That zincing feeling: the effect of EDTA on the behaviour of zinc-binding transcriptional regulators. *Biochem. J.* **381**, e3–e4 (2004).
27. Nicholas, B-J. *et al.* Rapid cycling of lipid raft markers between the cell surface and Golgi complex. *J. Cell Biol.* **153**, 529–541 (2001).
28. Simons, K. & Ikonen, E. Functional rafts in cell membranes. *Nature* **387**, 569–572 (1997).
29. Brown, D. A. & London, E. Functions of lipid rafts in biological membranes. *Annu. Rev. Cell Dev. Biol.* **14**, 111–136 (1998).
30. Varma, R. & Mayor, S. GPI-anchored proteins are organized in submicron domains at the cell surface. *Nature* **394**, 798–801(1998).
31. Hanada, K., Izawa, K., Nishijima, M. & Akamatsu, Y. Sphingolipid deficiency induces hypersensitivity of CD14, a glycosylphosphatidylinositol-anchored protein, to phosphatidylinositol-specific phospholipase C. *J. Biol. Chem.* **268**, 13820–13823 (1993).
32. Chen, R. *et al.* Mammalian glycosylphosphatidylinositol anchor transfer to proteins and posttransfer deacylation. *Proc. Natl. Acad. Sci. USA* **95**, 9512–9517 (1998).
33. Krege, J. H. *et al.* Male-female differences in fertility and blood pressure in ACE-deficient mice. *Nature* **375**, 146–148 (1995).
34. Hagaman, J. R., *et al.* Angiotensin-converting enzyme and male fertility. *Proc. Natl. Acad. Sci. USA* **95**, 2552–2557 (1998).
35. Honda, A., Yamagata, K., Sugiura, S., Watanabe, K. & Baba, T. A mouse serine protease TESP5 is selectively included into lipid rafts of sperm membrane presumably as a glycosylphosphatidylinositol-anchored protein. *J. Biol. Chem.* **277**, 16976–16984 (2002).
36. Lin, Y., Mahan, K., Lathrop, W. F., Myles, D. G. & Primakoff, P. A hyaluronidase activity of the sperm plasma membrane protein PH-20 enables sperm to penetrate the cumulus cell layer surrounding the egg. *J. Cell Biol.* **125**, 1157–1163 (1994).
37. Roberts, W. L., Myher, J. J., Kuksis, A., Low, M. G. & Rosenberry, T. L. Lipid analysis of the glycoinositol phospholipid membrane anchor of human erythrocyte acetylcholinesterase. Palmitoylation of inositol results in resistance to phosphatidylinositol-specific phospholipase C. *J. Biol. Chem.* **263**, 18766–18775 (1988).
38. McLeskey, S. B., Dowds, C., Carballada, R., White, R. R. & Saling, P. M. Molecules involved in mammalian sperm-egg interaction. *Int. Rev. Cytol.* **177**, 57–113 (1998).
39. Doan, T. N., Gletsu, N., Cole, J. & Bernstein, K. E. Genetic manipulation of the renin-angiotensin system. *Curr. Opin. Nephrol. Hypertens.* **10**, 483–491 (2001).
40. Bergaya, S., *et al.* Decreased flow-dependent dilation in carotid arteries of tissue kallikrein-knockout mice. *Circ. Res.* **88**, 593–599 (2001).
41. Meier, P., *et al.* Soluble dimeric prion protein binds PrP^{Sc} in vivo and antagonizes prion disease. *Cell* **113**, 49–60 (2003).
42. Tojo, H. Properties of an electrospray emitter coated with material of low surface energy. *J. Chromatogr. A* **1056**, 223–228 (2004).
43. Kasahara, Y. & Ashihara, Y. Colorimetry of angiotensin-I converting enzyme activity in serum. *Clin. Chem.* **27**, 1922–1925 (1981).
44. Kinoshita, T., Medof, M. E., Silber, R. & Nussenzweig, V. Distribution of decay-accelerating factor in the peripheral blood of normal individuals and patients with paroxysmal nocturnal hemoglobinuria. *J. Exp. Med.* **162**, 75–92 (1985).